

ORIGINAL ARTICLE

The narrow endemic Norwegian peat moss *Sphagnum troendelagicum* originated before the last glacial maximum

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It is commonly found that individual hybrid, polyploid species originate recurrently and that many polyploid species originated relatively recently. It has been previously hypothesized that the extremely rare allopolyploid peat moss *Sphagnum troendelagicum* has originated multiple times, possibly after the last glacial maximum in Scandinavia. This conclusion was based on low linkage disequilibrium in anonymous genetic markers within natural populations, in which sexual reproduction has never been observed. Here we employ microsatellite markers and chloroplast DNA (cpDNA)-encoded *trnG* sequence data to test hypotheses concerning the origin and evolution of this species. We find that *S. tenellum* is the maternal progenitor and *S. balticum* is the paternal progenitor of *S. troendelagicum*. Using various

Bayesian approaches, we estimate that *S. troendelagicum* originated before the Holocene but not before c. 80 000 years ago (median expected time since speciation 40 000 years before present). The observed lack of complete linkage disequilibrium in the genome of this species suggests cryptic sexual reproduction and recombination. Several lines of evidence suggest multiple origins for *S. troendelagicum*, but a single origin is supported by approximate Bayesian computation analyses. We hypothesize that *S. troendelagicum* originated in a peat-dominated refugium before last glacial maximum, and subsequently immigrated to central Norway by means of spore flow during the last thousands of years. *Heredity* (2011) **106**, 370–382; doi:10.1038/hdy.2010.96; published online 18 August 2010

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Introduction

Sympatric speciation can occur by interspecific hybridization associated with genome doubling, giving rise to allopolyploid daughter species. There is a broad consensus that this is an important mode of speciation in a range of organisms, plants in particular (Mallet, 2007; Soltis and Soltis, 2009). Although most or possibly all plant species may have polyploidization at some time in their ancestry (Soltis and Soltis, 2009; Wood *et al.*, 2009), the proportion of angiosperm and fern speciation events accompanied by ploidal increase has been estimated at 15 and 31%, respectively (Wood *et al.*, 2009). The proportion of polyploids has been estimated as somewhat lower for mosses (5–10%) and liverworts (6–19%), and these appear to be mainly allopolyploids (Såstad, 2004, but see Shaw (2009)). Many studies have shown that genome doubling may be accompanied by massive genome alterations, including loss of DNA and changes in gene expression (for example, Adams, 2007; Chen, 2007; Gaeta and Chris Pires, 2009).

Hybridization has several evolutionary consequences, including increased intraspecific genetic diversity and

transfer of genetic adaptations between taxa (Martin *et al.*, 2006; Whithney *et al.*, 2006; Castric *et al.*, 2008), origin of new ecotypes and species, as well as reinforcement or breakdown of reproductive barriers (reviewed by Rieseberg (1997) and Rieseberg and Willis (2007)). Furthermore, it is widely recognized that recurrent origins is the rule, not the exception for most polyploid species (Soltis and Soltis, 1999; Soltis *et al.*, 2003; Abbott and Lowe, 2004; Shaw, 2009). Recurrent polyploidization involving genetically different diploids can create a series of genetically distinct polyploid populations, and gene flow between polyploid populations of independent origin permits recombination and production of additional genotypes (Soltis and Soltis, 1999). Recurrent formation may be critical to counterbalance local extinction of small populations (Soltis *et al.*, 2003), and this may be one reason why there are few examples of extant polyploid taxa of vascular plants with single origins. Several vascular plant species have been shown not only to have originated multiple times, but also to have originated very recently, within hundreds or a few thousands of years (Abbott and Lowe, 2004; Soltis *et al.*, 2004; reviewed by Soltis and Soltis (2009)).

Sphagnum troendelagicum Flatb. (Flatberg, 1988) has been shown to be an allopolyploid derivative of *S. balticum* (Russ.) C. Jens and *S. tenellum* (Brid.) Brid. (Såstad *et al.*, 2001). *S. troendelagicum* is only known from five proximate areas in oceanic, boreal parts of central Norway (Flatberg, 1988), whereas the parental species

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are widespread throughout the Northern Hemisphere (Daniels and Eddy, 1985). It is one of the very few Norwegian endemic species, and it is classified as critically endangered (Flatberg *et al.*, 2006a,b). On the basis of studies of natural populations using random amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990), Stenøien and Flatberg (2000) hypothesized that *S. troendelagicum* may have originated multiple times. The main reason for this conclusion was the finding of relatively low linkage disequilibrium (LD) even though no sporophytes (that is, sexual reproduction) have ever been observed for these plants in the field. Still today, no sporophytes have been observed in natural populations of this species (KI Flatberg, personal observation). In addition, the total amount of genetic variability within *S. troendelagicum* is quite high even though local population sizes are small. On the basis of these observations, and given what we know about plant polyploidization (Soltis and Soltis, 1993; Soltis *et al.*, 2004), one can speculate that *S. troendelagicum* is a young species possibly having originated several times after the last ice age c. 11 000 years before present (bp), in or very close to localities where the plants are found today. Both parental species occur sympatrically with *S. troendelagicum* at most sites where it is found.

It has been shown that RAPD markers may have low reproducibility (Pérez *et al.*, 1998; Rabouam *et al.*, 1999, but see Ramos *et al.* (2008)), and previous results concerning levels of variability and inferred recombination in *S. troendelagicum* are therefore difficult to interpret with confidence. The aim of this study is to learn more about the evolutionary history of this species, more specifically to (1) determine which of the parental taxa are maternal and paternal progenitors, respectively; (2) quantify patterns of genetic variability and structuring in natural populations of *S. troendelagicum* and the parental species using variable markers with high reproducibility; (3) test the hypothesis that the species originated multiple times; (4) quantify historical parameters, most importantly time since speciation event(s), to determine whether the species likely originated in central Norway or whether it immigrated after the last ice age; and finally, (5) make suggestions concerning the continued preservation of *S. troendelagicum*.

Materials and methods

Sampling of material

Sphagnum troendelagicum, *S. balticum* and *S. tenellum* were sampled from mire localities at four of the five known areas it has been found, viz. Fosnes, Grong, Høylandet and Overhalla municipalities in the northern parts of central Norway (Supplementary Table S1). *S. balticum* and *S. tenellum* were also sampled from six mire localities outside the known distribution area of *S. troendelagicum* in more southern parts of central Norway. As far as possible, a minimum of 10 population samples of each species were sampled at each locality, mostly with a minimum distance of 10 m between samples. More samples were included from the Grong, Høylandet and Overhalla localities, particularly of *S. troendelagicum*, but in Fosnes and one of the localities from Selbu (S Haukåtjønna) only five or six samples were made because of smaller total population sizes. The samples

were air dried and stored in paper bags at room temperature. In addition, a number of selected herbarium specimens of the three species from Norway, Finland, Japan and North America were also included in the study (Supplementary Table S1).

Molecular analyses

Total DNA was extracted from dry capitulum tissue from each sample using the SP Plant DNA Mini Kit and the Plant DNA Kit (OMEGA bio-tek, <http://www.omegabiotek.com>) according to the manufacturer's protocols. Individuals were genotyped at 12 microsatellite loci previously described for *Sphagnum* (Shaw *et al.*, 2008b), that is, markers 4, 9, 10, 14, 17, 18, 19, 20, 22, 28, 29 and 30. Vouchers for each population, including the whole samples and the remaining part of the plants from which the DNA is extracted from are deposited at the herbarium in Trondheim (TRH). DNA and tissue material from *S. troendelagicum* is also deposited in the ColdGene genome resource bank (http://www.vm.ntnu.no/coldgene_en/).

