Evolution of Mating Systems in *Sphagnum* Peatmosses

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Dissertation submitted in partial fulfillment
of the requirements for the degree of Doctor of Philosophy
in the Department of Biology in the Graduate School of Duke University

2013

ABSTRACT

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Abstract

Bryophytes, by their haploid dominant life cycle, possess several unique qualities ideal for study of mating patterns. In particular, the possibility of intragametophytic selfing in some species and the vegetative propagation of gametes allow for a unique window into the haploid stage that is intractable in other groups. Despite these advantages, there have been relatively few studies on mating patterns bryophytes in natural populations. *Sphagnum* (peatmoss) is an excellent case study in the interactions between sexual condition, ecology, and mating patterns. In the first Chapter, I use microsatellites to characterize the genetic diversity and mating patterns in fourteen species of *Sphagnum*, diverse in sexual condition (separate vs. combined sexes in the haploid stage) and ecology (microhabitat variance along the water table). I find that genetic diversity and mating patterns are related only in species with separate sexes, that sexual condition and ecology have interacting effects on inbreeding coefficients, and that inbreeding depression is not a common phenomenon in *Sphagnum*. In the second Chapter, I conduct an intensive survey of one population of *Sphagnum macrophyllum* to detect whether variance in haploid fecundity and mating success is related to diploid fitness. I find a relationship between mating success and fecundity (a signal of sexual selection), and fitness of the diploid generation is connected to the parentage of the haploid generation. Finally, in Chapter 3 I use phylogenetic comparative methods to track the phylogenetic signal in microhabitat preference in *Sphagnum*. I find extremely fast rates of evolution along the micronutrient gradient, but high phylogenetic signal along a hydrological gradient. Given that *Sphagnum* species living high above the water table have reduced water availability, phylogenetic signal in the hydrological gradient has macroevolutionary implications for mating systems in *Sphagnum*.

For Mom and Dad, who let me read them all the biology facts at breakfast.

Love, Matt J.

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Introduction

The Bryophyte Life Cycle

Bryophytes, as land plants, have an alternation of generations between a diploid, spore producing stage—the sporophyte—and a haploid gamete producing stage—the gametophyte (Figure 1). What separates the bryophytes from the vascular plants is that the gametophyte is free-living and persistent, the dominant life cycle stage. Haploid spores germinate into haploid protonema, which then grow via clonal (vegetative) propagation into large gametophytes. Populations of byrophytes are made up of one or more genets growing intermixed.

Sexual reproduction in bryophytes is accomplished through production of haploid gametes from haploid gametophytes, via mitosis. Depending on the species, gametophytes may be unisexual (produce only sperm or egg) or bisexual (produce both sperm and egg), though most bryophyte species have unisexual gametophytes (Anderson & Wyatt 1984). Sperm are generated in antheridia (male sexual structures) and released into the water. They must swim to the egg, which is retained on the maternal gametophyte in archegonia (female sexual structure), for fertilization. Sperm transport may in some cases be facilitated by microscopic arthropods (Cronberg *et al.* 2006), but otherwise fertilization success depends on sperm longevity, limited to no more than a few days (Rosenstiel & Eppely 2009; Shortlidge *et al.* 2012).

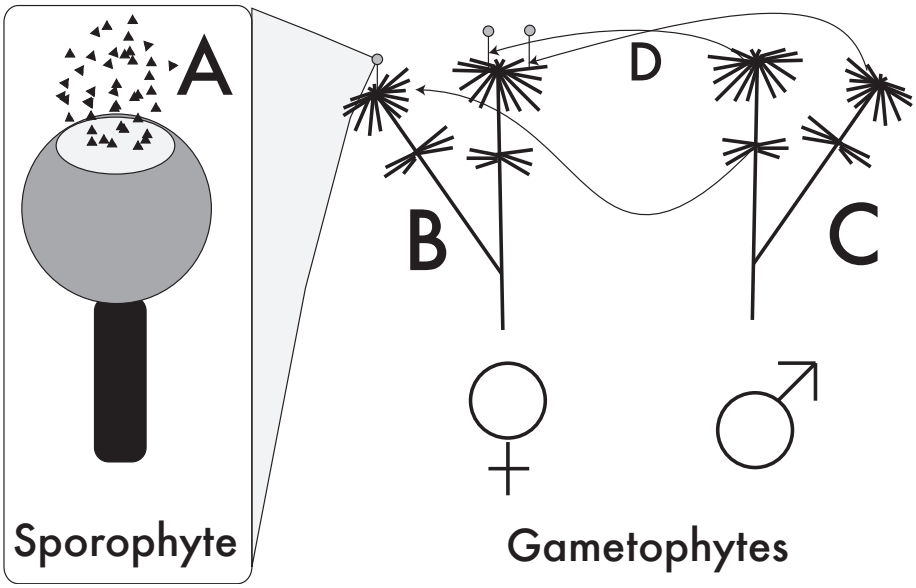

Figure 1 Alternation of generations in the haploid-dominant life cycle of *Sphagnum*. Haploid stages are in black, diploid stages in gray. *Left:* Haploid spores (A) are formed by meiosis within the spherical sporophyte. After dispersal they germinate into haploid gametophytes. *Right*: Gametophytes grow into large clones via vegetative propagation. In a unisexual species, such as *Sphagnum macrophyllum*, gametophytes are either female (egg producing, B) or male (sperm producing, C). Male gametophytes produce sperm via mitiosis, which swims (D) to the eggs, produced by female gametophytes, also via mitosis. In this diagram, of a single male genet and a single male genet, all three sporophytes shown will be genetically identical diploids. Sporophytes remain attached to the maternal gametophyte for the duration of their lives.

Diploid sporophytes are retained, attached to the maternal gametophyte, for the duration of their lives. Although they possess photosynthetic tissue, newly formed zygotes are typically supplied nutrients by the maternal gametophyte in the early stages (Ligrone & Gambardella 1988; Rushing & Anderson 1996). As sporophytes mature, they transition to the production of haploid spores via meiosis. Bryophytes are homosporous, and can produce several hundred thousand spores per sporophyte capsule (Longton & Miles 1982; Sundberg & Rydin 1998). These are released from the sporophyte into the air, usually by explosive force (Duckett *et al.* 2009; Sundberg 2010). These spores will germinate into new haploid gametophytes, completing the life cycle.

Implications for the study of bryophyte mating patterns

Intragametophytic Selfing

Several properties of the bryophyte life cycle create unique windows into bryophyte mating patterns that would be difficult or impossible to study in seed plants or animals. First, the mass production of haploid gametes by haploid gametophytes means that all gametes produced by the same gametophyte are genetically identical. The most obvious consequence of this is in bryophytes with bisexual gametophytes. Sperm and egg, each produced by mitosis from the same haploid gametophyte, are genetically identical. Bryophytes are therefore capable of true "self-fertilization," or intra-gametophytic selfing, which is distinct from the inter-gametophytic selfing possible in seed plants and hermaphroditic animals. Zygotes produced in this manner are completely homozygous at every locus.

The possibility of intragametophytic selfing has important consequences for the evolution of mating systems in bryophytes. Inbreeding depression, the decreased fitness of homozygous individuals, has traditionally been thought to be a powerful driving force in the evolution of inbreeding avoidance mechanisms (Charlesworth & Charlesworth 1999). The mechanism for inbreeding depression is most commonly thought to be the accumulation of deleterious recessive alleles (Barrett & Charlesworth 1991; Charlesworth & Charlesworth 1999). By contrast, hermaphroditic populations with mixed-mating approaches can purge deleterious alleles from the population, resulting in a reduced effect of inbreeding on fitness (Lande *et al.* 1994; Goodwillie *et al.* 2005).

For organisms with dominant free-living haploid stages, as with bryophytes, the consequences of inbreeding are less clear. Masking of deleterious recessive alleles can

only occur in the diploid sporophyte generation, and does not affect genes expressed exclusively in the haploid generation (Charlesworth & Charlesworth 1992). The overlap in gene expression between moss sporophytes and gametophytes is far more extensive compared to *Arabidopsis* (Szovenyi *et al.* 2011), and this could prevent the accumulation of deleterious alleles (Joseph & Kirkpatrick 2004).

Characterization of the mating patterns in populations can give key insight into the influence of inbreeding on mating system evolution. To date, only a handful of studies have investigated the extent of inbreeding in bryophyte sporophytes in nature (Eppely *et al.* 2006; Hutsemékers *et al.* 2013), or examined the extent of inbreeding depression (Taylor *et al.* 2007; Jesson *et al.* 2012), and only one has attempted to do both (Szovenyi *et al.* 2009). A survey of genetic variation, inbreeding, multiple paternity, and inbreeding depression in fifteen species of the peatmoss genus *Sphagnum* will be presented in Chapter 1.

Quantitative Fecundity of Gametophytes

In seed plants, every gametophyte (ovule or pollen) either participates in a fertilization event, or it does not. Therefore, gametophyte fecundity— the number of sporophytes produced by each gametophyte—is binary. In bryophytes, each gametophyte may produce many thousands of genetically identical gametes. Though each gamete may either participate in a fertilization event or not, the distribution of fecundity for the gametophyte is quantitative and continuous. Some gametophytes may have zero fecundity while others participate in dozens of fertilization events in a breeding season.

Bryophytes introduce another interesting complexity to their mating systems because of the vegetative propagation of the dominant gametophyte stage. Imagine a bryophyte population that begins with the germination of one male spore and one female spore. The haploid gametophytes of each grow and spread through the population, and the male and female gametophytes become intermingled. During the sexual stage, the male gametophyte releases sperm, which swim to the eggs produced by the female gametophyte. Because of the bryophyte life cycle, all of the zygotes in the population will have the exact same genotype. The implications of this for gametophyte fecundity and sporophyte fitness in bryophytes will be explored in Chapter 2.

***Sphagnum*: a model system for ecology and mating systems**

In the Northern Hemisphere, the boreal zone is dominated by peatlands, covering an estimated 5.8 million km^2, or roughly 3% of the Earth's total land mass (Måkilå & Saarnisto 2008). Rydin and Jeglum (2006) "boldy" assert that half of the biomass in these peatlands is *Sphagnum*, and estimate the dry mass of this peatmoss to be 300 Gt. Similar calculations led Clymo and Hayward (2009) to conclude "there may be more carbon in *Sphagnum* than any other genus of plants, vascular or non-vascular" (p 234). This tremendous biomass is split among 200-300 *Sphagnum* species (McQueen & Andrus 2009) and is divided into four major phylogentically distinct subgenera (Shaw, Cox, *et al*. 2010a). A single peatland may contain up to 25 *Sphagnum* intermingled species (Rydin & Jeglum 2006), sometimes outnumbering the species diversity of trees and shrubs.

Within the genus, there is considerable variation in ecology and sexual expression that makes *Sphagnum* a compelling case study on the variation of mating patterns in bryophytes. Of the 95 species recognized in North America, 16 are described as having bisexual gametophytes (McQueen & Andrus 2009), although there are disagreements in the literature on the nature of individual species (Crum 1984). Clearly, one may expect to observe greater outcrossing in the unisexual species, but the connection between mating patterns and sexual condition has never been tested within a group of closely related bryophyte species.

It has been well known that *Sphagnum* species tend to prefer a narrow range of microhabitats within peatlands (Clymo 1973). Species tend to occupy very narrow niches along two main gradients: a mineotrophic gradient of pH and other ionic nutrients; and a hydrological gradient, representing the height above the water table (Vitt & Slack 1984; Andrus 1986; Gignac 1992). The latter of these has potential consequences for mating patterns in *Sphagnum*. Because sperm must swim to effect fertilization, species preferring to live in hummocks high above the water table may have less access to dispersal opportunities. Additionally, if microhabitat preference is phylogenetically conserved, it might affect the evolution of mating systems for entire lineages of *Sphagnum*. The presence of phylogenetic signal in *Sphagnum* microhabitat preference is tested in Chapter 3.

Summary of Chapters

The primary goals of **Chapter 1** are to characterize the variation in mating patterns in fifteen species of *Sphagnum*, to investigate the connections between mating

patterns and species biology or ecology, and to test theoretical predictions about the presence of inbreeding depression in bryophytes.

In **Chapter 2**, I expand upon the work of the previous chapter with an intensive study of one natural population of *Sphagnum macrophyllum*, an aquatic species with unisexual gametophytes. The goals of the study are to 1) document the reproductive phenology, sex ratio, and clonal structure of the population; 2) use this detailed knowledge about population structure to show a connection between gametophyte fecundity and reproductive success, a hallmark of sexual selection; and 3) demonstrate a connection between gametophyte parental genotypes on sporophytic fitness.

Chapter 3 steps back from the fine scale of breeding seasons to investigate the macroevolutionary patterns in *Sphagnum* ecology. I combine an extensive record of *Sphagnum* microhabitat data with an 18-gene phylogeny of the genus to test for the presence of phylogenetic signal in *Sphagnum* microhabitat preferences. Using a variety of phylogenetic comparative methods, I test whether the evolution of *Sphagnum* niches conform to predicted models for quantitative trait evolution (such as Brownian motion), and whether the tempo of evolution has dramatically changed within the genus.

Chapter 1: Mating patterns in *Sphagnum* peatmosses are determined by genetic diversity, sexual condition, and microhabitat preference.

Introduction

Land plants have shown few themes as ubiquitous as the evolution of characters that reduce the probability of inbreeding. In angiosperms, self-incompatibility systems and dioecy both reduce the possibility of mating between gametes produced by the same sporophyte. The most common explanation for this extreme selection to avoid self-fertilization is, in outcrossing populations, slightly deleterious recessive alleles accumulate (Nei *et al.* 1975; Charlesworth & Charlesworth 1999). Inbreeding "exposes" these alleles in homozygotes that carry reduced fitness (inbreeding depression). However, in populations where inbreeding is common, deleterious alleles may be purged from the population, and subsequent inbreeding has a reduced fitness cost (Lande & Schemske 1985; Goodwillie *et al.* 2005). General surveys confirm that both outcrossing and selfing occur and are stable states in angiosperms (Barrett 2003).

The genetic consequences of inbreeding are potentially amplified in organisms with haploid-dominant life cycles, such as bryophytes. There is a wide diversity of sexual conditions in bryophytes, but most bryophytes have unisexual gametophytes (Anderson & Wyatt 1984). However, in some species, the gametophyte is bisexual, and produces both sperm and egg by mitosis. Should this gametophyte self-fertilize, it produces completely homozygous diploid sporophytes in a single round of mating. This "intragametophytic" mating cannot occur in heterosporous plants, all of which produce only one type of gamete per gametophyte. If this type of mating carried a corresponding

reduction in sporophyte fitness, it would create the same selection pressures for inbreeding avoidance mechanisms, as observed in angiosperms.

To date, only one study has investigated the prevalence of intragametophytic selfing in natural populations of bryophytes, finding a significantly higher inbreeding coefficient in mosses with bisexual gametophytes (Eppely *et al.* 2006). Although F_{IS} has frequently been interpreted as the "probability of identity by descent," this definition may be inappropriate for bryophytes. The clonal propagation of gametophytes in bryophyte populations can result in a skew in the number of gametes produced by each genotype (Szovenyi *et al.* 2009). This, in turn, can produce an excess of heterozygosity observed in the sporophyte, relative to expected heterozygosity. Indeed, inbreeding coefficients observed in natural populations of unisexual bryophytes often results in negative values (Eppely *et al.* 2006; Szovenyi *et al.* 2009). For bryophytes, it is useful to remember Wright's original definition of inbreeding coefficient as the "correlation between uniting gametes" (Wright 1922; 1965).

There is an even greater paucity of data regarding inbreeding depression in bryophytes: Taylor et al. (Taylor *et al.* 2007) found sporophytic inbreeding depression in a species with unisexual gametophytes, but not in a species with bisexual gametophytes. Jesson et al. (2012) did not observe reduced sporophyte fitness in self-fertilized sporophytes of *Atrichum undulatum*, a species with a mixed sexual condition. The only study of inbreeding depression in a natural population found a significant association between sporophyte size (fitness) and heterozygosity in *Sphagnum lescurii*, a unisexual species (Szovenyi *et al.* 2009). To our knowledge, there has been no demonstration of inbreeding depression in a natural population of a bryophyte with bisexual gametophytes.

Sphagnum (peatmosses) presents an excellent case study to investigate the role of sexual condition on mating systems in a group of closely related species. Of the 91 species that occur in North America, 14 are known to have bisexual gametophytes, 58 have unisexual gametophytes, and the rest have unknown sexual conditions (McQueen & Andrus 2009). To some extent, the diversity of sexual conditions is limited phylogenetically. The genus *Sphagnum* is separated in to six well-defined subgenera: *Acutifolia*, *Cuspidata*, *Rigida*, *Sphagnum*, *Subsecunda* and *Squarrosa*. There are no bisexual species within the subgenus *Sphagnum* or subgenus *Subsecunda,* but both species with section *Squarrosa* are bisexual. Sexual condition is variable in the other sections.

Study of mating patterns in *Sphagnum* is facilitated by a set of microsatellite markers that amplify in nearly every species in the genus (Shaw, Pokorny, *et al.* 2008c; Shaw *et al.* 2009; Karlin, Gardner, *et al.* 2010a; Karlin *et al.* 2011). As with all bryophytes, the sporophyte generation is short lived and remains attached to the maternal gametophyte throughout its life. It is therefore simple to determine the maternal haploid component of the diploid genotype, to infer the paternal haploid genotype by subtraction, and to characterize the levels of multiple paternity (Szovenyi *et al.* 2009).

Microhabitat preference is also likely to play a role in mating patterns in *Sphagnum*. Most species prefer to grow within narrow ranges along two ecological gradients—a mineotrophic gradient (pH and other micronutrients) and a hydrological gradient (height above the water table) (Vitt & Slack 1984; Andrus 1986; Gignac 1992). The hydrological gradient is of specific interest, because *Sphagnum* species that generate hummocks high above the water table are likely to experience limited water availability for dispersal of sperm. Additionally, species microhabitat preferences along the hydrological gradient are phylogenetically conserved in *Sphagnum* (Chapter 3), so any

connections between mating patterns and microhabitat preference are also likely to have macroevolutionary consequences.

In this study we investigate 18 populations across 14 species of *Sphagnum* with three main questions: 1) What is the diversity of mating patterns in *Sphagnum*? We test this using standard statistics to measure genetic diversity, inbreeding, and multiple paternity. 2) Is inbreeding depression common in unisexual species and absent in bisexual species, as predicted by population genetics theory? 3) Is there any connection between mating patterns and sexual condition or microhabitat preference?

Materials and Methods

Species and Population Sampling

In general, our sampling of "fruiting" populations of *Sphagnum* was subject to several factors: species are highly variable in sporophyte production (some produce sporophytes every year, others are more rare), only produce sporophytes at a certain time of year, and gametophytes sometimes difficult to distinguish from closely related species in the field. As a result, our sampling reflects our chance encounters with fruiting populations in the field; in some species, multiple populations could be collected, while in others there is only one population.

When a population with sporophytes was identified, our sampling strategy depended on the nature of the population. In populations where the sporophytes were spread along a large area, we sampled gametophytes and sporophytes along a transect. In other cases, when the populations consisted only of one or a few patches, we randomly sampled from within each patch. Populations are primarily from sites in Maine, Alaska, British Columbia, and the southeast United States. Detailed information

about each population, including DNA voucher and collection information, can be found in Appendix I. All specimens were deposited in the Duke University Herbarium, Durham, NC USA.

We attempted to sample species from each of the major subgenera, while also sampling both bisexual species and unisexual species. From subgenus *Acutifolia*, we sampled: *S. angustifolium* (1 population), *S. fallax* (2 populations), *S. molle* (1), and *S. warnstorfii* (1). From subgenus *Cuspidata* we sampled: *S. cuspidatum* (2), *S. tenellum* (1), and *S. pulchrum* (1). From subgenus *Rigida* we sampled: *S. compactum* (2) and *S. strictum* (2). From subgenus *Sphagnum* we sampled: *S. austinii* (1) and *S. magellanicum* (1). We sampled *S. squarrosum* from subgenus *Squarrosa.* Finally, we sampled *S. cribrosum* (1) and *S. macrophyllum* (2) from subgenus *Subsecunda*. Of these fourteen species, five (*S. molle*, *S. compactum*, *S. tenellum*, *S. strictum* and *S. squarrosum*) have been observed with bisexual gametophytes (Crum 1984; McQueen & Andrus 2009). Although allopolyploidy is a common feature in *Sphagnum*, all fourteen species sampled have haploid gametophytes.

Sphagnum species have narrow microhabitat preferences along two primary gradients: a mineral gradient (pH and other micronutrients) and a hydrological gradient (height above the water table). Since we hypothesize that the latter may affect sperm dispersal and therefore mating patterns, we assigned each species to a broad microhabitat group based on data collected from *Sphagnum* microhabitat surveys (Chapter 3). From this data we designated five "hummock" species (Height above water table > 10.0 cm): *S. angustifolium*, *S. austinii*, *S. fallax*, *S. magellanicum*, *S. squarrosum* and four "hollow" species: *S. cuspidatum*, *S. pulchrum*, *S. tenellum*, and *S. compactum*. The remaining species did not have data in the ecological surveys; however, *S. macrophyllum*

and *S. cribrosum* are both aquatic species (Anderson *et al.* 2009), and are classified here as "hollow," while *S. molle* and *S. strictum* both form cushions, and are classified here as "hummock."

Genotyping

For DNA extractions of gametophyte tissue, a portion of the gametophyte's capitulum (the dense cluster of branches at the apex of *Sphagnum* plants) was removed. In addition, all sporophytes attached to the female gametophyte were sampled, and the entire capsule was used. Extraction followed a CTAB protocol (Shaw, Cox, & Boles 2003b). In preparation for PCR amplification, we diluted the DNA of gametophytes 7:1, and diluted sporophytes 2:1.

All plants were genotyped at nineteen microsatellite loci, via previously described protocols (Shaw, Cao, *et al.* 2008b). Primers were multiplexed in five sets: Set 1: p17, p22, p65, p78; Set 2: p1, p7, p12, p68; Set 3: p4, p10, p30; Set 4: p18, p19, p29, p93; Set 5: p9, p14, p20, p56. These microsatellite loci have been shown to be variable in a multitude of *Sphagnum* species from every subgenus. Not all loci were successful in each species, but using this standard set of loci, we ensured that at least 10-12 loci consistently amplified in each species.

Mating Patterns: Statistics

To characterize mating patterns in *Sphagnum*, we focused on three main properties of the sporophyte generation in each species: genetic diversity, inbreeding, and multiple paternity. Sporophytes attached to the same maternal gametophyte necessarily share half of their multilocus genotype, and therefore are no longer

independent draws from the mating events in the population. Particularly fecund maternal gametophytes (those with many sporophytes) will introduce a bias when estimating allele frequency.

To address this bias, we randomly sampled one sporophyte from each of the maternal shoots to generate 1000 subset populations. We used these data sets to calculate to measures of sporophyte genetic diversity at each locus: The effective number of alleles (also referred to as Simpson's Index), $1/\ \Sigma(p_i^2)$, where p_i is the frequency of the *i*-th allele at the locus. We also calculated Shannon's Diversity Index, $1/\Sigma(p - \ln(p))$, and the inbreeding coefficient F_{IS} ($1 - H_o/H_e$, where H_o is the observed heterozygosity and H_e is the expected heterozygosity in the subsampled population).

We estimated the extent of sporophytic inbreeding depression via the linear regression of heterozygosity on fitness (sporophyte size). Multiple paternity occurs when there is more than one paternal genotype present in the "brood" of sporophytes raised by one maternal gametophyte. To assess multiple paternity, we first inferred the haploid paternal genotype from every sporophyte, by subtracting the haploid genotype of the maternal gametophyte. Inferred paternal genotypes were grouped into "paternal genets" via a custom Python script. We quantified multiple paternity with three measures: K, the average number of paternal genotypes per brood, K_e, the effective number of paternal genotypes per brood, and K_e/K, a measure of paternity skew (Szovenyi *et al.* 2009). Paternity skew has a theoretical maximum of 1, which occurs if all paternal genotypes are found in equal proportions in the population. Confidence intervals for each species were calculated using 1000 bootstrap replicates. All statistical calculations were conducted in R version 2.15 (Team 2012).

Results

Bisexual species

Seven populations of five bisexual *Sphagnum* species show high levels of intragametophytic selfing and low genetic diversity (Table 1). Inbreeding was lowest (F_{IS} = 0.41 95% CI 0.27-0.62) in *S. tenellum*, in which 46% of sporophytes were completely homozygous despite moderate genetic diversity (I = 0.63, 95% CI 0.55-0.69). Intragametophytic selfing was highest in one population of *S. strictum* from North Carolina; 100% of sporophytes were homozygous at every locus, although there was also zero genetic diversity (I = 0) in this population. The other population of *S. strictum* we sampled, from South Carolina, had a low, but non-zero genetic diversity (I = 0.19, 95% CI 0.12-0.30), and also had several sporophytes with heterozygosity at exactly one locus. We also observed high intragametophyte selfing rates in *S. molle*, despite a comparatively larger amount of genetic diversity. Although the population of *S. squarrosum* has the highest genetic diversity in populations of bisexual species (I = 0.81, 95% CI 0.75-0.88), it also has a very high inbreeding coefficient (F_{IS} = 0.71, 95% CI 0.65 – 0.88).

In most bisexual populations, homozygous sporophytes have a large range in fitness that is equivalent to the ranges observed for heterozygous sporophytes.

The linear regression of heterozygosity on sporophyte fitness was significant for only two populations, both of *S. compactum* (Figure 2). In one population (AK-PH), 23 of 39 (59%) of the sporophytes are completely homozygous. These sporophytes are, on average, larger than the heterozygous sporophytes in the population (b = -25.1, r^2=0.12, $p < 0.05$), suggesting outbreeding depression.

By contrast, the other population of *S. compactum* (AK-PC) shows a strong *positive* association between heterozygosity and fitness (inbreeding depression, Figure 2); the 14 of 29 homozygous capsules are significantly smaller than the heterozygous sporophytes ($b = 74.1$, $r^2 = 0.43$, $p < 0.001$). In this population, the 15 heterozygous sporophytes are sired by 15 unique inferred paternal genotypes. Six unique maternal genotypes are present, and five of these raised sporophytes that were self-fertilized.

Multiple paternity was highest in the AK-PC population of *S. compactum* (2.10 paternal genotypes per brood, 95% CI 1.80-2.40) and lowest in the SC36 population of *S. strictum* (1.13, 95% CI 1.00-1.38), with similar trends in the effective number of fathers. The amount of skew in paternity, as measured by K_e/K, did not deviate from the theoretical maximum (1.0, no skew) in most populations; the two exceptions are *S. molle* ($K_e/K = 0.77$, 95% CI 0.69-0.86) and the SC36 population of *S. strictum* ($K_e/K = 0.80$, 95% CI 0.78-0.82).

Table 1. Genetic diversity, mating patterns, and multiple paternity in bisexual *Sphagnum* species. Significant relationships between heterozygosity and size indicate inbreeding depression (IN, positive correlation) or outbreeding depression (OUT, negative correlation). Multiple paternity statistics calculated at the "brood" level—sporophytes attached to the same maternal gametophyte. Key to subgenera: Rig = *Rigida*, Acut = *Acutifolia*, Cusp = *Cuspidata*, Squa = *Squarrosa*. Key to microhabitat preference: Hol = "Hollow," Hum = "Hummock."

Species	*S. compactum*	*S. compactum*	*S. molle*	*S. tenellum*	*S. squarrosum*	*S. strictum*	*S. strictum*
Subgenus	Rig	Rig	Acut	Cusp	Squ	Rig	Rig
Population	AK-PH	AK-PC	SC-36	ME-W	AK-W	SC-36	NC-LL
Ecology	Hol	Hol	Hum	Hol	Hum	Hum	Hum
Female Gametophytes	16	10	10	15	23	8	6
Sporophytes	39	29	32	37	58	36	48
Maternal Genotypes	5	6	4	10	11	2	1
Inferred Paternal Genotypes	8	17	7	23	15	4	1
Homozygous Sporophytes	23	14	28	23	42	32	48

Effective Alleles (Ne)	1.54	1.9	1.52	1.78	2.22	1.23	1
Shannon's Diversity (I)	0.51	0.59	0.34	0.63	0.81	0.19	0
Inbreeding Coefficient (F_{IS})	0.35	0.42	0.86	0.41	0.71	0.6	N/A
Heterozygosity vs Size	**OUT**	**IN**	NO	NO	NO	NO	N/A
Number of Fathers/Brood (K)	1.31	2.1	1.3	1.87	1.3	1.13	1
Effective Fathers (Ke)	1.26	1.97	1.2	1.7	1.24	1.08	1
Paternity Skew (Ke/K)	0.9	0.93	0.77	0.92	0.89	0.8	1

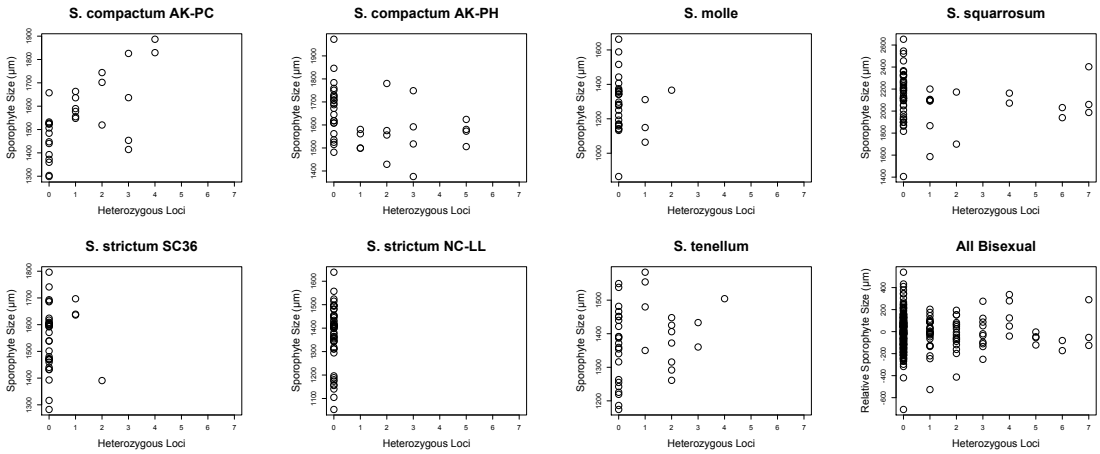

Figure 2. Relationship between heterozygosity and sporophyte size in seven populations of bisexual bryophytes. A significant positive correlation indicates inbreeding depression, while a significant negative correlation indicates outbreeding depression. In the bottom right, sporophyte sizes have been standardized within populations (mean of zero), in order to compare all bisexual populations.