Thirteen samples of *S. troendelagicum* in addition to 12 samples of *S. balticum* and 16 samples of *S. tenellum* from across the species distribution ranges (Supplementary Table S1) were sequenced for the chloroplast area *trnG* (for primer information, see Pacak and Szweykowska-Kulinska, 2000). PCR amplification and sequencing were accomplished using protocols described in Shaw *et al.* (2003).

Population genetic analyses

Overall summary statistics were estimated for DNA sequence and microsatellite data. For the *trnG* data, we estimated several within- and between-group statistics, including numbers of segregating sites (*S*), numbers of mutations, haplotypic diversity and nucleotide diversity (π) within and between taxa using the DnaSP 5.00.07 software (Librado and Rozas, 2009). Microsatellite diversity was assessed by calculating the proportion of polymorphic loci, mean number of pairwise differences between individuals and average gene diversity over loci (H_E ; Nei, 1987). The presence of LD between all pairs of loci was tested for all three species using an exact test of LD (Slatkin, 1994). For *S. troendelagicum*, the 'gametic disequilibrium' was estimated based on genotypes instead of alleles without them needing to specify gametic phase. Loci with missing alleles were excluded from LD analyses to obtain maximally conservative estimates. The percentage of linked loci in relation to the maximum number of linked loci (P_d) was estimated for each population as described in Stenøien and Sæstad (1999). We estimated fixation indices, F_{ST} , among *S. troendelagicum* populations, and the corrected numbers of pairwise differences between *S. troendelagicum* populations, and between *S. troendelagicum* and parental species populations (Weir and Cockerham, 1984; Michalakis and Excoffier, 1996). The significance of differentiation measures was tested using the non-parametric permutation approach described by Excoffier *et al.* (1992). Overall genetic diversity, LD and genetic structure were analysed with the aid of the software Arlequin ver. 3.0 (Excoffier *et al.*, 2005).

To further explore genetic structuring of the three species, we performed Bayesian cluster analysis on

genotypic data using the program Structure ver. 2.3.1 (Pritchard *et al.*, 2000). We evaluated the most likely number of clusters (K) by aid of the ΔK statistics (Evanno *et al.*, 2005). We assumed an admixture model with independent allele frequencies with sampling locations as prior information to assist in the clustering (Hubisz *et al.*, 2009). Ten runs were performed for each K from 1 to 15, with 100 000 steps of burn-in and 100 000 steps per run.

Hypothesis testing

We wanted to test the hypothesis that *S. troendelagicum* originated once, twice or four times for the four populations investigated (that is, for Fosnes, Grong, Høylandet and Overhalla), and this was carried out in a coalescence framework using approximate Bayesian computation (ABC; Beaumont *et al.*, 2002). The three historical scenarios evaluated are presented in Figures 1a–c. In scenario 1 (Figure 1a), it is assumed that *S. troendelagicum* originated only once by hybridization between *S. balticum* and *S. tenellum* t_4 years in the past. After that, the *S. troendelagicum* lineage split three times to form the four populations investigated in this study; that is, at t_3 , t_2 and t_1 years in the past, respectively. *S. balticum* and *S. tenellum* diverged from each other t_A years in the past. It is assumed that the effective population sizes of the two parental species have been similar and constant through time (N_A). It is

also assumed that the four *S. troendelagicum* populations have had similar effective population sizes through their histories (N_t). *S. troendelagicum* consists of 50% *S. balticum* alleles and 50% *S. tenellum* alleles found in the parental species populations at time t_4 . For a relatively short period after this speciation event (equal to x years), the effective population size of the *S. troendelagicum* population (N_0) is assumed to be low. Note that time is not to scale in Figure 1, and also that even though t_4 must have occurred before t_3 , there are no assumptions in the model with regard to the relative order of t_2 and t_1 (see below for an overview of different assumptions in the models).

In scenario 2 (Figure 1b), *S. troendelagicum* originated twice, first at time t_4 and second (independently) at time t_3 . After that, the two *S. troendelagicum* lineages split at times t_2 and t_1 , respectively, to form the four populations. Again, the parental species split at t_A , 50% of each of the parental genomes forms the initial *S. troendelagicum* populations at t_4 and t_3 , and population sizes of the newly formed *S. troendelagicum* populations are N_0 in a period of x years after the speciation events. There are no assumptions with regard to the relative order of t_3 and t_4 , but $t_4 > t_2$ and $t_3 > t_1$.

Finally, in scenario 3 (Figure 1c), all *S. troendelagicum* populations originated independently of one another at times t_1 – t_4 in the past. Moreover, here each speciation event is associated with the parameters x and N_0 as above, each parental species contributed 50% of the genetic material to the newly formed populations and

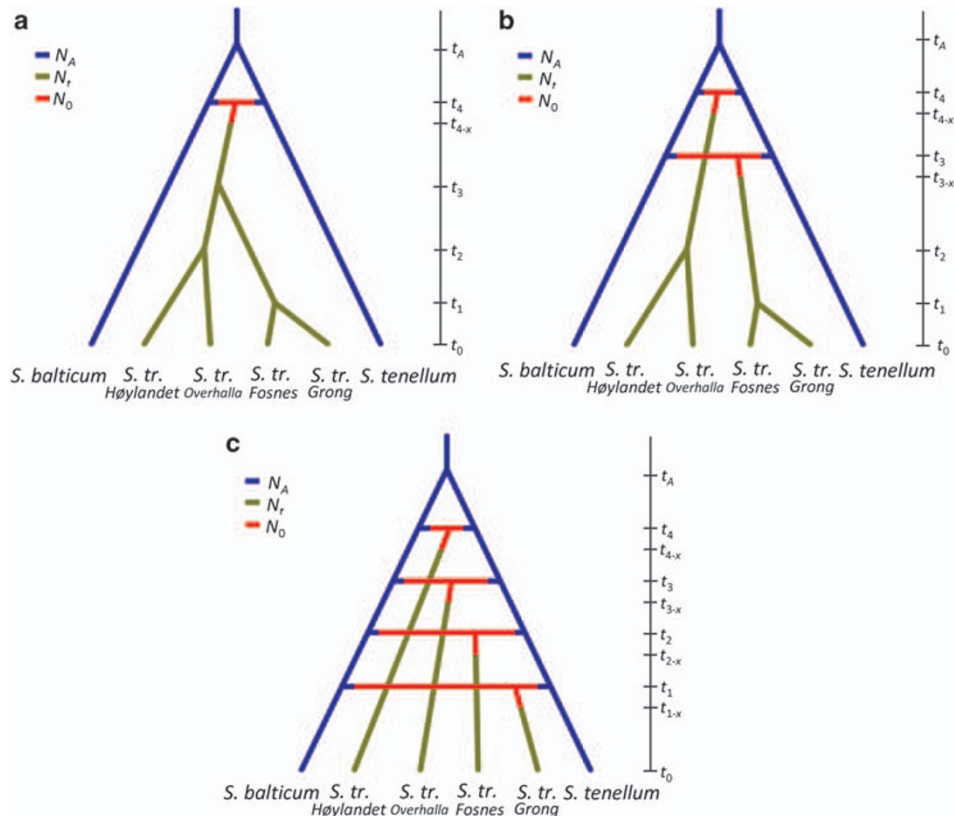


Figure 1 Three scenarios for the evolution of *S. troendelagicum* populations were studied here, the major difference being the number of times the species has independently originated (once under scenario 1 (a), twice under scenario 2 (b) and four times under scenario 3 (c); see text above for details). The relative branching in scenarios 1 and 2, that is, the assumption that Overhalla and Høylandet are more closely related than Fosnes and Grong, are based on results of the Bayesian cluster analyses.

the relative orders of t_1 – t_4 are not determined *a priori*. The parental species are coded as diploids in the input data set in these analyses, and all the populations of each parental species have been grouped together in single global populations.