Unisexual species

We observe a much greater variance in genetic diversity and mating patterns among *Sphagnum* species with separate sexes (

Table 2). Five of the eleven populations have genetic diversity estimates (N_e and I) greater than any of the bisexual populations. One population, of *S. austinii*, shows no genetic variation at any locus in the gametophytes, and all sporophytes are completely

homozygous. Other than this population, genetic diversity is lowest in *S. pulchrum* (I = 0.49, 95% CI 0.43-0.56) and highest in the GA28 population of *S. cuspidatum* (I = 1.29, 95% CI 1.23-1.36).

The populations vary from mixed mating in the GA28 *S. cuspidatum* population (F_{IS} = 0.56, 95% CI 0.50-0.63) to *S. magellanicum* (F_{IS} = -0.98, 95% CI -0.98 to -1.00) in which nearly all sporophytes were heterozygous at all variable loci (Figure 3). With the exception of the GA28 *S. cuspidatum* population, the SC36 *S. macrophyllum* population, and *S. cribrosum*, all other populations have significantly negative inbreeding coefficients. Excepting *S. austinii*, the only unisexual population with any homozygous sporophytes is the GA28 population of *S. cuspidatum*; three sporophytes, all attached to the same maternal ramet, had zero heterozygosity.

There is a strong positive, significant relationship between genetic diversity (I) and inbreeding coefficient in unisexual populations (Figure 4; $r^2 = 0.44$, $p < 0.05$). The relationship is even stronger when considering the effective number of alleles ($r^2 = 0.53$, $p < 0.01$).

Inbreeding depression is absent in all but two unisexual populations, both of *S. macrophyllum*—the SC36 population has a stronger association between heterozygosity and fitness (b = 30.5, r^2 – 0.15, $p < 0.01$) than the SC39 population (b = 13.5, $r^2 = 0.02$, $p < 0.05$). A highly significant *negative* relationship (outbreeding depression) between heterozygosity and size is found in *S. cribrosum* (b = -64.7, $r^2 = 0.31$, $p < 0.0001$).

In two populations, genetic diversity in the inferred paternal genotypes is low or absent. For *S. magellanicum*, only two unique paternal genotypes are inferred; one of these is found in only two sporophytes, and differs from the other genotype at only one

locus. In *S. austinii*, all sporophytes are genetically identical and homozygous, so one paternal genotype (equal to the maternal genotype) is inferred.

High levels of multiple paternity are found in each of the other populations, ranging from the ME-S population of *S. fallax* (K = 1.86, 95% CI 1.14-2.43) to the SC39 population of *S. macrophyllum* (K = 4.39, 95% CI 3.74-5.21). Notably, the other population of *S. fallax* had higher levels of multiple paternity (K = 3.40, 95% CI 2.70-4.10).

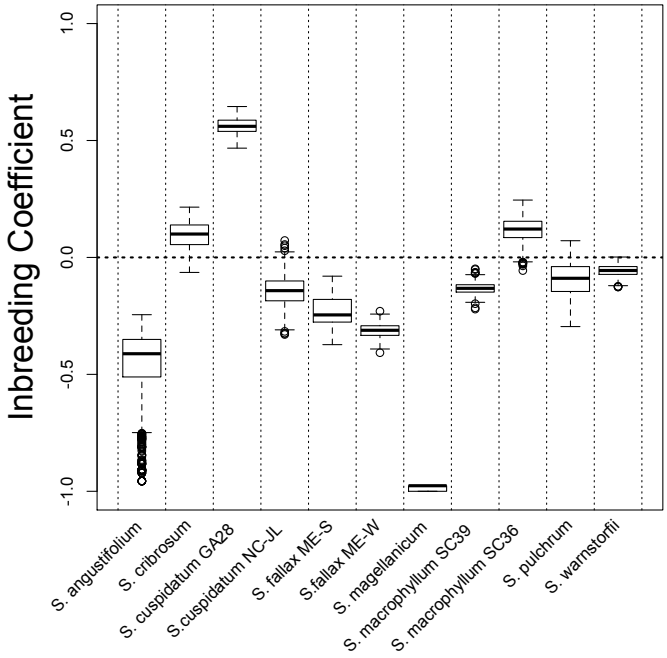

Figure 3. Variation in inbreeding coefficients (F_{IS}) among unisexual *Sphagnum* populations.Inbreeding coefficients are calculated by randomly sampling one sporophyte from each maternal gametophyte. The boxplot shows means and standard variations for 1000 subsamples of each population.

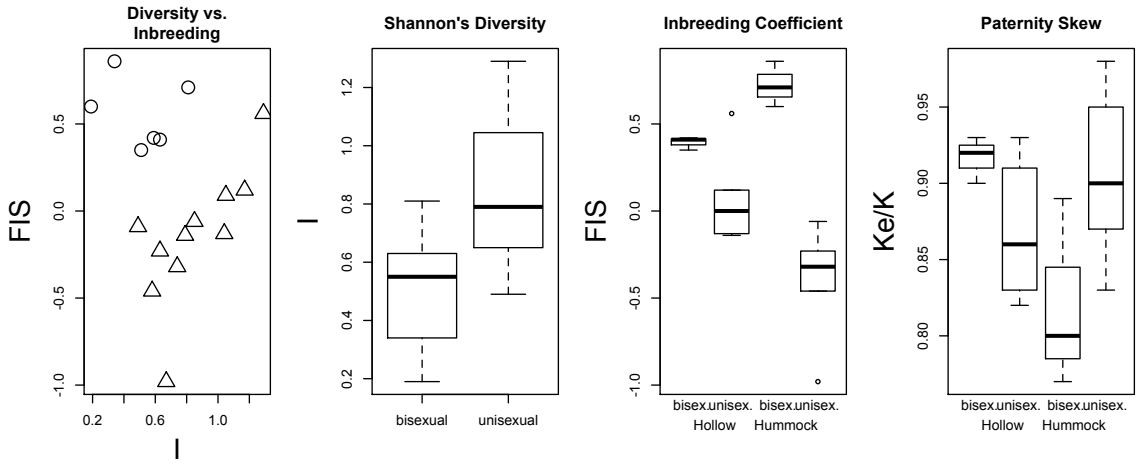

Figure 4. Effect of sexual condition and microhabitat preferences on mating pattern and genetic diversity statistics.Far left—relationship between genetic diversity (I) and inbreeding coefficient (F_{IS}) for bisexual (circles) and unisexual (triangles) populations. Left center- Effect of sexual condition on genetic diversity. Right center- Interaction effect between sexual condition (unisexual vs bisexual) and ecology (hummock vs hollow) on F_{IS}. Far right—interaction effect between sexual condition and ecology on paternity skew.

Table 2. Genetic diversity, mating patterns, and multiple paternity in unisexual *Sphagnum* species. Significant relationships between heterozygosity and size indicate inbreeding depression (IN, positive correlation), outbreeding depression (OUT, negative correlation) or no significant correlation. Multiple paternity statistics calculated at the "brood" level—sporophytes attached to the same maternal gametophyte. Key to subgenera: Acut = *Acutifolia*, Cusp = *Cuspidata*, Sph = *Sphagnum*, Subs = *Subsecunda*. Hol = "Hollow," Hum = "Hummock."

Species	*S. angustifolium*	*S. austinii*	*S. cribrosum*	*S. cuspidatum*	*S. cuspidatum*	*S. fallax*	*S. fallax*
Subgenus	Acut	Sph	Subs	Cusp	Cusp	Acut	Acut
Population	ME-S	VI	GA-32	GA-28	NC-JL	ME-S	ME-W
Ecology	Hum	Hum	Hol	Hol	Hol	Hum	Hum
Female Gametophytes	7	24	9	18	8	7	10
Sporophytes	36	48	46	68	39	31	36
Maternal Genotypes	2	1	8	17	2	3	1
Paternal Genotypes	14	1	25	43	12	8	27
Homozygous Sporophytes	0	48	0	3	0	0	0
Effective Alleles (Ne)	1.78	1	2.73	3.78	2.13	1.96	1.90
Shannon's Diversity (I)	0.58	0	1.08	1.29	0.79	0.63	0.74

Inbreeding Coefficient (F_{IS})	-0.46	N/A	0.09	0.56	-0.14	-0.23	-0.32
Heterozygosity vs Size	NO	N/A	**OUT**	NO	NO	NO	NO
Fathers/Brood (K)	3.29	1	2.51	2.56	2.88	1.86	3.40
Effective Fathers (Ke)	2.77	1	2.18	2.39	2.50	1.65	3.31
Paternity Skew (Ke/K)	0.83	1	0.82	0.91	0.86	0.87	0.98

Species	*S. macrophyllum*	*S. macrophyllum*	*S. magellanicum*	*S. pulchrum*	*S. warnstorfii*
Subgenus	Subs	Subs	Sph	Cusp	Acut
Population	SC-36	SC-39	NC-JL	ME-C	AK-M
Ecology	Hol	Hol	Hum	Hol	Hum
Female Gametophytes	12	41	6	8	18
Sporophytes	46	275	32	35	43
Maternal Genotypes	10	11	1	7	8
Paternal Genotypes	91	33	2	13	22
Homozygous Sporophytes	0	0	0	0	0
Effective Alleles (Ne)	3.18	2.65	2.01	1.77	2.35
Shannon's Diversity (I)	1.17	1.04	0.67	0.49	0.85
Inbreeding Coefficient (F_{IS})	0.12	-0.13	-0.98	-0.09	-0.06
Heterozygosity vs Size	**IN**	**IN**	NO	NO	NO
Fathers/Brood (K)	2.75	4.39	1.17	2.13	1.72
Effective Fathers (Ke)	2.55	3.55	1.13	1.91	1.67
Paternity Skew (Ke/K)	0.93	0.83	0.9	0.86	0.95

Effect of Ecology and Sexual Condition on Mating Patterns

In addition to assignment based on sexual condition (bisexual vs unisexual), species were assigned to ecological groups based on microhabitat preference (hummock vs hollow). We investigated the relationship between each of the genetic diversity and

mating pattern statistics, and find four associations related to ecology and sexual condition. First, as mentioned above, there is a significant correlation between genetic diversity and inbreeding coefficient, but only for unisexual populations (Figure XX).

Genetic diversity (Shannon's Diversity) is significantly affected by sexual condition (Figure 4, ANOVA Type III SSS $F_1 = 7.66$, $p < 0.05$), but not ecology ($F_1 = 3.09$, $p > 0.1$), and there was no interaction effect ($F_1 = 0.41$, $p > 0.5$). A similar pattern is found for effective number of alleles (results not shown). Inbreeding coefficients are also affected by sexual condition ($F_1 = 30.4$, $p < 10^{-4}$), but not ecology ($F_1 = 0.31$, $p > 0.5$). However, there is a significant interaction effect (Figure 4, $F_1 = 9.3$, $p < 0.01$). F_{IS} is likely to be higher in bisexual hummock populations than bisexual hollow populations, but the reverse is true for unisexual populations.

Multiple paternity is not affected by sexual condition ($F_1 = 0.56$, $p > 0.4$) or ecology ($F_1 = 1.4$, $p > 0.2$) but there is a significant interaction effect ($F_1 = 7.0$, $p < 0.05$). Paternity skew is higher in bisexual hummock populations than bisexual hollow populations, but this trend is reversed in unisexual populations (Figure 4). Neither sexual condition nor ecology had any effect on multiple paternity (either K or K_e, results not shown).

The LL population of *S. strictum* and *S. austinii* were excluded from these analyses, due to the absence of genetic variation; with zero expected heterozygosity and only one genetically distinct inferred father, both F_{IS} and K_e/K would be meaningless for these populations.

Discussion

Inbreeding Depression

Only three studies have thus far investigated the effects of inbreeding on sporophyte fitness (Taylor *et al.* 2007; Szovenyi *et al.* 2009; Jesson *et al.* 2012). Taylor et al. (Taylor *et al.* 2007) found that heterozygosity was associated with reduced sporophyte size, but not reduced spore output in a unisexual moss. In *Sphagnum lescurii,* an aquatic unisexual species, Szovenyi et al. (Szovenyi *et al.* 2009) also found a significant increase in sporophyte size with increased heterozygosity in a natural population (b = 35.7). Our data suggests that these studies should not be extrapolated to a general conclusion that unisexual moss populations will show inbreeding depression. Only two of our unisexual populations, both in the aquatic species *S. macrophyllum*, showed a significant relationship between size and heterozygosity. In both of our populations, the inbreeding depression effect is smaller than was found in *S. lescurii*.

Several authors have suggested that inbreeding depression may not be a universal feature in outcrossing populations, when there is considerable overlap in gene expression between haploid and diploid generations (Shaw & Beer 1997; Joseph & Kirkpatrick 2004). In fact, about 70% of genes expressed in the haploid stage of the bisexual moss *F. hygrometrica* are also expressed in the diploid stage, compared to just 3% in *Arabidopsis* (Szovenyi *et al.* 2011). If the same were true for *Sphagnum*, then deleterious alleles would be purged from the population through the dominant haploid stage, and there would be a reduced cost to intergametophytic selfing.

Taylor et al. (Taylor *et al.* 2007) also show no inbreeding depression in a bisexual moss species, *Funaria hygrometrica*. Their findings agree with the predominating theory, that populations with intragametophytic selfing will quickly purge deleterious alleles,

and therefore subsequent self-fertilization does not carry a fitness cost (Charlesworth & Charlesworth 1992). Indeed, six of our seven populations of bisexual moss species show no evidence of inbreeding depression (Figure 2). However, in one population of *S. compactum*, heterozygous sporophytes are significantly larger than homozygous sporophytes. As far as we are aware, this is the first evidence of sporophytic inbreeding depression in a species capable of intragametophytic selfing.

It is difficult to imagine a scenario in which inbreeding depression can evolve in a population where nearly 50% of the sporophytes are completely homozygous. It is possible that the effect is the specific combination of parental genotypes, rather than related to heterozygosity *per se*. This has been found to be the case in another species, *S. macrophyllum* (Chapter 2).

Outbreeding depression (a negative relationship between heterozygosity and fitness) is observed in two populations: in the AK-PH population of *S. compactum*, and in the unisexual *S. cribrosum*. Outbreeding depression is feasible in populations of predominantly selfing plants, because many generations of local adaptation may result in epistasis, broken up by hybridization between inbred lines (Fischer & Matthies 1997; Edmands 2006). If the intragametophytic selfing rate observed in *S. compactum* is typical, this would explain why heterozygous sporophytes are less fit, but a multi-generation study would be necessary to confirm this.

More typically, outbreeding depression is seen between distant populations, such as between populations of the moss *Ceratadon purpureus* from New York and Ecuador (McDaniel & Shaw 2003). Genetic incompatibilities developed in isolation have been suggested as a type of postzygotic isolation important in speciation (Dobzhansky 1950; Lynch 1991). Interestingly, the *S. cribrosum* population studied here is known to

have the highest genetic diversity of any population in the species (Johnson *et al.* 2012); that study suggested that the GA32 population is the source of genetic diversity for the species. However, if the population were instead a sink, receiving migrants from all over the distribution of *S. cribrosum*, it would make outbreeding depression more likely. Similarly, outbreeding depression was seen in natural immigrants to an inbred population of song sparrows (Marr *et al.* 2002) and among genetically diverse individuals in a population of *Ipomopsis aggregata* (Waser *et al.* 2000).

Overall, we find a lack of association between heterozygosity and fitness in natural populations of *Sphagnum*. This suggests that, in contrast to flowering plants, selection for inbreeding avoidance mechanisms would be very weak.

Sexual Condition, Mating Patterns, and Sex Determination

Sexual condition is the major contributing factor to mating patterns in *Sphagnum*. Most of the unisexual populations had inbreeding coefficients near or below zero (Figure 3), while the bisexual populations had significantly higher inbreeding coefficients (Figure 4). In unisexual populations, there is a strong correlation between genetic diversity and inbreeding, as predicted by theory (Charlesworth & Charlesworth 1999). This correlation is, however, absent in bisexual populations.

For bisexual species, the F_{IS} values are consistent with values found in a survey of several moss species (Eppely *et al.* 2006), and indicates a very high level of intragametophytic selfing in *Sphagnum*. It appears as though mixed mating (F_{IS} near 0.50) is a common feature in bisexual *Sphagnum* populations, in contrast to angiosperms, where only outcrossing (F_{IS} near zero) and inbreeding (F_{IS} near 1) are stable states (Lande & Schemske 1985).

As discussed earlier, the negative values of F_{IS} in most unisexual populations are to be expected when there is a large skew in gametophyte fecundity, producing an excess of heterozygotes. The two exceptions are the GA28 population of *S. cuspidatum*, and *S. austinii*. These outliers are likely explained by a) incorrect assignment of sexual condition, or b) very low genetic diversity, limiting the ability to distinguish maternal and paternal genotypes in the sporophytes. For *S. austinii*, the latter is more likely to be correct, because there is only one allele at every locus, and all sporophytes are 100% homozygous. A broader survey of *S. austinii* in North America also revealed very low diversity compared to other members of subgenus *Sphagnum* (M. Kyrkjeeide, pers. comm.). Finally, there are no known bisexual species within subgenus *Sphagnum*, making misdiagnosis of sexual condition very unlikely.

By contrast, the GA28 population of *S. cuspidatum* has a very high degree of genetic variability (Table 2). Despite this, the population inbreeding coefficient is more similar to the bisexual populations (Table 1) than to other unisexual populations. In addition, three sporophytes, all attached to the same maternal ramet, are completely homozygous. One explanation is that these sporophytes are heterozygous, but undifferentiated at these loci; given the high number of alleles at each locus in the population, this seems unlikely.

An alternative explanation is that *S. cuspidatum* is not completely unisexual. In fact, there has been some disagreement about the sexual condition of this species. McQueen and Andrus (2009), whose treatment in *Flora North America* we followed for this study, state that *S. cuspidatum* is dioicous (unisexual gametophytes). However, an earlier treatment by Crum (* Crum 1984) indicates that *S. cuspidatum* is "monoicous, and

apparently also dioicous." There are several other discrepancies between the two treatments; most notably for this study, *S. tenellum* is classified as unisexual by Crum and bisexual by McQueen and Andrus. Our results here indicate the latter is correct, based on high rates of intragametophytic selfing.

However, the possibility remains that sexual determination in *Sphagnum* is not fixed for some species. Many bryophyte species have chromosomal sex determination; the first ever sex chromosomes in plants were discovered in the complex thalloid liverwort *Sphaerocarpus* (Allen 1919). In the so-called "UV" system (haploid phase determination), male gametophytes have one U chromosome, female gametophyetes have one V chromosome, and all sporophytes would be UV (Bachtrog *et al.* 2011). In this scenario, allopolyploidy is expected to generate gametophytes with both U and V chromosomes, generating bisexual gametophytes, a trend supported by phylogenetic reconstruction in mosses (Crawford *et al.* 2009).

If sexual condition in *Sphagnum* were determined by a UV system, polyploid species should be bisexual. However, of the fourteen bisexual *Sphagnum* species found in North America (McQueen & Andrus 2009), only one (*S. arcticum*) is known to be polyploid (see Appendix II). There has also been extensive work recently on the origins the allopolyploid species of *Sphagnum* (Sastad *et al.* 2001; Ricca *et al.* 2008; Karlin, Gardner, *et al.* 2010a); in most cases one or more extant haploid parents can be identified, and a corresponding change in sexual condition has not been observed. For example, the allodiploid hybrid *S. missouricum* and its two haploid parents, *S. lescurii* and *S. subsecundum*, all have unisexual gametophytes (Ricca & Shaw 2010). From this evidence we can conclude that *Sphagnum* does not have a UV chromosomal sex determination.

Instead, sexual condition may be labile within species or populations, especially in subgenus *Cuspidata* and subgenus *Acutifolia*. There are several disagreements between McQueen and Andrus (2009) and Crum (1984) in these subgenera. Gene expression analysis, along with careful tracking of individual ramets within populations, would be very beneficial to understanding the differences between male and female gametophytes within populations, and to discover the basis of sex determination. Our results suggest that *S. cuspidatum* would be a very good choice for this type of study.

Effect of microhabitat on mating patterns

Two statistics related to mating pattern—inbreeding coefficient and paternity skew—are significantly affected by species microhabitat preferences along the hummock-hollow gradient (Figure 4). The effects are not direct, but rather an interaction with sexual condition. Inbreeding coefficient is higher in bisexual hummock populations than bisexual hollow populations. The limited water availability atop hummocks means that sperm—which much swim to effect fertilization— may have fewer opportunities to fertilize genetically diverse eggs. This assumes that genetic diversity within a hummock is low. Our data supports this, because inferred paternal genotypes are rarely found in sporophytes attached to mothers from different parts of the population, in hummock species.

The opposite effect is found in unisexual populations: hummock populations have lower inbreeding coefficients than hollow populations. This pattern could, intriguingly, be explained by the same phenomenon of limited water availability. As discussed earlier, an overabundance of one type of gamete in the mating pool would increase observed heterozygosity above expected, generating negative values of F_{IS}. By

definition a unisexual gametophyte must mate with a different genotype to produce sporophytes; if these opportunities are limited to local genotypes, it could explain the extremely negative values found in unisexual hummock populations.

This hypothesis is supported by the significant interaction between sexual condition, ecology, and paternity skew. Paternity is much more highly skewed in unisexual hummock populations, and is most reduced in bisexual hollow populations. It seems that each hummock is an island of mating opportunities, restricted from other hummocks by limited water availability.

Conclusions

We find that sexual condition, genetic diversity, and microhabitat preference all affect mating patterns in *Sphagnum* populations. Sexual condition is the most prevalent, and the effect of microhabitat is subtler. We have greatly expanded the knowledge of inbreeding depression in natural populations, and have revealed it to not be a universal phenomenon in either unisexual or bisexual populations. Multiple paternity appears to be very common in *Sphagnum*, but paternity skew is most pronounced in unisexual populations living high above the water table. The possibility of labile sexual conditions within some species, such as *S. cuspidatum*, requires future study into the genetic components of sexual determination in *Sphagnum*.

Chapter 2: Fecundity, fitness, and a signature of sexual selection in the peatmoss *Sphagnum macrophyllum*.

Introduction

In its original formulation, sexual selection was defined on the basis of extreme secondary sexual characteristics, and competition between individuals to mate and produce offpsring (Darwin 1859). The quantitative description of sexual selection later became synonymous with the relationship between fecundity (the number of offspring) and mating success (the number of different mates) (Bateman 1948). When the strength of this relationship differs between the sexes, the sex with the smaller variance benefits from the ability to choose among many potential mates (Arnold 1994). Competition is not limited to showy traits or behaviors-- recent studies on post-copulatory sperm competition instead highlight the importance of the haploid stage in the evolution of sexual selection (e.g. Firman & Simmons 2009; Gasparini *et al.* 2010; Boschetto *et al.* 2011). Sexual selection, then, is not limited to the diploid stage.

The relevance of the haploid stage in studies of mating patterns has received increased attention recently. In sea urchins, gamete recognition drives high selection pressures (Levitan 2008) and extremely fast rates of molecular evolution in bindin genes expressed only in the haploid stage (Zigler & Lessios 2003). In addition to their importance for speciation (reviewed in Palumbi 2008), gamete recognition also drives intrasexual competition and intersexual conflict among sexes (Levitan 1996).

In seed plants, pollen competition experiments have shown fertilization success among pollen donors is consistent across mothers (Snow & Spira 1991; Marshall &

Diggle 2001) and that success is correlated with faster pollen tube growth rate (Hormaza & Herrero 1996; Aronen *et al.* 2002). The presence of multiple donors has been shown to increase the fitness of diploid offspring in several angiosperms (Mulcahy & Mulcahy 1975; Winsor *et al.* 2000; Zhang *et al.* 2010). One mechanism for development of intrasexual competition is the "good sperm" hypothesis, which suggests that the most successful male gametes also contain genes for more fit diploids (García-González 2008). A drawback of these studies is that every fertilization event is unique, and by necessity studies are limited to the lumped effects of pollen or sperm donors. It would be beneficial to instead study the effect of individual gametes on fertilization success and sporophyte (seed) fitness, but such a study would be biologically intractable in seed plants.

Bryophytes, because of their unique haploid dominant life cycle, present an opportunity to extend the understanding of the role of the haploid stage on mating success. As with all land plants, bryophytes have an alternation of generations between a spore-producing stage (sporophyte) and a gamete-producing stage (gametophyte) (Figure 1). The relatively short-lived sporophyte produces haploid spores via meiosis. The spores germinate and grow into large, persistent gametophytes via clonal vegetative propagation. Therefore, potentially hundreds of independent gametophytes (ramets) may be have identical genotypes, and therefore are clones in the same genet. Depending on the clonal structure of the population, genets may be isolated or grow intermixed with other genets.

Most bryophyte species possess unisexual gametophytes (Anderson & Wyatt 1984), and in these species gametophytes can be characterized as male or female by the presence of specialized gamete-producing structures (gametangia). Male plants produce

sperm in antheridia, while female plants produce eggs in archegonia; both types of gametes are produced by mitosis.

Figure 1 (p. 2) represents a population containing exactly one male and one female genet. The male genet grows into a large clone via vegetative propagation, and each of the many ramets produces sperm by mitosis, giving rise to genetically identical sperm. The same is true of the large female clone, which produces many genetically identical eggs. Fertilization between gametes produced the one male clone and one female clone would produce diploid sporophytes that are genetically identical. An important consequence of this life cycle is that fertilization events can be replicated in the population. Additionally, the fecundity of each male (and female) gametophyte need not be binary, as in seed plants and animals. Instead, fecundity is a quantitative trait. For example, one haploid male gametophyte may participate in one to many fertilization events, or none.

Bryophyte reproduction also allows for unequivocal assignment of diploid sporophyte genotypes into two haploid parental genotypes. Bryophytes are quasi-broadcast spawners; sperm is dispersed into the water, while eggs are retained on the female plants. After fertilization in *Sphagnum*, the sporophyte (zygote) is essentially a sphere containing meiotic spores, and is dependent upon the maternal gametophyte for nourishment, and for exertion, via a pseudopodium of gametophyte tissue, into the air for eventual spore release. Because the sporophyte remains physically attached to the maternal gametophyte throughout its life, maternity is easily determined, and paternity is inferred by subtracting the maternal haploid genotype from the sporophyte diploid genotype (Szovenyi *et al*. 2009).

Taken together, these traits allow for an insight into haploid fecundity and mating success that would be difficult or impossible in seed plants or animals. Despite this advantage, very little is known about mating patterns in bryophytes. Previous studies have focused on the fecundity of just one sex (Szovenyi *et al.* 2009), or have only focused on inbreeding as a source of fitness variance (Taylor *et al.* 2007). In this study, we survey gametophytes and sporophytes of the peatmoss *Sphagnum macrophyllum*, an aquatic bryophyte with separate sexes. With our study we aim to 1) characterize the variance in and relationship between fecundity and mating success in a natural population of gametophytes, 2) demonstrate/estimate the contribution of the haploid genotype to sporophyte fitness.

Materials and Methods

Study Site and Species

The study site is in the Hell Hole Bay Wilderness Area in the Francis Marion National Forest, in Berkeley County, South Carolina (33.218˚ N 79.712˚ W). The sampling site is a break in the otherwise very dense swamp pocosin: an open canopy of *Taxodium* trees surrounded by less than a meter of standing water. The understory contains several shrubs such as *Nyssa biflora*, *Lyonia lucida*, and *Vaccinium formosum*. Aquatic plants, including *Dulichium arundinaceum*, *Carex striata*, and *Nympaea odorata*, can be found scattered in the open water surrounded by several peatmoss species, including *Sphagnum cuspidatum*, *S. magellanicum*, and *S. portoricense* . However, the dominant plant, found floating in the water in nearly continuous mats covering an area of roughly 2000 m^2, is the aquatic peat moss *Sphagnum macrophyllum*.