Several additional assumptions are included in the models. First, the times for the various events (that is, the various t 's) in the three scenarios are not dependent on one another. For example, t_4 in scenario 1 is calculated independently from t_4 in scenarios 2 and 3 (technically, t_4 is treated as separate parameters t_5 and t_7 in scenarios 2 and 3, respectively, and for technical reasons the same approach is followed for other parameters). Second, it is assumed that t_A must be greater than t_1 , t_2 , t_3 and t_4 , that $t_4 > t_3$ and t_3 is larger than both t_2 and t_1 in scenario 1 and that $t_4 > t_2$ and $t_3 > t_1$ in scenario 2. Previous information about the parameters are given, with N_A and N_t limits set to 1000–1 000 000, whereas N_0 limits set to 100–100 000, the events t_1 – t_4 are occurring between 1000–1 000 000 years ago, while t_A occurred 10 000–10 000 000 years ago, and x lasted between 1 and 1000 years. We assumed a stepwise mutation model (Ohta and Kimura, 1973) with no indel mutations. In plants, microsatellite mutation rates have been found to vary widely (for example, 1×10^{-2} in chickpeas (Udupa and Baum, 2001) and 1×10^{-6} in *Dictyostelium discoideum* (McConnell *et al.*, 2007). Previous information on mutation rates will highly influence estimated divergence times. On the basis of previous estimates of microsatellite mutation rates varying between 10^{-6} and 10^{-4} per year in *S. fimbriatum* (Szövényi *et al.*, 2008), we used 10^{-5} as an average mutation rate per year for the 12 simulated loci. Each locus were allowed to mutate to a number of repeats equal to the maximum observed numbers for each of the loci; thus, 40, 165, 104, 92, 134, 44, 56, 101, 77, 54, 94, 117 repeats for loci 22, 28, 4, 9, 10, 18, 17, 20, 29, 30, 19, 14, respectively (Shaw *et al.*, 2008b). In each simulation, the populations are sampled at t_0 and summary statistics are computed for each sample; that is, mean number of alleles, mean genetic diversity, mean size variance for each individual sample, mean M index (ratio of number of alleles to range size) across loci (Garza and Williamson, 2001; Excoffier *et al.*, 2005) and mean index of two-sample classification (Rannala and Mountain, 1997; Pascual *et al.*, 2007). Data sets (500 000) were generated for each scenario; that is, 1 500 000 simulations were performed in total. The scenarios were compared using two approaches: one by directly comparing the summary statistics with the observed diversity in the data set and counting the frequency of the various scenarios among the most similar simulated data sets (direct estimate approach; Miller *et al.*, 2005; Pascual *et al.*, 2007), and one by doing a logistic regression of each scenario probability for the most similar simulated data sets on the deviations between simulated and observed summary statistics (Fagundes *et al.*, 2007; Beaumont, 2008). In these two comparisons, 0.1 and 1% simulated data closest to the observed values were used, respectively. The ABC computations and parameter estimations were performed using the DIY-ABC ver. 0.72 software (Cornuet *et al.*, 2008).

Estimation of historical parameters

Two approaches were used to estimate historical parameters. First, the 1% simulated data sets from the ABC

analyses (see above) that fit observed data most closely were used to inform the posterior distribution of parameter values (that is, t_1 – t_4 , t_A , N_A , N_0 , N_t and x) using logit-transformation of original and composite parameter values (Beaumont *et al.*, 2002). As suggested previously (Excoffier *et al.*, 2005), the reference tables were used together with pseudo-observed data having known parameter values to compute estimation bias of parameter estimates. The average relative bias was used for this, defined as the difference between estimates and true values averaged over all test data sets. One per cent of simulated data sets was used in the evaluation, with logit-transformed data and fixed parameter values. Second, the isolation-with-migration (IM) model was used to estimate time since divergence (that is, allopolyploidization) between *S. troendelagicum* and each of the parental species using the IM software (Nielsen and Wakeley, 2001; Hey and Nielsen, 2004). The IM model assumes an ancestral population being split into two daughter populations at some time while allowing subsequent migration between these two populations. Coalescence simulations are used to determine maximum-likelihood parameter estimates given the genetic data and prior information. This model is not ideal for exploring time since polyploidization events, because two populations are merged rather than one population being split into two. To use this approach, it is necessary to split the *S. troendelagicum* genome into 'tenellum' and 'balticum' alleles for the various loci (that is, assess from which parental species the alleles have been derived), and then use this information in two IM analyses: one involving *S. tenellum* and one involving *S. balticum*. It was possible to use six loci in the comparison between *S. troendelagicum* and *S. balticum*, in which it was clear that alleles in *S. troendelagicum* must have been derived from *S. balticum*. In the comparison involving *S. tenellum*, only four loci could be used. Only individuals containing no missing values for any of these loci were used for any of the data sets. Three independent IM runs were performed on each of the data sets (six runs in total), with generation time arbitrarily set to 5 years. Generation times in peat mosses may be larger than 5 years (for example, Flatberg *et al.*, 2006a, b), but this prior value has no influence on divergence time estimates. In the IM runs, we assumed no subsequent migration after speciation (that is, divergence). This latter approach would tend to underestimate divergence time if subsequent introgression has occurred to any significant extent. It must also be assumed that only one speciation event has occurred in the history of the sample. We used 10^{-5} as an average mutation rate per year in the IM analyses (Szövényi *et al.*, 2008). The two latter runs for each species pair was set to a minimum of 61 million steps after burn-in, with effective sample size (ESS) values being at least 600.

Results

Data considerations

In total, 326 individuals were genotyped (126 *S. tenellum*, 86 *S. balticum* and 114 *S. troendelagicum*). A high number of *S. troendelagicum* individuals exhibited one or several homozygous genotypes for individual loci, or missing data, and a few individuals of the parental taxa exhibited

single heterozygous loci. Only two chromosome numbers, $N=19$ and 38, have been reported in *Sphagnum* (Fritsch, 1991). On the basis of chromosome counts, flow cytometric estimates of genome sizes, isozyme patterns and microsatellite allelic profiles, *S. balticum* and *S. tenellum* are known to have haploid gametophytes (that is, $N=19$), whereas *S. troendelagicum* has diploid gametophytes (Såstad *et al.*, 2001). As allopolyploid origin and fixed heterozygosity have been shown for isozyme markers (Såstad *et al.*, 2001), we have chosen to adopt a conservative strategy by treating one allele as missing data in homozygous *S. troendelagicum* if the parental species do not possess the same allele. Similarly, we deleted one allele in heterozygous *S. balticum* and *S. tenellum* loci, using the most common allele in the population in further analyses. We observe relatively high number of missing data, and inferred levels of variability should thus be interpreted with care.