Several properties of *S. macrophyllum* at Hell Hole Swamp made it an excellent choice for our study. After visiting more than 50 sites of the species, Hell Hole Swamp

has the largest biomass of the species we have seen. It is also the only site that shows consistent sexual reproduction, producing sporophytes every year since we began sampling the site in 2005 (Johnson et al, unpubl.)

Sphagnum macrophyllum is part of a distinct clade within the subgenus *Subsecunda* (Shaw, Boles, *et al.* 2008a), and can be found in North America along the coastal plains of the Atlantic Ocean and Gulf of Mexico, from Newfoundland to Texas (McQueen & Andrus 2009) It usually grows in acidic waters such as roadside ditches, the margins of small ponds, and in pine savannahs, especially where there is underlying sandy soil (Anderson *et al.* 2009).

Sampling of sporophytes and gametophytes

To describe the sex ratio and reproductive phenology of the population, we sampled gametophytes throughout a breeding season, from December 2009 to June 2010. We also sampled mature sporophytes in two seasons: April-May 2009 and March-June 2010. *Sphagnum macrophyllum*, like all members of the subgenus *Subsecunda*, has unisexual gametophytes. There is little sexual differentiation, but male gametophytes are identified by antheridia (the male gametangia, which produce sperm). Our observations indicate that antheridia are produced and visible between December and March; plants are not identifiable as male outside of this range.

Female gametophytes are more challenging to identify; archegonia (the female gametangia, which produce eggs) are often not visible even by careful dissection. The most reliable way to identify a gametophyte as female is by the presence of sporophytes, which remain attached to their maternal gametophyte. Archegonia may turn a reddish color when recently fertilized, allowing for easier identification. Females are identifiable

beginning in late December, and young, macroscopic sporophytes are easily seen by February.

Sporophytes mature from April through June, and all spores are released by July. *Sphagnum* sporophytes are roughly spherical capsules containing, at maturity, haploid spores (products of meiosis). The diameter of the capsule is a reliable predictor of spore number in *Sphagnum* (Sundberg & Rydin 1998). We measured the diameters of all sporophytes in the study, when they were at full maturity, and we use this measure as a proxy for sporophyte fitness (Szovenyi *et al.* 2009).

Female gametophytes and sporophytes were collected in two breeding seasons: April and May 2009, and March through June 2010. We also wished to characterize genetic diversity in male gametophytes as well as those gametophytes with no evidence of sexual expression ("sterile"). However, male and female plants cannot always be simultaneously identified, so we sampled plants throughout the breeding season, from December through June. We marked eight positions (neighborhoods) throughout the site (Figure 5). Each month, we randomly sampled twelve gametophytes (ramets) within a 3m radius at each neighborhood.

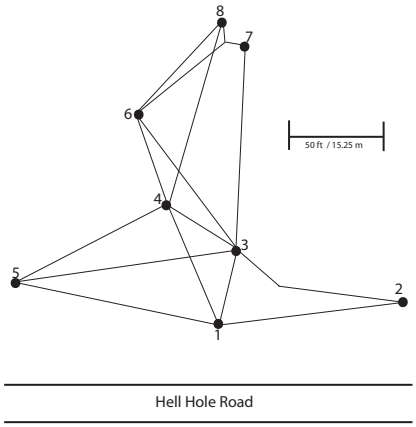

Figure 5. Sampling neighborhoods at Hell Hole Swamp. Twelve gametophytes were randomly sampled from within a 3m radius in each neighborhood, each month between December 2009 and June 2010.

We determined sex of each gametophyte by microscopic dissection, classifying each gametophyte as "male" (presence of antheridia), "female" (presence of archegonia or attached sporophytes), or "sterile" (neither gametangia nor sporophytes observed). All gametophytes identified as female were transferred to individual tubes containing sterile water. All female plants eventually produced sporophytes in the lab, indicating that each had already mated when they were collected.

For genotyping, we selected every gametophyte identified in the phenology survey as female (75 samples). We also selected a representative sample of male (58 samples) and sterile (65 samples) gametophytes from each neighborhood each month. We also genotyped all mature sporophytes (321 samples) collected in April-May 2009 and April-June 2010.

Genotyping

For DNA extractions of gametophyte tissue, a portion of the gametophyte's capitulum (the dense cluster of branches at the apex of *Sphagnum* plants) was removed. For sporophytes, the entire capsule was used. DNA extraction followed a CTAB protocol (Shaw, Cox, & Boles 2003b). Briefly, tissue was flash frozen in tubes using liquid nitrogen and ground into a powder using steel beads. Cellulose and other proteins were removed using CTAB and chloroform mixed with β-mercaptoethanol in a 24:1 ratio. DNA pellets were precipitated using cold isopropanol and eluted in 50 μl of T.E. buffer. In preparation for PCR amplification, we diluted the DNA of gametophytes 7:1, and diluted sporophytes 2:1.

All plants were genotyped at fifteen microsatellite loci, via previously described protocols (Shaw, Cao, *et al.* 2008b): 1, 4, 7, 9, 10, 14, 17, 18, 19, 20, 29, 65, 68, 75, and 93.

Paternity and clone assignment

Because all bryophyte sporophytes remain attached to their maternal gametophyte, determining the multilocus paternal haploid genotype is straightforward. Given a sporophyte's diploid genotype, the maternal haploid genotype may be subtracted, leaving only alleles that must have come from the father. In the case of homozygous loci, the paternal and maternal genotypes were assumed to be identical. The inferred haploid paternal multilocus genotype for every sporophyte was determined by a custom Python script.

To characterize the clonal structure of the population, a second Python script was used to assign every sample to unique multilocus genotypes. All samples were included: female, male and sterile gametophytes, as well as the inferred paternal genotypes. Genotypes with missing data were also classified, and were included in further analysis only if they unambiguously matched exactly one unique multilocus genotype.

Genetic analyses

Population genetic statistics, including PhiPT (the multilocus equivalent of F_{ST}), Analysis of Molecular Variance (AMOVA), and principal coordinate analysis (PCoA) were conducted using GenAlEx version 6.5 (Peakall & Smouse 2012). Missing data was not interpolated in any analysis using GenAlEx.

Sporophytes attached to the same maternal gametophyte are not random draws from the population. Because of this bias, to calculate F_{IS} for this population, we

employed a modified version of bootstrap method of Szovenyi et al. (2009). The script, written in R (Team 2012) samples one sporophyte at random from each distinct maternal genotype to create a population subset. F_{IS} is calculated from the observed and expected heterozygosity of sporophytes within this subset, and this was repeated 1000 times, to estimate a range of inbreeding coefficients for the whole population.

All other statistical analyses, including analysis of variance, analysis of covariance, and correlations, were conducted in R.

Results

Hell Hole Swamp: Phenology and Genetic Diversity

The survey of sex expression in *S. macrophyllum* in the 2010 breeding season reveals a heavily male-biased sex ratio (Table 3). Gametophytes can be identified as male (by observing the presence of antheridia) in five of the seven months; in each of these, the number of samples classified as male exceeds those classified as female. In most months, a large percentage of gametophyte samples cannot be determined as either male or female. Though this could reflect the increased difficulty of identifying female plants, the sterile samples we genotyped were more likely to be male (see below).

Table 3. Phenology of sexual expression and sporophyte production in *S. macrophyllum* at Hell Hole Swamp.

Month	Male	Female	Sterile	Sporophytes
Dec-09	46	7	43	0
Jan-10	40	16	40	66
Feb-10	44	16	36	76
Mar-10	40	31	25	182
Apr-10	28	18	50	82
May-10	0	24	72	151

Jun-10	0	14	82	45
Total	**198**	**126**	**348**	**602**

Of the 197 gametophytes genotyped, 149 are unambiguously assigned to one of the 65 unique multilocus haploid genotypes. There are seventeen unique genotypes that contained at least one sample identified as male (by presence of antheridia), and 22 unique genotypes were identified as female (by the presence of sporophytes). Of the sterile samples, 47 can be unambiguously assigned to a multilocus genotype—twenty-one sterile samples match a male clone, while eight samples match a female clone.

Among the female samples, half (37 of 75) belong to a single clone that occurs in six of the eight neighborhoods. The next most common clone is found in just four samples. One clone comprising 25 of 78 samples dominates the male plants.

The genetic diversity in the population is characterized by high divergence between male and female genotypes (Figure 6). Female clones are significantly differentiated from male clones (PHIPT = 0.103, $p < 0.001$) and from sterile clones (PHIPT = 0.039, $p < 0.05$). Male clones are not differentiated from sterile clones (PHIPT = 0.00). A significant proportion of genetic differentiation in all gametophytes is among neighborhoods (PHIPT = 0.088, df=7, $p < 0.01$).

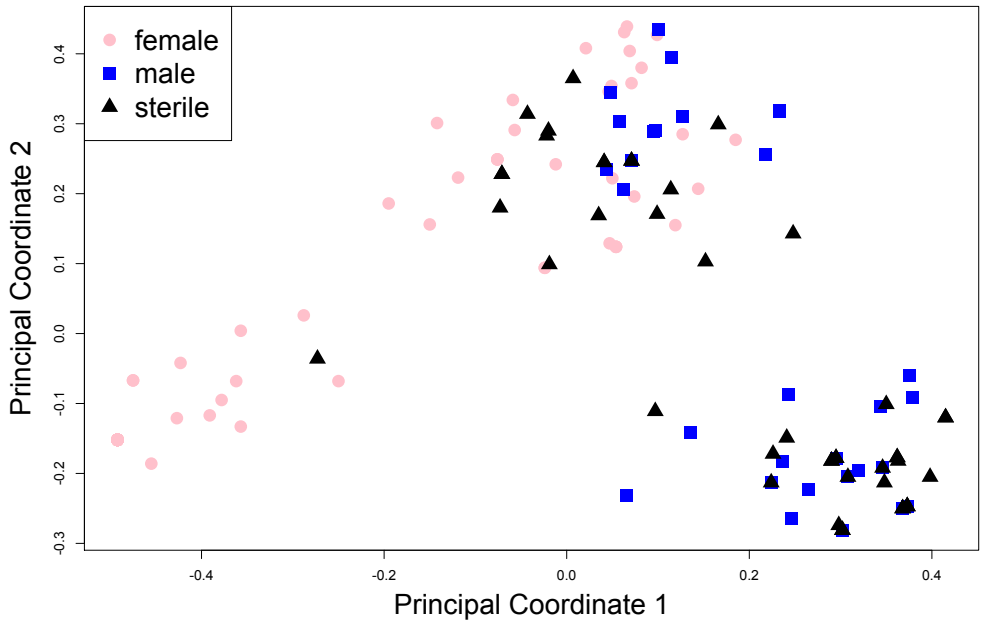

Figure 6. Principal coordinate analysis of genetic diversity in gametophytes. All gametophytes were classified by whether the clone contained any samples identified as male or female. All other samples are classified as sterile. When a sterile plant (black) has the same multilocus genotype as a male (blue) or female (pink) plant, the points overlap. Percent of total variance: Axis 1- 39.0%, Axis 2- 23.5%.

Fecundity and Mating Success

A total of 321 mature sporophytes were surveyed and represent ten unique maternal genotypes and ninety-one unique inferred paternal genotypes. One single female genotype, mentioned above, mated with 60 different paternal genotypes to produce 68.5% (220 of 321) of the sporophytes (Figure 7, top). Each of the other maternal genotypes also mated with multiple paternal genotypes.

The skew in fecundity is also pronounced for the paternal genotypes (Figure 7, bottom). Three of the most commonly inferred genotypes, together, account for 36% of the sporophytes. Comparatively few of the inferred paternal genotypes matched gametophytes we sampled: only seven of the 91 inferred paternal genotypes match any

gametophytes. There are 19 paternal genotypes found in at least three sporophytes; six of these fathers mated with more than one maternal genotype.

The correlation between fecundity (number of offspring) and mating success (number of different genetic mates) was significant for both males ($r^2 = 0.644$, $p < 2 \times 10^{-16}$) and females ($r^2 = 0.983$, $p < 2.2 \times 10^{-8}$) (Figure 8Figure 9). The slope of the male gradient (11.25) was more than three times as steep as the female gradient (slope = 3.70), a significant difference (ANCOVA df=98, F = 63.14, $p < 3.3 \times 10^{-8}$). The fecundity/mating success relationship for females, and the difference in slopes are both significant even if excluding the most common female. (results not shown).

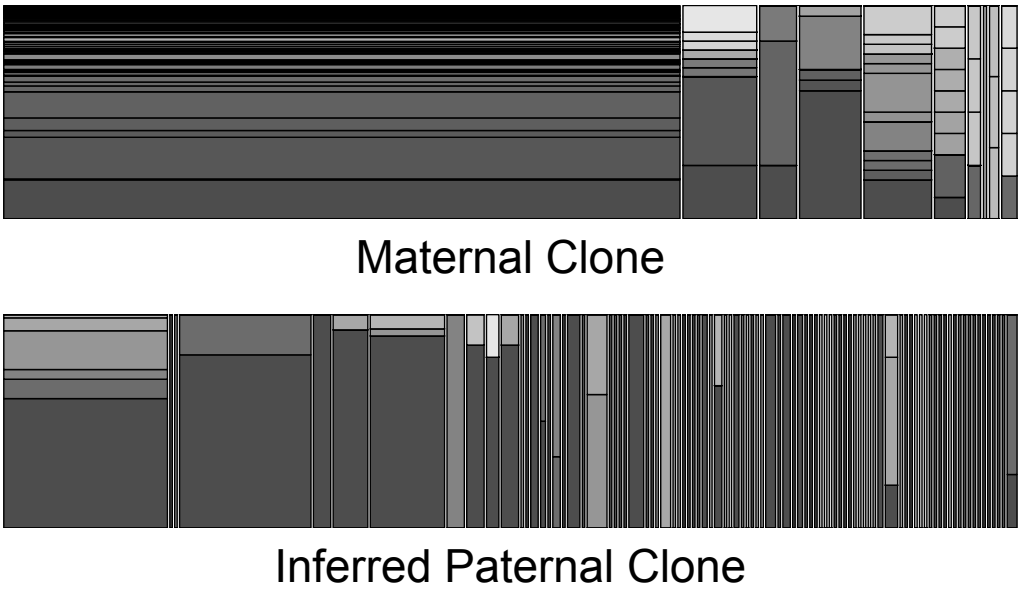

Figure 7. Spineplot showing the variance in fecundity and mating success in *Sphagnum macrophyllum* at Hell Hole Swamp. The width of each box corresponds to the fecundity (number of sporophyte offspring) for each unique haploid multilocus genotype. Horizontal bars within each box correspond to the mating success (number of genetically distinct mates) for each unique genotype. *Top*: female gametophyte fecundity, dominated by a single female gametophyte (left), which produced over 200 sporophtyes with over 60 different genetic fathers. *Bottom*: male gametophyte fecundity; there are many more successful male genotypes, with a narrower variance in both fecundity and mating success.

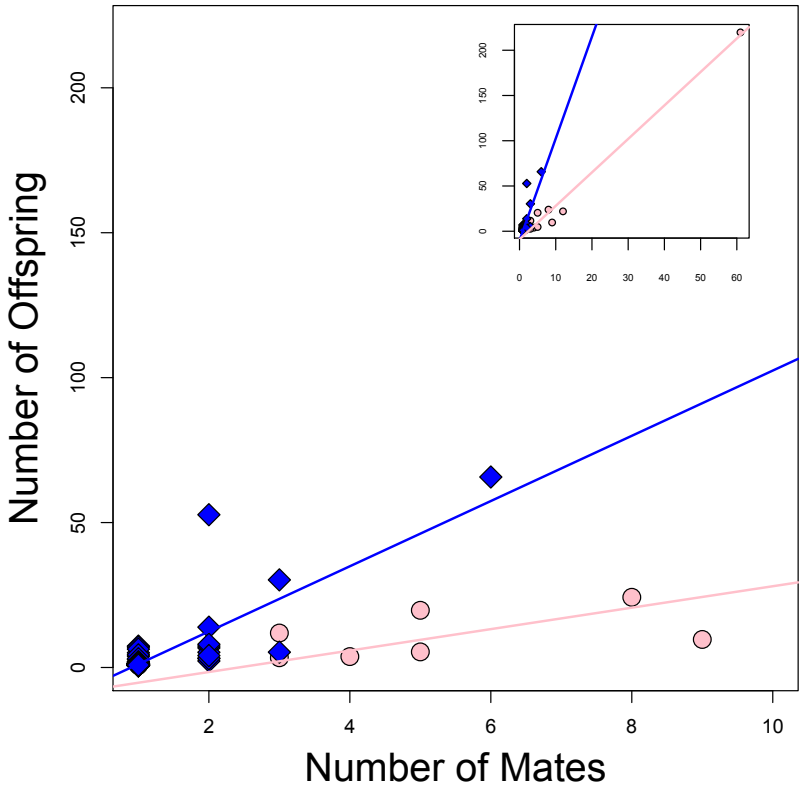

Figure 8. Bateman gradient for *Sphagnum macrophyllum* at Hell Hole Bay. The effect of mating success (number of different mates) on fecundity (number of sporophyte offspring) is shown for male (blue diamonds) and female (pink circles) gametophytes. The inset shows the full dataset, including the very large female clone (top right), while the main plot is zoomed in to show the differentiation between in male and female fecundity. Trend lines for each sex include all datapoints in both plots.

Sporophyte Fitness

The mean sporophyte size (our proxy for offspring fitness) in the population is 1508 μm (± 108 μm SD). We investigated several sources of variance in sporophyte size: inbreeding, limitation of resources in the maternal gametophyte, and the genotype of the maternal and paternal gametophyte parents.

Using 1000 subsampled populations, the average F_{IS} is -0.11 (± 0.04 s.d.). In the three neighborhoods where sporophytes are most common, F_{IS} is even more negative: Neighborhood 3 (F_{IS} = -0.28, ± 0.06 s.d.); Neighborhood 4 (F_{IS} = -0.34 , ± 0.05 s.d.);

Neighborhood 6 (F_{IS}= -0.14 , ± 0.02 s.d.). The average multilocus heterozygosity is 54%, and individual sporophytes range from 20 to 100% heterozygous loci.

Despite the high degree of outcrossing in the population, there is only a very small, but significant, effect of heterozygosity on sporophyte fitness (Figure 9A). Increasing the number of sporophytes attached to a single female gametophyte ramet ("brood size") could limit the amount of maternal resources available to each sporophyte, potentially reducing the average fitness of the brood. However, there is no effect of brood size on the average brood fitness (Figure 9B, range 2-18, n = 76, $r^2 = 0$).

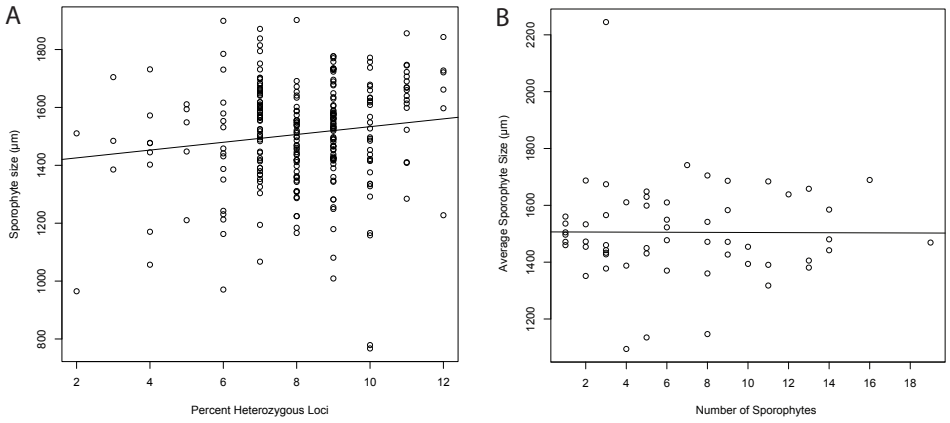

Figure 9. Effects of inbreeding depression and resource limitation on sporophyte fitness. *A:* Relationship between percent heterozygosity and sporophyte size ($r^2 = 0.018$, $p < 0.05$). *B:* Effect of "brood size" (number of sporophytes supported by a maternal gametophyte) on average brood fitness ($r^2 = 0.00$).

Instead, the genetic identity of the maternal (d.f. = 5, F = 4.24, $p < 0.01$) and paternal (d.f. = 22, F = 3.58, $p < 0.001$) parents drives much of the variance in sporophyte size (ANOVA, Type III SSS, singletons removed). We detect no interaction effect (d.f. = 8, F = 1.13, $p > 0.1$), and paternal identity (9.2%) accounts for greater variance compared maternal identity (3.6%). Due to low sample size, the effects of specific maternal or paternal genotypes cannot be assessed.

To illustrate, in Figure 10 we show only those sporophytes mothered by the most common female clone, separated by paternal identity. All sporophytes within one column in Figure 10 have the same haploid mother and the same haploid father, generating repeated fertilization events across the population. Despite the variance found within each box, there is a highly significant effect of paternal identity on sporophyte fitness for this subset (ANOVA, $F = 5.25$, $df = 16$, $p < 10^{-7}$), indicating the effect of haploid paternal identity on sporophyte fitness must have a large genetic component.

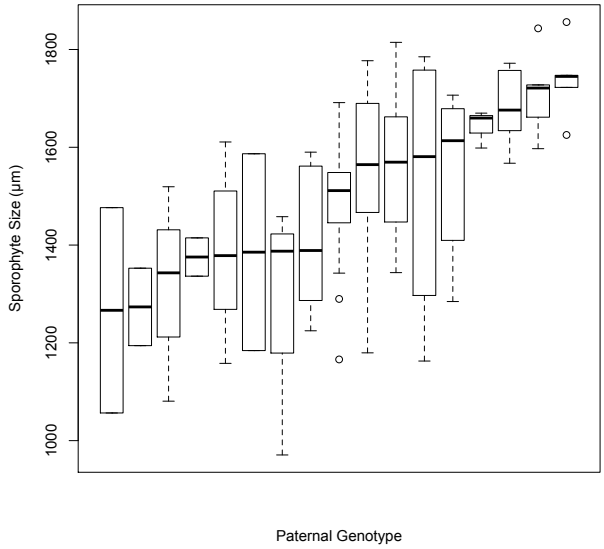

Figure 10. Distribution of sporophyte fitness by genetic father. All sporophytes shown have the same genetic mother. Each box represents identical diploid sporophytes formed throughout the population; all variance within a box is environmental. Paternal identity is a significant predictor of sporophyte fitness (singletons removed, ANOVA, $F = 5.25$, $df = 16$, $p < 10^{-7}$).

Discussion

Phenology and Genetic Structure

Sexual expression in *Sphagnum macrophyllum* at Hell Hole Swamp appears to be extremely male-biased (3:1 male:female ratio among sampled gametophytes). Most bryophytes, by contrast, have female biased sex expression in natural populations (reviewed in Bisang & Hedenäs 2005). In bryophytes, the lack of male sex expression has been attributed to a "shy male hypothesis," in which genetically male gametophytes do not always produce antheridia, obscuring a truly even sex ratio (Stark *et al.* 2010).

If the distorted sex ratio among gametophytes is due to "shy females," we would have scored them as "sterile," whether they failed to produce archegonia or were simply unsuccessful at fertilization. However, sterile gametophytes matched the microsatellite profiles of *male* plants three times as often as they matched female clones. Further, the population has significant genetic structure attributable to sex. Male and female gametophytes form distinct clusters on the PCoA (Figure 6), and plants we classified as sterile are distinct from the female, but not the male cluster. Finally, the information from the inferred paternal genotypes in the sporophyte generation (91 distinct fathers) implies that we have severely under sampled the genetic diversity of male gametophytes. It is therefore much more likely that male gametophytes are more common at both the ramet and genet levels in this population. Because the population is perennial plants that can reproduce asexually via vegetative propogation, the sex ratio distortion is likely to be stable over many years and breeding seasons.

Variance in fecundity and mating success

We find a high variance in the number of different mates, and the number of offspring they yield, for gametophytes of both sexes. Multiple paternity in sporophyte "broods" raised by the same gametophyte mother is very high, as has been previously described in *Sphagnum lescurii* (Szovenyi *et al.* 2009). Most paternal genotypes in *S. macrophyllum* paired found to have mated with multiple maternal genotypes. This represents the first demonstration of promiscuity in both sexes in a bryophyte population.

The high variance in fecundity and mating success in both sexes represents an opportunity to test for the Bateman gradient in the population. The strength of the association in males compared to the association in females implies the underlying mating system for the population (Arnold & Duvall 1994). In *S. macrophyllum*, the significant association between fecundity and mating success in both sexes (Figure 8). The greater strength of the gradient in male gametophytes, suggests a polygamous mating system. In most other organisms, this association has been interpreted as a signal of sexual selection (Levitan 1998; Becher & Magurran 2004; Tatarenkov *et al.* 2008; Anthes *et al.* 2010).

It is not clear what role, if any, sexual selection could play in a population of aquatic mosses. Sexual selection is expected to be most effective in mating systems where one sex has an upper fecundity limit, which would put strong pressure on the other sex to compete for fertilization success (Arnold & Duvall 1994). For *S. macrophyllum*, the Bateman gradient is linear for both sexes, although steeper for males (Figure 8). Primarily, the trend in females is due to one very large female clone, which accounts for 50% of female gametophytes and 68% of sporophytes produced. Therefore,

an alternative explanation for the relationship between fecundity and mating success is natural selection for clone size, rather than competition for fertilization success within sexes.

However, there could also be sexual selection for clonal growth patterns. Given equivalent biomass, a clone that creates a large mat in one location may not contact as many potential mates as a clone that spreads outward. Clonal growth patterns are known to be extremely variable to in *Sphagnum cribrosum*, the sister species to *Sphagnum macrophyllum* (Johnson *et al.* 2012). Given the larger slope of the Bateman gradient in males (Figure 8), there may be greater competition for exposure to multiple females, in an effort to increase mating success, and thus fecundity. In this study, it was not possible to determine the effect of clone breadth (vs. clone size), because male gametophyte diversity was poorly represented in the random sampling.

Although purely speculative, the possibility that sexual selection could operate on gamete recognition in bryophytes is certainly feasible, given that sperm must swim to the eggs. Experimental tests could determine whether, as in seed plants (Snow & Spira 1991) and urchins (Levitan 2004), the number of available sperm donors affects fertilization success. In bryophytes, there may be competition between genetically sperm produced by the same genetic individual and among genotypes, which reveals an additional level of sperm competition not present in other organisms. Experimental manipulation of sperm availability could test not only whether there is sperm competition, but whether female genotypes choose among sperm, whether there is variance in chosen sperm genotypes, and whether the selection is carried over into increased sporophyte fitness.

Sporophyte Fitness

The most pronounced characteristic in the mature sporophyte population is an excess of heterozygosity, resulting in an estimate of F_{IS} that is significantly below zero. Using similar methods, Szovenyi et al. (2009) also found a slightly negative F_{IS} for *Sphagnum lescurii*. Other *Sphagnum* species with unisexual gametophytes generally exhibit inbreeding coefficients far below zero (Chapter 1). Using isozymes, Eppely et al. (2006) found low (though positive) inbreeding coefficients in several other bryophytes with unisexual gametophytes.

A highly outbred population is more likely to show evidence of inbreeding depression at equilibrium, due to the inability to purge deleterious alleles from the population (Lande & Schemske 1985). Sporophyte size does increase with heterozygosity in the study population, but the effect is very weak (Figure 9A). However, a large majority of sporophytes in this study show more than 50% heterozygous loci. The excess of observed heterozygosity may result from homozygous sporophytes that did not reach maturity. The only other study to investigate the effect of inbreeding depression in a natural bryophyte population found a much larger relationship between heterozygosity and sporophyte fitness (Szovenyi *et al.* 2009), but results from other unisexual species of *Sphagnum* suggest that inbreeding depression is not universal in *Sphagnum* (Chapter 1).

In bryophyte sporophytes, there is the potential for intergenome conflict among parental genotypes: maternal gametophytes aim for high fitness for all sporophytes, and paternal genomes try to procure additional resources (Haig & Wilczek 2006). The maternal gametophyte is probably responsible for supplying nutrients to young sporophytes (Duckett *et al.* 2009),and exerts the capsule upon a stalk of gametophyte

tissue, which may affect spore dispersal. There does not appear to be any evidence of maternal resource limitation in the population. Though the large number of sporophytes (up to 18) found attached to some maternal ramets is extreme for *Sphagnum* (Johnson et al., unpublished) brood size does not significantly affect average brood fitness. This may be because the maternal ramets are not limited in their resources and are therefore able to support very large broods.

The most compelling determinant of sporophyte fitness is the identity of the maternal and paternal haploid genotypes. This finding would be intractable or impossible to dissect in seed plants or animals, because it relies on the potential for quantitative fecundity of the haploid generation. The clonal propagation of gametophytes throughout the population, and the production of gametes via mitosis, allows for multiple, repeated fertilization events. In the most extreme case, one haploid female was fertilized by the same haploid male 55 times, in six of the eight neighborhoods. All of these diploid sporophytes are genetically identical; any size variance must be environmental. Despite this, both maternal and paternal identity explained a significant proportion of sporophyte fitness variance (Figure 10).