Genetic variability

Forty-one chloroplast DNA (cpDNA; *trnG*) sequences are deposited to GenBank (accession numbers HM439652–HM439692), and results from genetic analyses are presented in Tables 1–3. The only haplotype found among *S. troendelagicum* individuals is identical to the most common haplotype found in *S. tenellum* (Table 1).

Table 1 Various haplotypes of the *trnG* chloroplast gene found in this study from the three species

Species	Haplotype	Sequence
<i>S. troendelagicum</i>	S-troe-1	---TTAGCCATATC-AGAAACGCAC----TC
<i>S. tenellum</i>	S-tene-1	-----
	S-tene-2	-----
	S-tene-3	gct-----
	S-tene-4	-----a-----
	S-tene-5	-----ta-----
<i>S. balticum</i>	S-balt-1	---cg.t...g.....t.c.tc.at.t
	S-balt-2	---cg.t...g.....t.c.tc.at.t
	S-balt-3	gct.cg.t...g.....t.c.tc.at.t
	S-balt-4	---cg.....a.....c.----t
	S-balt-5	---.t.tgac.--tttc.cgca---at

Both nucleotide polymorphisms and indel variants are shown. The only haplotype found in *S. troendelagicum* individuals is identical to the most common *S. tenellum* haplotype (S-tene-1). The other *S. tenellum* haplotypes found differ from this most common type by 1–3 nucleotide polymorphisms or one indel. *S. balticum* haplotypes differ from the *S. troendelagicum* haplotypes by 5–11 nucleotide polymorphisms and 1–2 indels.

DNA sequence diversities are presented in Table 2. *S. troendelagicum* is monomorphic in the *trnG* region, whereas *S. balticum* is more variable than *S. tenellum* for various diversity statistics, including number of segregating sites (16 versus 1, respectively) and nucleotide diversity ($\pi \pm$ s.d. equal to 0.0066 ± 0.0028 and 0.0002 ± 0.0001 , respectively). Levels of differentiation between the three species for the *trnG* region are summarized in Table 3. *S. troendelagicum* and *S. tenellum* are not differentiated to any significant extent, whereas *S. balticum* is more differentiated from both of the two other species with four fixed nucleotide differences between *S. balticum* and both *S. tenellum* and *S. troendelagicum*.

In the microsatellite analyses, loci with less than 50% missing data were included in the analyses of genetic diversity; levels of genetic variability within populations of the three species are presented in Table 4. Genetic variability is found in parental species populations, with an average 4.4 and 3.0 polymorphic loci per population of *S. balticum* and *S. tenellum*, respectively, implying an average proportion of polymorphic loci of 51 and 41%, respectively. Average H_E is 0.18 (± 0.04 s.e.) and 0.11 (± 0.02 s.e.) per population in *S. balticum* and *S. tenellum*, respectively, whereas the mean number of pairwise differences is 1.46 (± 0.38 s.e.) and 0.88 (± 0.21 s.e.), respectively. Levels of LD are low in populations of the parental species (average P_d values found to be 7 and 6% in *S. balticum* and *S. tenellum*, respectively). Levels of genetic variability are high in diploid *S. troendelagicum* compared with the parental species because of fixed heterozygosities. On average, 10.8 polymorphic loci occur per population, yielding an average proportion of polymorphic loci of 69%. Expected H_E is 0.34 (± 0.20 s.e.) and mean pairwise differences are 3.99 (± 0.22 s.e.) per population. Levels of LD are less than possible maxima in *S. troendelagicum*, with P_d values ranging from 47 to 100% within populations (66% on average), and an average P_d for the whole species is 43%.

Genetic structure

There is significant genetic differentiation among *S. troendelagicum* populations (F_{ST} ranging from 0.12 to 0.16; Table 5). Average F_{ST} values between *S. troendelagicum* and the parental species are 0.09 to *S. balticum* and 0.19 to *S. tenellum*, whereas F_{ST} between *S. balticum* and *S. tenellum* is 0.46 (all P -values < 0.001). Thus, F_{ST} values between *S. troendelagicum* and each of the parental species are relatively similar to F_{ST} values estimated between *S. troendelagicum* populations. In Table 6, corrected numbers of pairwise differences between individuals in different *S. troendelagicum* populations

Table 2 Summary statistics for within-species diversities in *trnG* sequences of *Sphagnum troendelagicum*, *S. tenellum* and *S. balticum* surveyed

Species	No. of sequences	No. of sites	No. of sites excluding indels	No. of segregating sites (S)	No. of mutations	No. of haplotypes	Haplotype diversity	s.d.	Nucleotide diversity (π)	s.d.
<i>S. troendelagicum</i>	13	797	788	0	0	1	0	0	0	0
<i>S. tenellum</i>	16	797	788	1	1	2	0.125	0.106	0.00016	0.00014
<i>S. balticum</i>	12	797	787	16	16	3	0.439	0.158	0.00657	0.00284

Sites with indels are not included in the analyses. No variability is found in *S. troendelagicum*, while *S. balticum* is more variable than *S. tenellum*.

Table 3 Differentiation between the three species studied in the *trnG* region with regard to the number of polymorphic sites, mutations found to be polymorphic in species 1, but monomorphic in species 2 and *vice versa*, number of shared mutations and the average number of nucleotide differences between the two species

Species pairs	No. of seqs.	Polym. sites	No fixed diff.	Mutations poly 1, mono 2	Mutations poly 2, mono 1	Shared mutations	Average no. of nucleotide diff. between 1 and 2
<i>Sphagnum troendelagicum</i> – <i>S. tenellum</i>	29	1	0	0	1	0	0.063
<i>S. troendelagicum</i> – <i>S. balticum</i>	25	20	4	0	16	0	8.417
<i>S. tenellum</i> – <i>S. balticum</i>	28	21	4	1	16	0	8.479

S. troendelagicum and *S. tenellum* is relatively similar, while *S. balticum* is more differentiated from the two other species.

Table 4 Genetic diversity in studied populations of *Sphagnum balticum*, *S. tenellum* and *S. troendelagicum* as revealed by the number of polymorphic loci