This effect is in some ways analogous to General and Specific Combining Ability in breeding experiments (Falconer & Mackay 1996). For example, a male parent would have high General Combining Ability if, when crossed with multiple females, his offspring are on average more fit than the rest of the population. In quantitative genetics, this effect is important because it can be used to estimate the additive genetic variance in the population Specific Combining Ability is the performance of one particular cross, and represents non-additive genetic variance. We do observe a higher relative performance of certain males in the population when they crossed with the most

common female clone (Figure 5). However, because this is a natural population, and not a designed cross, we did not observe all possible combinations of males and females. Unfortunately, this means there was not enough power to detect whether specific males or females produced larger offspring. This could be further studied with a more detailed analysis that accounts for gene expression differences between the maternal and paternal components of the sporophyte genome (as suggested by Haig and Wilczek, 2006).

Conclusions

In a population of aquatic, unisexual, haploid peatmosses, we find a large variance in the fecundity and mating success of gametophytes. The relationship between the number of mates and the number of offspring is characteristic of a Bateman gradient suggesting the potential for sexual selection. Although it is unclear what traits may be selected, the extremely biased male sex ratio, and the connection between gametophyte parental identity and sporophyte fitness both suggest that intrasexual competition for fertilization success is likely. To distinguish between natural and sexual selection, further experiments are necessary to determine the major factors that determine fecundity, mating success, and fitness. We have demonstrated the utility of the bryophyte system to provide unique windows into the often hidden haploid component of mating patterns in natural populations.

Chapter 3: Evolution of niche preference in *Sphagnum* peatmosses

Introduction

Hierarchical inheritance of species traits results in related species more closely resembling each other, compared to randomly selected species on a phylogeny (Blomberg & Garland 2002). This pattern, "phylogenetic signal," is not universal, but rather an assumption to be tested, and may be unsupported in labile traits such as behavior (Blomberg *et al.* 2003) and ecological niche (Losos 2008). Sufficient phylogenetic signal (the pattern) must be demonstrated in the niche descriptors under investigation, before any inferences such as community assembly and niche conservatism (the process) can be made. The most basic pattern of phylogenetic signal is Brownian motion, where trait variance is correlated with phylogenetic distance (Felsenstein 1985). However, this pattern may be obscured by more complex patterns of trait evolution: if the correlation between trait distance and phylogenetic distance is not perfect (Pagel 1994), if there are multiple rates of trait evolution on the tree (O'Meara *et al.* 2006), or if stabilizing selection has constrained trait evolution (Hansen 1997; Butler & King 2004).

When considering the evolution of ecological niche descriptors, it is useful to distinguish between β-niche (climatic tolerances or macrohabitat affinity) and α-niche (within-community microhabitat affinity) (Ackerly *et al.* 2006). Many studies model ecological niches using macrohabitat data, climatic variables from databases such as BIOCLIM (e.g. Boucher *et al.* 2012) and focus on β-niche because α-niche is unavailable or impractical to collect. In cases where α-niche is considered, phylogenetic signal can suggest whether habitat preferences underlie community assembly (Cavender Bares *et*

al. 2004) or whether plastic traits portray phylogenetic signal potentially overwhelmed by natural selection (Eterovick *et al.* 2010).

A genus with many cosmopolitan and potentially co-occurring species would be the most appropriate study system for the evolution of α-niche differentiation, but precise microhabitat data are often lacking. However, *Sphagnum* (peatmosses) offers an excellent model of microhabitat differentiation. Many species have circumboreal distributions, and very similar assemblages of species can be found in peatlands in both North America and northern Europe. Both within and between peatlands, vegetation (dominated by *Sphagnum*) varies on two important ordinations: wetness and a combined gradient of pH and ion concentrations. The wetness axis is typically characterized as a gradient between hummock species — those that build cushions high above the water table, and hollow species — those that live near the water table. Ionic concentrations, including pH, vary from ombotrophic mires (bogs, pH ~4), to minerotrophic mires (fens, with pH up to 8 in calcareous rich fens). The sorting of *Sphagnum* species along these gradients is well documented, with data both for their average position and for the ranges they cover, i.e., the niche breadth.

Sphagnum species do not simply inhabit these peatland gradients; the gradients are also formed by the specific physiological traits that enable *Sphagnum* to engineer its local environment (van Breemen 1995; Granath *et al.* 2010) through their 'extended phenotypes'. *Sphagnum* is an unparalleled group for investigating the community and ecosystem consequences of the species traits (Whitham *et al.* 2003). Certain species both create and inhabit the raised microtopographic features (hummocks) because of their growth forms, water transport abilities and low decay rates (Rydin *et al.* 2006). By producing large amounts of organic acids, they lower the pH, and yet maintain an

effective uptake of minerals through cation exchange in extremely nutrient-poor environments (Hemond 1980). By creating an environment that is wet, acid and anoxic, *Sphagnum* decomposes slowly and thereby triggers peat accumulation; *Sphagnum*-peat represents a carbon store of global importance (Turunen *et al.* 2002; Rydin & Jeglum 2006).

Compared to other plants, we therefore have an unusually good basic understanding in *Sphagnum* of trait functions and the mechanisms by which morphology and physiology enable species to endure certain habitat conditions. For example, the morphology that allows hummock species to withstand desiccation is well described (Rydin *et al.* 2006). In contrast to vascular plants, many of the same *Sphagnum* species dominate peatlands in Eurasia and in North America, where they exhibit the same relationships to habitat conditions (Rydin 1993). Comparable niche data are available for many peatland species from the temperate, boreal and arctic-alpine zones across the Holarctic.

Subgeneric classification in *Sphagnum* already gives some clue about inheritance of microhabitats in the genus. Of the 250-300 species of Sphangum found worldwide, approximately 40 of these species have circumboreal distributions and can be commonly found in peatlands throughout the high latitudes of the Northern Hemisphere. Compared to the age of the entire Sphagnopsida (~250 my), all of these originate from a radiation approximately 15 million years ago (Shaw, Devos, *et al.* 2010b). Two monophyletic subgenera, *Cuspidata* and *Subsecunda*, are generally characterized by species living at or near the water table (hollow), while members of subgenera *Acutifolia* and *Sphagnum* (also monophyletic) are more likely to form hummocks high above the

water table. This suggests phylogenetic inheritance, may be more prevalant in hummock/hollow microhabitat descriptors, compared to the pH/ionic gradient.

If one or more of the microhabitat descriptors is phylogenetically inherited, it follows that the related functional traits (such as decomposition rate or cation exchange rate) would also show similar patterns. Demonstration of signal would guide the focus of future studies on the evolution of peatland ecosystems. In this study, we consider whether *Sphagnum* microhabitat descriptors are phylogenetically inherited, using a variety of comparative models testing the tempo, direction, and heterogeneity of microhabitat niche preference evolution in the genus. To do this we used ecological niche data for 39 *Sphagnum* species from three large published studies in northern Europe and North America, constructed a phylogenetic tree using sequences from 18 genes, and analyzed the comparative dataset containing eight univariate niche descriptors and two principal components representing the gradients. Using methods designed to account for phylogenetic uncertainty and within-species measurement error, we test whether any of the niche descriptors a) has phylogenetic signal; b) whether this signal corresponds to or deviates from BM; and c) if changes in evolutionary rates can be detected within *Sphagnum*.

Materials and Methods

Niche Differentiation

Peatland ecologists have noted the specificity of *Sphagnum* species along electrochemical and hydrological gradients for more than forty years (Clymo 1973; Vitt & Slack 1984; Andrus 1986), and ideally, we would have used the microhabitat data from all available studies. However, we chose to focus on three recent major surveys of

Sphagnum microhabitat specificity, in order to ensure consistent measurements, the largest selection of species, and the most modern *Sphagnum* taxonomy. The three large surveys of peatland ecology spanning Europe and Canada employed similar collection techniques. Each recorded data from eight niche descriptors (Height above Water Table (HWT), percent vascular plant cover (as an indicator of shade), pH, electrical conductivity (EC), nutrient concentrations (Ca, K, Mg, Na) and species abundance were measured. Each study represents a number of sites, and within each site, date was recorded for a number of 0.5 m^2 plots along transects. In each plot, the eight niche descriptors were recorded, as well as the presence and relative abundance of each species in the plot. Each plot, therefore, may represent a data point for one or more species.

The first survey covered 498 sites in eastern (2647 data points) and western (944 data points) Canada, with samples collected according to the procedures outlined in (Gignac *et al.* 2004). The second study also included two areas of Canada: 19 sites in Quebec and 4 sites in New Brunswick (1369 data points) and one area in Estonia (Europe) where 11 sites were surveyed (389 data points) (Pouliot 2011). The last study included 36 sites (714 data points across 29 mire complexes) in eastern Finland located in the mid boreal zone (Tahvanainen 2004). Two of the mire complexes were sampled intensively in a separate sub-study of 270 plots (258 data points) (Tahvanainen *et al.* 2002). Taken together, our data represents 6,533 observations of *Sphagnum* microhabitat, by far the most comprehensive data set of its kind.

The fusion of the three major studies allows us to be confident that if a species was not observed in any plots, it is not a major contributor to boreal peatland diversity in Canada or northern Europe. In total, 40 species were recorded, but we excluded *S.*

auriculatum because of low sample size (N=1), and we ended up with 39 species in the final data set. Data were summarized across the five studies by weighting the means and standard deviations of each species by percent cover of the sampled plots, i.e. giving more weight to plots where the species covered a larger area.

The niche descriptor (trait) for each species was transformed so that its mean was zero and its standard deviation across the genus was one. In addition to univariate descriptors, we also investigated the evolution of microhabitat niche in a multivariate sense, using a principal components transformation on all eight niche descriptors. The PCA scores from the first two ordinations (Fig 1) were included in the analyses below. The first two PC axes accounted for 47.3% and 17.7% of the total variance, respectively.

DNA Extraction, Amplification, and Sequencing

For each of the 39 *Sphagnum* species that were sampled for microhabitat data, we sampled representative DNA sequences from published sequences in GenBank and from a database maintained by AJS; most sequences have been submitted previously, newly generated samples are identified as such in Table S1. We also selected one sample each of *Flatbergium serecium* and *Eosphagnum inretortum*, representing early diverging members of class Sphagnopsida, to serve as outgroups (Shaw, Cox, *et al.* 2010a). We followed protocols described in (Shaw, Cox, & Boles 2003a) to sample sequences from the following genes, used for the phylogeny reconstruction: photosystem II (PSII) reaction center protein D1 (*psbA*), photosystem II (PSII) reaction center protein T (*psbT-H*), ribulose-bisphosphate carboxylase gene (*rbcL*), plastid ribosomal gene (*rpl16*), RNA polymerase subunit beta (*rpoC1*), ribosomal small protein 4 (*rps4*), tRNA(Gly) (UCC) (*trnG*), and the *trnL* (UAA) 59 exon- *trnF* (GAA) region (*trnL*) from the plastid genome; introns within NADH protein-coding subunits 5 and 7 (*nad5*,

nad7, respectively) from the mitochondrial genome; the nuclear ribosomal internal transcribed spacer region (ITS), two introns in the nuclear LEAFY/FLO gene (*LL* and *LS*), three anonymous nuclear regions (*rapdA*, *rapdB*, *rapdF*) and two sequenced nuclear microsatellite loci (*ATGc89* and *A15*) from the nuclear genome. Primer sequences for amplifying and sequencing the loci for most loci were provided in (Shaw, Cox, & Boles 2003a). For *rpoC1* we used primers described in the Royal Botanic Gardens, Kew, web page: DNA Barcoding, phase 2 protocols (http://www.kew.org/barcoding/protocols.html). For the two microsatellite-containing loci, we used primer sequences: A15— F: 5'TGTGGAGACCCAAGTGAATG3' R: 5'GGTGATGCTCAAAGGGCTTA3'; ATGc89— F: 5'CGTCGAACGGATTCAAAAAT3' R: 5'AGGGGAAGAGACCATCAGGT3'. We used the Duke University Sequencing facility for Sanger sequencing of all samples.

Phylogenetic Reconstruction

Individual genes were aligned using MUSCLE (v 3.8.31) (Edgar 2004) and adjusted manually using PhyDE (Muller *et al.* 2010). When concatenated, the dataset contained 14918 characters, of which 636 were parsimony informative (Table S1). To obtain ultrametric trees required for the phylogenetic comparative methods, we reconstructed the *Sphagnum* phylogeny via Bayesian inference on a concatenated 18-gene alignment, using BEAST (v 1.7.1) (Drummond *et al.* 2012). For each gene we chose a substitution model using the Bayesian Information Criterion from jModelTest (Guindon & Gascuel 2003; Posada 2008) (Table S1). Branch lengths were inferred using uncorrelated relaxed clock model and a lognormal branch length prior, one model for each gene separately. We confirmed convergence to the same joint posterior distribution by replicating the BEAST analysis (N=2). In each analysis, the chain ran for 200 million

generations, sampling every 10000 steps following a 20 million generation burnin. We summarized the 18,000 trees from the posterior distribution using a maximum credibility tree calculated by TreeAnnotator (v 1.7.1) (Drummond *et al.* 2012), with node heights set to the median branch lengths. In order to marginalize phylogenetic uncertainty (topology and branch lengths) in the comparative methods, we randomly selected 1000 trees from the posterior density for most analyses.

Evolution of Niches: Model Choice

Testing models of comparative evolution has become much easier through use of the statistical programming environment R, because all of the models can be implemented and connected using the R phylogenetic package ape (Paradis *et al.* 2004). On each ecological niche descriptor, we tested six models of evolution (Table 1). 1) White Noise (**WN**)-- the trait is evolving independently of the phylogeny; this represents our baseline model. Practically, all internodes on the phylogeny are set to zero length, creating a star phylogeny—all trait evolution occurs at the tips, and phylogeny and trait variance are completely unrelated. By using WN as a baseline, we assert that alternative models (below) must demonstrate better fit to the data than a model where the phylogeny does not contribute to trait evolution. Any model with a sample-size corrected Akiake Information Criterion (AICc) score exceeding the score for WN is not a plausible alternative.