Populations	No. of individuals	No. of loci <50% missing data	No. of polym. loci	PPL (%)	H _E	s.d. (s.e.*)	Mean pairwise diff.	s.d. (s.e.*)	P _d (%)
<i>S. balticum</i> Fosnes	2	10	0	0	0	0	0	0	NA
<i>S. balticum</i> Grong	12	6	5	83	0.10	0.09	0.59	0.49	5
<i>S. balticum</i> Høylandet	8	6	4	67	0.34	0.22	2.03	1.20	15
<i>S. balticum</i> Klæbu 1	8	6	2	33	0.09	0.09	0.57	0.49	0
<i>S. balticum</i> Klæbu 2	3	7	5	71	0.38	0.27	2.67	1.65	0
<i>S. balticum</i> Melhus	10	7	4	57	0.16	0.12	1.13	0.77	0
<i>S. balticum</i> Midtre Gauldal	1	11	NA	NA	NA	NA	NA	NA	NA
<i>S. balticum</i> North America	4	12	6	50	0.25	0.17	3.00	1.75	NA
<i>S. balticum</i> Overhalla	12	10	9	90	0.35	0.21	3.53	1.86	10
<i>S. balticum</i> Selbu	15	7	3	43	0.12	0.10	0.86	0.62	17
<i>S. balticum</i> Trondheim	10	5	1	20	0.04	0.06	0.21	0.27	NA
<i>S. balticum</i> Verdal	1	12	NA	NA	NA	NA	NA	NA	NA
Mean <i>S. balticum</i>	86 (total)	8.3	3.9	51	0.18	0.04*	1.46	0.38*	7
<i>S. tenellum</i> Finland	1	10	NA	NA	NA	NA	NA	NA	NA
<i>S. tenellum</i> Fosnes	2	6	0	0	0	0	0	0	NA
<i>S. tenellum</i> Grong	18	8	7	88	0.21	0.14	1.71	1.02	1
<i>S. tenellum</i> Høylandet	13	7	5	71	0.19	0.13	1.32	0.85	2
<i>S. tenellum</i> Japan	2	10	1	10	0.07	0.07	0.67	0.63	NA
<i>S. tenellum</i> Klæbu 1	10	3	1	33	0.04	0.07	0.13	0.20	NA
<i>S. tenellum</i> Klæbu 2	3	7	0	0	0	0	0	0	NA
<i>S. tenellum</i> Melhus	9	6	0	0	0	0	0	0	NA
<i>S. tenellum</i> Midtre Gauldal	2	11	3	27	0.18	0.15	2.00	1.41	NA
<i>S. tenellum</i> North America	19	11	6	55	0.16	0.11	1.81	1.07	NA
<i>S. tenellum</i> Overhalla	22	9	7	78	0.20	0.13	1.78	1.05	11
<i>S. tenellum</i> Selbu	14	6	5	83	0.16	0.12	0.94	0.67	17
<i>S. tenellum</i> Trondheim	9	4	2	50	0.06	0.08	0.24	0.28	0
<i>S. tenellum</i> Verdal	2	11	1	9	0.06	0.07	0.67	0.63	NA
Mean <i>S. tenellum</i>	126 (total)	7.5	3.1	41	0.11	0.02*	0.88	0.21*	6
<i>S. troendelagicum</i> Fosnes	4	12	10	83	0.31	0.20	3.68	2.08	NA
<i>S. troendelagicum</i> Grong	32	11	10	91	0.32	0.19	3.56	1.84	47
<i>S. troendelagicum</i> Høylandet	32	12	12	100	0.38	0.21	4.51	2.25	100
<i>S. troendelagicum</i> Overhalla	46	12	11	92	0.35	0.20	4.22	2.11	50
Mean <i>S. troendelagicum</i>	114 (total)	11.8	10.8	69	0.34	0.20*	3.99	0.22*	66

Abbreviations: NA, not applicable; PPL, proportion of polymorphic loci. H_E, expected heterozygosity (\pm s.d. for individual populations and \pm s.e. (*) for species averages taken over all studied populations); P_d, proportion of loci in significant linkage disequilibrium compared to maximum number of polymorphic locus pairs.

and local populations of the parental species are presented, as well as the average pairwise differences between *S. troendelagicum* and parental species populations. *S. troendelagicum* populations are not more similar to local *S. balticum* populations than to other populations of this species. However, there is a tendency for *S. troendelagicum* populations to be more similar to local *S. tenellum* populations than to other populations of *S. tenellum*.

In the Bayesian cluster analysis, ΔK had the highest estimated value for $K=2$ (Figure 2). When $K=2$,

S. balticum populations are mostly assigned to one group, all *S. tenellum* populations are mostly assigned to the second group and all *S. troendelagicum* populations are assigned to both the groups (approximately 40–60% to each group for each *S. troendelagicum* population; Figure 3). At $K=3$, the *S. troendelagicum* populations are divided into two groups: Fosnes and Grong to one, and Høylandet and Overhalla to the other. Some populations of the parental species are also assigned to this third group, in particular *S. tenellum* individuals at Grong (results not shown). At $K=4$, both *S. troendelagicum*

Table 5 Genetic structure in *Sphagnum troendelagicum* populations sampled from four localities

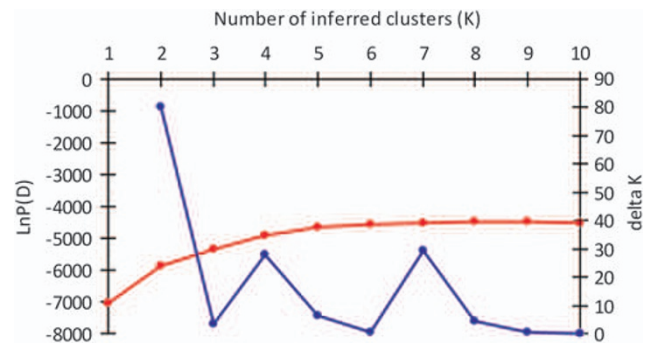
	Fosnes	Grong	Høylandet	Overhalla
Fosnes	—	-0.76	-2.48	-1.32
Grong	-0.02	—	2.62	3.46
Høylandet	-0.12	0.12	—	-0.16
Overhalla	-0.06	0.16	-0.01	—

Below-diagonal are pairwise F_{ST} values between populations, while above-diagonal are corrected average pairwise differences between individuals in the various populations. Bold numerals indicate significance at the 0.05 level (all significant P -values are found to be <0.001).

Table 6 Corrected average pairwise differences between four *Sphagnum troendelagicum* populations (Fosnes, Grong, Høylandet and Overhalla) and *S. balticum* and *S. tenellum* populations

	Local <i>S. balticum</i>	Average <i>S. balticum</i>	s.d. (s.e.*)	Local <i>S. tenellum</i>	Average <i>S. tenellum</i>	s.d. (s.e.*)
Fosnes	2.67	1.46	1.27	NA	2.92	1.63
Grong	4.64	3.38	2.50	4.64	5.51	2.19
Høylandet	2.64	2.61	1.56	3.21	4.11	1.81
Overhalla	1.99	3.41	1.67	3.03	4.72	1.96
Average	2.99	2.72	0.26*	3.63	4.31	0.12*

Comparisons are made with both *S. balticum* and *S. tenellum* populations at the four localities. *S. troendelagicum* has been sampled, and also average distances averaged across all *S. balticum* and *S. tenellum* populations, respectively (NA: not significant at the 0.05 level, standard error estimates indicated by *).

**Figure 2** Results of *Structure* analyses, where probabilities of different K values ($\ln P(D)$; red line) and ΔK values (blue line) are plotted against values of K from 1 to 10 (results from K values up to 15 not shown). $\ln P(D)$ increases for increasing values of K and it is hard to determine the exact breaking point. ΔK on the other hand clearly peaks at $K=2$.

and *S. balticum* populations are separated into two major groups each (Figure 4).

Number of independent origins of *S. troendelagicum*

In the ABC analyses, the numbers of simulated data sets with summary statistics similar to observed values based on the various scenarios are treated as posterior probabilities for the three scenarios. The direct approach does not reveal clearcut differences in probabilities of the three scenarios (results not shown). The logistic regression approach supports scenario 1, that is, a single origin of *S. troendelagicum* ($P > 0.9846$).

Estimation of historical parameters

In the ABC approach, simulations based on scenario 1 with summary statistics close to observed values were used to estimate parameters included in the model. Results from these calculations are presented in Table 7. The parental species seem to have diverged approximately 270 000 years bp (t_A , median value). Around 40 000 years ago (t_4), *S. troendelagicum* was formed by hybridization between these two diverging species. In a period after this initial formation, most likely ~ 450 years (x), the effective size of this newly formed species, N_0 , was $\sim 50 000$, and long-term effective population size (N_l) is $>110 000$. The effective sizes of the parental species are approximately $>60 000$ individuals each. At approximately 11 000 years bp, the species split into two major lineages (t_3), and at 900 years bp (t_2), the Høylandet and Overhalla populations split, whereas somewhat later, at 600 years bp (t_1), the Fosnes and Grong populations split.

The mean relative bias varied among parameters (Table 8), with extreme median values ranging from 0.03 (N_A) to 3.85 (t_1) and 63.83 (N_0). All other median values were in the range 0.15–1.49, with t_4 having a median mean relative bias of 0.37. This means that an estimated t_4 of 40 000 years implies that the true value could be wrong by approximately 10 000 years, and one can speculate that 30 000 years may be a better estimate of time since origin.

The results from the IM analyses are summarized in Figure 5, with divergence time between *S. troendelagicum* and *S. balticum* having a highest probability for $t = 14 200$ years bp (averaged over two long runs, 95% confidence interval between 4750 and 73 850), and divergence time between *S. troendelagicum* and *S. tenellum* having a highest probability for $t = 21 000$ years bp (also averaged over two long runs, 95% confidence interval between 6650 and 85 750). Divergence times before the last glacial maximum and up to 40 000 years bp thus have relatively high probabilities in both IM analyses. On the basis of the combined ABC and IM results, although, it seems unlikely that *S. troendelagicum* is younger than the last glacial maximum (c. 11 600 years) or older than 80 000 years.

Discussion

Stenøien and Flatberg (2000) reported relatively high genetic variability within *S. troendelagicum* compared with other studied peat mosses (for example, gene diversity comparable with levels found in the common circumboreal *S. fallax*; Stenøien and Sæstad, 1999). They also found very low levels of LD (average P_d equal to 4.8%) and low differentiation between *S. troendelagicum* populations (average F_{ST} equal to 0.07). The high variability and low LD in an apparently asexual species led the authors to suggest that *S. troendelagicum* may have originated several times, possibly at each locality it is found presently. This latter would imply very recent speciation events, as current central Norwegian peatlands may have originated 5000–8000 years bp (Solem, 1986). However, a scenario involving multiple origins, perhaps one origin at each local site, should yield high genetic differentiation among populations if there is limited gene flow. A peat moss without sexual reproduction and spore production should have an extremely

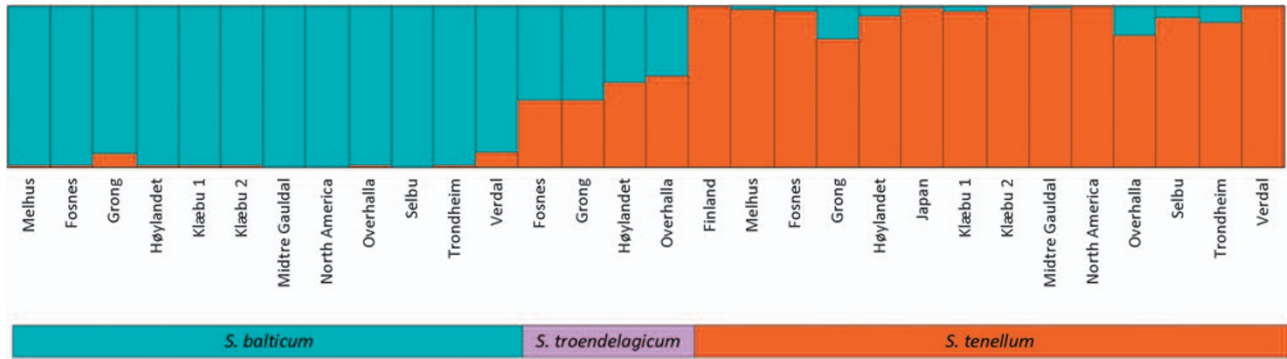


Figure 3 Results from clustering of individuals of the three species at the most probable value of K ($K=2$), as measured by the delta K statistics. *S. balticum* populations are mostly assigned to cluster 1 (91–99%), *S. tenellum* populations are mostly assigned to cluster 2 (90–100%, except Grong and Overhalla with 80 and 82%, respectively) and *S. troendelagicum* populations assigned approximately one-half to each of the clusters (at least 41% assigned to one of the clusters).

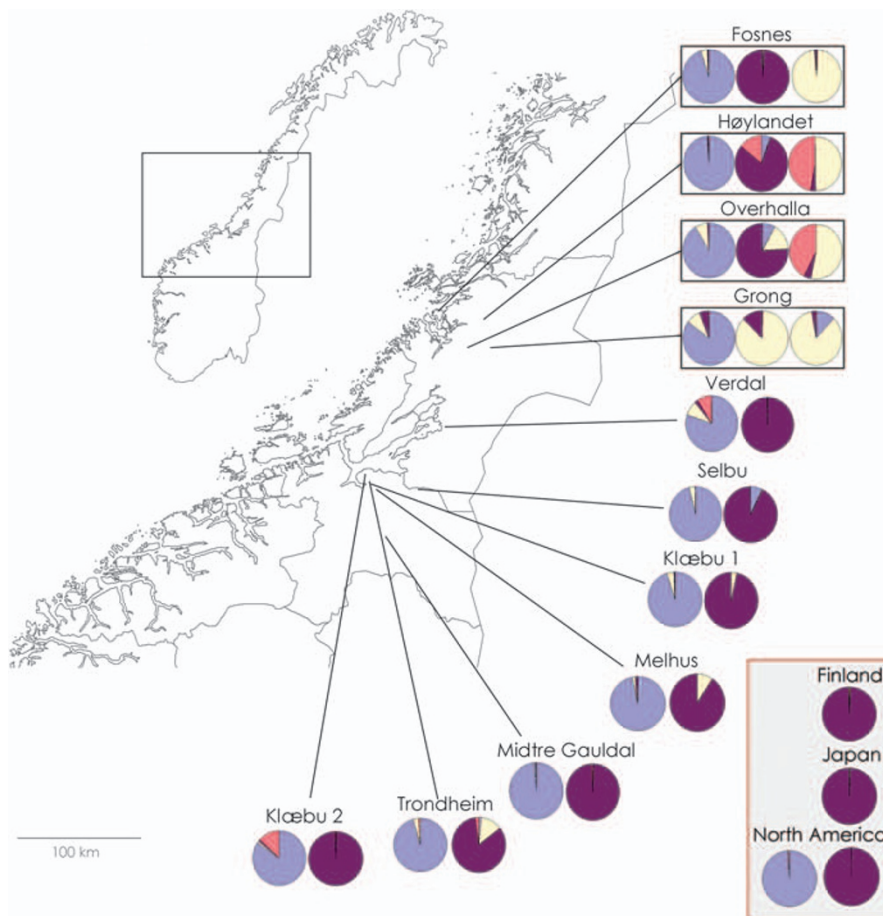


Figure 4 Results of *Structure* analyses run for the different *S. balticum*, *S. tenellum* and *S. troendelagicum* populations when $K=4$ clusters are considered. For each locality, *S. balticum* is presented first, followed by *S. tenellum* and third by *S. troendelagicum* at Fosnes, Høylandet, Overhalla and Grong localities. *S. troendelagicum* populations can be separated into two main groups (Fosnes-Grong versus Høylandet-Overhalla), and *S. troendelagicum* and *S. tenellum* are genetically similar at the Grong locality.

limited gene flow, even though limited dispersal by vegetative fragments could in theory be possible over short distances (Stenøien and Sæstad, 1999). The results reported by Stenøien and Flatberg (2000) could therefore perhaps equally likely fit a scenario of a single origin of *S. troendelagicum*, given sufficient time since origin for the accumulation of genetic variation by mutations, some

sexual reproduction and some gene flow between populations. However, distinguishing between these different scenarios becomes problematic since the reproducibility of the anonymous genetic markers (RAPDs) employed by Stenøien and Flatberg (2000) is uncertain.

In this study, we find higher levels of within-population variability than reported earlier. Average frequencies

Table 7 Results of ABC analyses for estimating historical parameters of *Sphagnum troendelagicum* based on the present data sets (see Figure 1a)

Parameter	Mean	Median	Mode	q ₀₂₅	q ₀₅₀	q ₉₅₀	q ₉₇₅
t_1	871	610	431	186	226	1813	2319
t_2	1132	890	709	279	339	2328	2866
t_3	11 438	11 358	11 294	11 235	11 247	11 703	11 858
t_4	41 844	41 539	41 444	41 399	41 411	42 590	43 683
t_A	315 678	268 906	240 461	192 571	201 359	506 836	687 804
N_t	137 582	111 494	89 562	49 624	55 875	306 561	425 540
N_A	64 525	61 528	60 948	42 971	45 684	82 919	92 569
N_0	52 715	54 443	88 544	2076	4609	95 848	97 959
x	472	457	248	23	44	936	971

Abbreviation: ABC, approximate Bayesian computation. Mean, median and modal values of most likely parameter values are presented, given scenario 1 above, as well as 2.5, 5, 95 and 97.5% quintiles of estimated values.

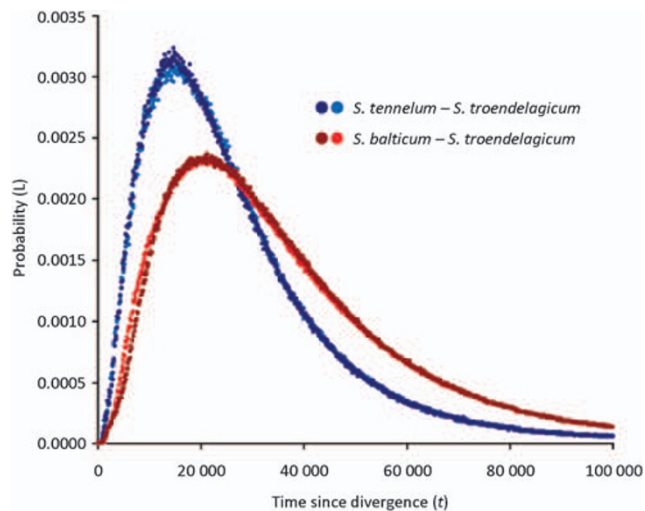
Table 8 Mean relative bias of the ABC analyses for estimated historical parameters of *Sphagnum troendelagicum* based on the present data sets (see Figure 1a and text for details)

Parameter	Mean	Median	Mode
t_1	5.50	3.85	2.08
t_2	0.86	0.50	0.31
t_3	1.41	1.19	1.00
t_4	0.66	0.37	0.22
t_A	1.44	1.04	0.74
N_A	0.06	0.03	0.02
N_t	1.78	1.49	1.03
N_0	58.48	63.83	94.48
X	0.10	0.15	0.79

Abbreviation: ABC, approximate Bayesian computation. Mean, median and modal values of most relative biases, defined as the average difference between estimates and true values based on simulation analyses, are presented.

of polymorphic loci are almost five times higher in microsatellites compared with RAPDs, and average gene diversity is approximately 10 times higher for the Fosnes and Høylandet *S. troendelagicum* populations, and >500 times higher for the Overhalla population compared with earlier reports (Stenøien and Flatberg, 2000). The levels of genetic variability within populations are comparable with the selfing model angiosperm *Arabidopsis thaliana* (for example, Stenøien et al., 2005). This means that *S. troendelagicum* harbours appreciable levels of genetic variability, even though the absolute levels are not very high compared with widespread outcrossing species, as pointed out also by Stenøien and Flatberg (2000). Interestingly, the level of microsatellite variability is much lower than found in *S. beringiense*, another peat moss not yet found with sporophytes (Shaw et al., 2008a). It seems that mating system is not a major determinant on levels of genetic variability in many bryophytes (see Stenøien and Sæstad, 2001). Similar cpDNA haplotypes are found in *S. troendelagicum* and *S. tenellum*, and as cpDNA is maternally inherited in *Sphagnum* (Natcheva and Cronberg, 2007), our results suggest that *S. tenellum* is the maternal species in the hybridization event or events leading to the present *S. troendelagicum* populations.

Boatman and Lark (1971) hypothesized that sexual reproduction may be of limited ecological importance in *Sphagnum*, as establishment from spores have never been

**Figure 5** Results of IM runs comparing divergence between *S. troendelagicum* and *S. balticum* (red lines) and *S. balticum* (blue lines), respectively. Results from two runs for each species are presented (represented with different colours). Time since divergence is measured in years before present, and the y-axis represents likelihood of the estimated distribution.

observed in the field, and as spores are hard to germinate in mire water. We are interested in judging whether patterns of genetic variability in *S. troendelagicum* are due to sexual recombination, multiple origins or both. Our results concerning LD and inferred recombination in *S. troendelagicum* must be interpreted with caution because polymorphisms are often represented as relatively few individuals with low-frequency genotypes, making it difficult to perform statistical tests of association. Our results do, however, support earlier claims that either recombination must have occurred in the history of *S. troendelagicum*, or the species must have been formed multiple times. This is evidenced by the observation that only about 66% of maximum LD is encountered in sampled microsatellite loci. Such levels are frequently found even in populations of the highly selfing model angiosperm *Arabidopsis thaliana* (Lundemo et al., 2009) and does not necessarily imply very recent or frequent recombination within populations.

We believe that the low levels of LD reflect recombination rather than multiple speciation events for two reasons. First, in mosses dispersal occurs most effectively via spores (Van Zanten, 1978; Longton, 1997; Sundberg, 2005), and even movement between local populations in central Norway is difficult to imagine without the aid of spores. Second, lack of LD at the species level can be explained by recurrent speciation, but one would then expect complete or almost complete LD within populations if migration is limited between populations due to lack of spore-mediated gene flow. This is not the case in the present populations, as P_A values are relatively low also within populations. We therefore conclude that sexual recombination has occurred repeatedly throughout history of *S. troendelagicum*. This does not preclude the possibility that this species may have originated multiple times, though.

This study reveals somewhat higher genetic structuring than that reported earlier, with average F_{ST} values being twice as high for microsatellites compared with

RAPD markers (F_{ST} being on average equal to 0.14 in this study compared with 0.07 in Stenøien and Flatberg (2000)). The differentiation between *S. troendelagicum* populations is approximately the same as the differentiation between *S. troendelagicum* and each of the parental species. *S. troendelagicum* populations are not more similar to local *S. balticum* populations than other populations of this species. However, there is a tendency for *S. troendelagicum* populations to be more similar to local *S. tenellum* populations than other populations of *S. tenellum* (Table 6), especially at the Grong locality (Figure 4). This could be so because *S. troendelagicum* at least at some sites has been formed by the local populations of the maternal species. Alternatively, one cannot rule out the possibility that backcrossing between *S. troendelagicum* and *S. tenellum* has taken place at these sites. Such backcrossing between an allopolyploid and one of its parents was documented by Flatberg *et al.* (2006a, b) for species in *Sphagnum* section *Acutifolia* and by Ricca (unpublished) for species in section *Subsecunda*.

Interestingly, cluster analysis reveals two main clusters in the total microsatellite data set, with the parental species largely belonging to one of the clusters each and *S. troendelagicum* individuals admixed. It is clear from Figure 3 that *S. troendelagicum* populations group into two subgroups, with Fosnes and Grong forming one subgroup and Høylandet and Overhalla forming the other. This is even clearer for the results of the cluster analysis resolving four main clusters (Figure 4). The parental species are clearly genetically differentiated at each of the localities where they co-occur and where *S. troendelagicum* is not found. On localities where *S. troendelagicum* is also found, the parental species are also genetically dissimilar, suggesting that hybridization is not regularly occurring at these sites either. Nevertheless, the two subgroups found in *S. troendelagicum* may suggest at least two independent speciation events. There are no indications of *S. troendelagicum* having immigrated to the Trøndelag region from other parts of the world (that is, North America or Asia) at some time in the past, as parental species genotypes from these localities are not genetically more similar to *S. troendelagicum* than those found within the Trøndelag region.

From the analyses presented above, it is possible to formulate various hypotheses concerning the evolutionary history of *S. troendelagicum*. First, it seems reasonable to assume that it is a fairly young species; this is supported by the relatively low genetic divergence between *S. troendelagicum* and the parental species. Furthermore, the lack of cpDNA nucleotide differences between *S. troendelagicum* and the maternal progenitor species, *S. tenellum*, supports a hypothesis of relatively recent speciation. It must be remembered, though, that for a sequence of 1000 years bp (approximate size of *trnG*) with a substitution rate of up to 3×10^{-9} per nucleotide site per year (Wolfe *et al.*, 1987), one would not expect an interspecific substitution to take place before 3 million years after divergence. In addition, the conservative approach taken when scoring microsatellite data will tend to underestimate genetic structuring and levels of genetic variability, and one cannot rule out the possibility that populations contain more variability than what we document based on our approach.

Second, it is clear that *S. troendelagicum* populations are grouped into two major clusters (Fosnes and Grong in one cluster, and Høylandet and Overhalla in the other cluster). This is not easily reconciled with a hypothesis of this species being formed multiple times at local sites by hybridization of *S. balticum* and *S. tenellum* populations located there. This latter scenario would perhaps imply stronger genetic structuring between *S. troendelagicum* populations found at the four localities, and it would also imply that one should find lower genetic differentiation between *S. troendelagicum* and the parental species at local sites compared with parental species' populations found elsewhere. As mentioned, measures of pairwise population differentiation do not indicate that local *S. balticum* individuals have been involved in the formation of local *S. troendelagicum* populations, even though our results do not strongly contradict this possibility. It cannot be ruled out that local *S. tenellum* has been involved in such multiple speciation events. In any case, the presence of two *S. troendelagicum* clusters raises the question as to whether the species has been formed by at least two independent hybridization events.

Here we tested the three scenarios presented in Figure 1 using the ABC approach (Beaumont *et al.*, 2002), and based on these analyses we can conclude that the present data set support a hypothesis of one single origin for *S. troendelagicum* (Figure 1a). The assumptions in this test are violated for polyploids (see below), and the results must therefore be interpreted with caution. Nevertheless, a single origin is not an implausible scenario since there is no strong genetic structuring observed between *S. troendelagicum* populations. Such structuring would have been expected if different populations had separate evolutionary histories. If different parental genotypes had contributed to different *S. troendelagicum* clades, then the presence of different alleles in various clades would yield F_{ST} 's of 1 between them. Nevertheless, if extensive gene flow has occurred between the populations throughout history, then any signal of independent origins may have been lost. We can therefore not entirely rule out the possibility that *S. troendelagicum* has originated several times. What we can conclude is that if we consider one speciation event as the null hypothesis, our data cannot reject this hypothesis.

The IM and ABC approaches yield different estimates for the time since origin of *S. troendelagicum*. This is as expected given the differences in assumptions in the two models, as well as the differences in data manipulations previous to the analyses. The stringent criteria concerning choice of homologous alleles to be included in the IM analyses most certainly pushes divergence time estimates down to the lower end of the scale, and IM results should therefore be viewed as minimum estimates of divergence. It is interesting to note that the estimated time since divergence between *S. troendelagicum* and the two parental taxa are quite similar, though. In the ABC analyses, one uses a coalescence approach based on the Wright–Fisher model in the simulation of gene trees to be compared with the real data set. In this model, alleles in each generation drawn from a gamete pool of infinite size and assembled into genotypes to form the next generation. The assumptions of the model are not fulfilled in an allodiploid species lacking free recombination, as alleles are paired in the gametes, that is,

a random draw of one allele causes the associated allele to also be drawn. This should cause estimated coalescence times to be inflated, and estimated parameters should therefore be viewed as maximum estimates of the historical events. The inflated coalescence times are clearly seen in the estimated effective population sizes in *S. troendelagicum* populations seen presently (c. 110 000 individuals, compared with the census sizes of a few thousand individuals observed in natural populations). Therefore, it is likely that *S. troendelagicum* originated somewhat earlier than the modal value of the posterior estimates suggests. As the maximum age based on IM analyses and the minimum age of the ABC analyses are somewhat similar, we suggest that the species originated probably before the start-up of the Late Weichselian glaciation in Scandinavia (26 000 years bp) and no later than 80 000 years ago.

Conclusion

The parental species *S. tenellum* has a suboceanic distribution, whereas *S. balticum* occupies more continental areas, but they do coexist at many peatland localities in boreal areas of the Northern Hemisphere, particularly in Scandinavia. Both species are dioicous, but are commonly found with antheridia and sporophytes, even when they are found growing close together in the field. It is therefore surprising that so few populations of *S. troendelagicum* exist. Sástad *et al.* (2001) speculated that the difference in timing of gamete production in *S. balticum* and *S. tenellum* throughout most of their geographic distribution ranges could prevent hybridization events to occur and thus explain the limited distribution of *S. troendelagicum*. For hybridization to take place, the parental individuals must grow close together (Cronberg, 1996), but it is also possible that the spermatozooids can travel over longer distances with snow melting or rainwater, especially in sloped terrain. In any case, more work is needed to understand the unique occurrence of *S. troendelagicum* in central Norway.

The present results suggest that *S. troendelagicum* originated before the start of the Late Weichselian glacial period c. 26 000 years bp, implying that it must have had a different distribution previously than it does today. It could have survived the Late Weichselian glacial period in more southern or south-western parts of Europe not covered by ice. In any case, *S. troendelagicum* must have immigrated to central Norway quite recently, and then probably experienced a reduction in distribution area for some unknown reason, but most likely not because of its short evolutionary age. We have little reason to assume that this species will originate again in the future in the area that it is found today. To the contrary, habitat fragmentation and human pressure may cause the species to go extinct in foreseeable future. On the basis of our results, we suggest that all populations of the species be prioritized in future conservation programmes.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Heredity website (<http://www.nature.com/hdy>)