2) Brownian motion (**BM**)-- the trait increases in variance through evolutionary time at a constant rate (beta). Although this is the standard phylogenetic comparative model, signal may be masked by several other patterns of trait evolution, which are addressed with three additional models. 3) **Lambda** model (Pagel 1994)— the trait has

phylogenetic signal, but deviates from a pure Brownian motion process. Specifically, the covariance between branch lengths and trait difference is multiplied by a scalar, which is inferred via maximum likelihood. The WN model (lambda = 0) and the BM model (lambda = 1) are nested within the lambda model, in which lambda is inferred as a free parameter. Values between 0 and 1 correspond to an "imperfect" Brownian motion model, where only some proportion (lambda) of the trait variance can be explained by phylogeny. 4) **Delta** model (Pagel 1997) – All node depths are raised to the power delta—values less than one provide evidence that much/most trait evolution occurred deep (early) in the phylogeny, while values greater than one indicate trait evolution concentrated in the tips. 5) Ornstein-Uhlenbeck (**OU**) model (Martins & Hansen 1997)—although the trait is evolving like BM, evolution is constrained by a strength parameter (alpha), causing the trait to trend towards an optimum value (theta). The BM model is also nested within the OU model (alpha = 0). We tested the WN, BM, and lambda models using the R package phytools (Revell 2011), the delta model with geiger (Harmon *et al.* 2011), and the OU model was tested using the pmc_fit method of the package pmc (Beaulieu *et al.* 2012).

Rate changes within Sphagnum

All of the methods above assume constant conditions on the entire *Sphagnum* phylogeny. To incorporate the possibility of different rates of niche evolution within the tree, which would mask the pattern when considering the entire genus, we used two different methods. In our first approach, we pruned the phylogeny to contain only members of subgenera *Acutifolia* and *Cuspidata*, which represented the two largest subgenera sampled. Every branch on the phylogeny was classified as an *Acutifolia* or a

Cuspidata lineage. We tested whether a model allowing different rates of niche evolution in the two lineages (**BM2**) was supported over a single-rate model (**BM1**, the "Brownie" model; (O'Meara *et al.* 2006)), using the brownie.lite method in phytools. We also tested whether an OU model with different trait optima for the *Acutifolia* and *Cuspdiata* lineages (**OU2**) was supported over a single-optimum **OU1** model, using pmc.

In our second approach, we used a Bayesian MCMC approach on the full *Sphagnum* phylogeny to identify nodes where rate changes have occurred (Revell *et al.* 2012). This method samples evolutionary rates and locations of exceptional rate shifts in proportion to their posterior probability, which does not require any *a priori* hypothesis about the location of rate shifts. We ran the MCMC implemented in phytools, under the default priors for evolutionary rate and proposal frequency, for 10,000 generations, sampling every 100 generations (the first 20 samples were discarded as burnin).

Within-Species Error and Phylogenetic Uncertainty

Many sources of error exist in the estimation of mean trait values for species, and phylogenetic comparative methods are improved when they account for measurement error (Ives *et al.* 2007). For each niche descriptor, we used methods in phytools for the Brownian motion, lambda, and delta models to incorporate measurement error (standard deviation and sample size); incorporation of measurement error is not implemented in pmc_fit, so it is absent from the OU models.

We conducted a sensitivity analysis to ask whether the wide variance in sampling frequency among *Sphagnum* species affects model selection. Using the maximum credibility tree from BEAST, we analyzed each model for each descriptor 39

times, deleting one *Sphagnum* species each time. We compared the support for each model on the reduced trees to the WN model to assess the sensitivity for each descriptor.

Most phylogenetic comparative methods also unrealistically assume that the tree (topology and branch lengths) is known without error. To incorporate phylogenetic uncertainty into the model fitting procedure, we tested each model on 1000 trees randomly sampled from the BEAST posterior distribution. We recorded, for each descriptor and tree, the AICc scores for each model. The distribution of the AICc scores for each model and descriptor is an indication of model fit, averaged over phylogenetic uncertainty (Boucher *et al.* 2012). For the Bayesian MCMC approach, we used only the maximum credibility tree from BEAST.

For descriptors found to have significant phylogenetic signal, we used a phylogenetic generalized least squares (PGLS) model (Freckleton *et al.* 2002) to compare these descriptors for correlated evolution, using the R package caper (Orme *et al.* 2011).

Results

Niche differentiation

There is a large variation among species in sample sizes, from three (*S. wulfianum*) to 1055 (*S. girgensohnii*), reflecting the relative abundances of species in the study sites (Table 4). Standard deviation was lowest for pH (Table 5) and highest for shade (Table 4). The PCA analysis of the niche data (Figure 11) shows that PC1 is predominantly "pH - ionic gradient" while PC2 reflects a "hummock-hollow gradient". The covariation of Shade and HWT (Height above Water Table) mainly reflects the abundance of dwarf-shrubs on hummocks and the relative scarcity of vascular plants in hollow habitats. The differentiation among subgenera shows that *Acutifolia* are largely hummock species (higher on PC2), and *Cuspidata* largely hollow inhabitants (lower on

PC2), but there are some species deviating from this general pattern. For example, *S. subfulvum* (subgenus *Acutifolia*) has a high PC2 score, while *S. flexuosum* (subgenus *Cuspidata*) is low on that scale. The subgenus *Sphagnum* is quite variable in HWT, and the four species with highest PC1 scores (pH) all represent different subgenera.

Table 4. Subgenus assignment, microhabitat preference for hydrological traits, and average sample size across all microhabitat traits in 39 species of *Sphagnum*.

Species	Subgenus	Percent Shade	Height Above Water Table	Average Sample Size
Sphagnum affine	*Sphagnum*	25.18 (13.71)	29.98 (9.2)	6
Sphagnum angermanicum	*Acutifolia*	62.75 (36.18)	10.5 (1.5)	3
Sphagnum angustifolium	*Cuspidata*	34.09 (27.05)	19.21 (12.86)	712.4
Sphagnum annulatum	*Cuspidata*	9.62 (7.9)	6.69 (3.95)	50.9
Sphagnum aongstroemii	*Acutifolia*	20.05 (25.28)	12.6 (4.13)	2.9
Sphagnum austinii	*Sphagnum*	12.9 (22.34)	48.62 (9.86)	34.3
Sphagnum balticum	*Cuspidata*	15.25 (13.27)	7.69 (4.29)	122.3
Sphagnum capillifolium	*Acutifolia*	27.52 (22.18)	22.96 (16.48)	768
Sphagnum centrale	*Sphagnum*	41.38 (23.06)	17.93 (14.49)	20.4
Sphagnum compactum	*Cuspidata*	22.14 (17.07)	8.07 (5.14)	20.4
Sphagnum contortum	*Subsecunda*	25.87 (15.75)	6.75 (8.23)	18.6
Sphagnum cuspidatum	*Cuspidata*	20.34 (23.33)	2.9 (4.59)	139.8
Sphagnum fallax	*Cuspidata*	26.68 (24.85)	15.43 (19.78)	323.9
Sphagnum fimbriatum	*Acutifolia*	49.17 (29.6)	21.55 (11.15)	23.9
Sphagnum flavicomans	*Acutifolia*	28 (21.06)	22.6 (17.09)	85
Sphagnum flexuosum	*Cuspidata*	39.21 (24.33)	19.29 (10.78)	17.3
Sphagnum fuscum	*Acutifolia*	38.06 (24.04)	33.28 (15.68)	1055.5
Sphagnum girgensohnii	*Acutifolia*	95.38 (74.25)	28.62 (23.23)	13.6

Sphagnum jensenii	*Cuspidata*	6.83 (10.99)	5.19 (2.3)	82.4
Sphagnum lenense	*Cuspidata*	8.05 (9.17)	28.33 (31.75)	4
Sphagnum lindbergii	*Cuspidata*	8.99 (12.73)	6.16 (4.59)	47.9
Sphagnum magellanicum	*Sphagnum*	32.91 (30.06)	18.86 (11.69)	1013.4
Sphagnum majus	*Cuspidata*	11.1 (14.19)	2.65 (6.38)	196.1
Sphagnum obtusum	*Cuspidata*	36.13 (15.27)	9.59 (2.93)	7.6
Sphagnum pacificum	*Cuspidata*	4.89 (2.02)	20.64 (2.91)	16
Sphagnum papillosum	*Sphagnum*	14.89 (16.08)	13.05 (8.66)	341.9
Sphagnum platyphyllum	*Subsecunda*	8.51 (12.9)	0.61 (3.02)	38.9
Sphagnum pulchrum	*Cuspidata*	14.99 (13.42)	5.48 (2.88)	129.5
Sphagnum riparium	*Cuspidata*	27.11 (25.64)	7.47 (13.99)	45.9
Sphagnum rubellum	*Acutifolia*	48.49 (34.73)	24.71 (18.45)	457.9
Sphagnum russowii	*Acutifolia*	38.65 (20.08)	19.94 (10.44)	145.8
Sphagnum squarrosum	*Acutifolia*	48.16 (45.8)	13.08 (6.27)	16
Sphagnum subfulvum	*Acutifolia*	22.34 (19.29)	13.64 (8.33)	19.6
Sphagnum subnitens	*Acutifolia*	44.41 (30.63)	9.43 (3.6)	10.1
Sphagnum subsecundum	*Subsecunda*	32.55 (34.34)	9.83 (4.58)	62.4
Sphagnum tenellum	*Cuspidata*	18.69 (14.35)	5.55 (3.51)	47.6
Sphagnum teres	*Acutifolia*	26.94 (28.05)	13.15 (7.93)	35.8
Sphagnum warnstorfii	*Acutifolia*	40.46 (26.64)	21.22 (14.55)	227.9
Sphagnum wulfianum	*Acutifolia*	66.11 (22.16)	31.42 (9.6)	2.3

Table 5 *Sphagnum* microhabitat preferences for pH and micronutrients; mean and standard deviation across all studies.

Species	pH	EC	Ca	Mg	Na	K

Sphagnum affine	4.4 (0.58)	30.88 (10.07)	1 (0.45)	0.27 (0.14)	1.06 (0.34)	0.6 (0.14)
Sphagnum angermanicum	4.63 (0.74)	0.03 (n/a)	0.51 (0.07)	0.34 (0.25)	6.14 (2.75)	0.22 (0.1)
Sphagnum angustifolium	4.47 (0.76)	33.46 (38.23)	2.67 (5.76)	0.89 (1.42)	1.69 (2.17)	1.09 (1.61)
Sphagnum annulatum	4.46 (0.4)	29.14 (34.68)	0.87 (0.4)	0.53 (0.3)	1.42 (1.09)	0.68 (0.51)
Sphagnum aongstroemii	4.86 (0.16)	18.04 (0.88)	1.44 (0.58)	0.72 (0.07)	1.29 (0.05)	0.45 (0.22)
Sphagnum austinii	4.26 (0.76)	38.95 (28.68)	1.63 (0.92)	0.64 (0.29)	3.81 (1.46)	1.33 (1.07)
Sphagnum balticum	4.11 (0.21)	41.42 (41.47)	1.14 (0.61)	0.53 (0.25)	1.6 (1.03)	1.21 (1.26)
Sphagnum capillifolium	4.1 (0.41)	21.48 (28.67)	0.77 (2.41)	0.23 (0.55)	1.78 (2.37)	0.42 (0.5)
Sphagnum centrale	5.75 (0.68)	51.57 (23.23)	7.67 (3.21)	1.27 (0.59)	1.75 (1.94)	1.3 (0.57)
Sphagnum compactum	4.13 (0.36)	13.21 (16.04)	1.17 (0.57)	0.45 (0.21)	2.07 (1.52)	1.2 (1.18)
Sphagnum contortum	6.23 (0.49)	147.67 (51.74)	18.46 (9.43)	3.22 (1.19)	4.73 (1.44)	1.29 (0.31)
Sphagnum cuspidatum	3.99 (0.2)	45.94 (35.02)	1.22 (1.53)	0.52 (0.71)	2.4 (2.63)	1.19 (1.13)
Sphagnum fallax	4.39 (0.5)	25.45 (27.07)	0.92 (1.03)	0.26 (0.31)	1.09 (2.06)	0.65 (0.9)
Sphagnum fimbriatum	4.81 (0.73)	76.73 (63.63)	7.86 (11.12)	1.96 (1.23)	2.95 (4.19)	1.77 (1.17)
Sphagnum flavicomans	4.33 (0.57)	12.1 (15.33)	0.61 (0.4)	0.3 (0.25)	3.55 (2.81)	0.25 (0.25)
Sphagnum flexuosum	4.92 (0.75)	24.08 (25.1)	2.1 (2.57)	0.43 (0.46)	2.11 (1.37)	1.05 (1.04)
Sphagnum fuscum	4.36 (0.83)	47.72 (82.37)	4.31 (14.47)	1.14 (2.85)	2.23 (2.58)	1.01 (1.56)
Sphagnum girgensohnii	4.63 (0.81)	49.62 (25.97)	2.77 (1.98)	1.29 (0.96)	3.97 (5.2)	1.04 (0.61)
Sphagnum jensenii	4.31 (0.22)	12.68 (7.81)	1.14 (2.13)	0.6 (0.34)	1.23 (0.53)	0.58 (0.5)
Sphagnum lenense	3.82 (0.1)	61.28 (6.37)	2.03 (0.09)	0.15 (0.07)	1.2 (0.1)	1.17 (0.22)
Sphagnum lindbergii	4.57 (0.52)	23.32 (24.62)	1.96 (2.04)	0.64 (0.63)	2.88 (3.07)	0.75 (0.91)
Sphagnum magellanicum	4.15 (0.47)	41.31 (37.8)	1.27 (1.94)	0.51 (0.77)	2.12 (2.99)	1.09 (1.19)
Sphagnum majus	4.41 (0.49)	28.16 (26.29)	0.96 (0.8)	0.37 (0.31)	1.6 (2.41)	0.96 (1.12)
Sphagnum obtusum	5.19 (0.57)	93.32 (32.89)	10.51 (1.41)	1.75 (0.41)	3.43 (1.04)	2.18 (0.33)
Sphagnum	4.22	45.09	3.57	1.36	3.59	2.2

pacificum	(0.23)	(16.13)	(0.83)	(0.44)	(0.92)	(0.29)
Sphagnum papillosum	4.73 (0.57)	23.99 (24.06)	1.85 (2.47)	0.6 (0.6)	2.42 (2.41)	0.84 (0.96)
Sphagnum platyphyllum	4.67 (0.34)	22.39 (16.2)	1.25 (0.67)	0.67 (0.31)	1.32 (0.72)	0.67 (0.32)
Sphagnum pulchrum	4.66 (0.59)	13.62 (18.23)	0.93 (0.55)	0.35 (0.23)	1.43 (1.8)	0.59 (0.67)
Sphagnum riparium	4.57 (0.53)	52.7 (20.12)	2.84 (1.99)	1.09 (1.2)	2.38 (1.52)	7.6 (8.68)
Sphagnum rubellum	3.98 (0.57)	46.48 (35.17)	1.25 (1.89)	0.53 (0.7)	3 (3.8)	1.03 (0.96)
Sphagnum russowii	4.66 (0.62)	30.45 (27.98)	2.01 (2.49)	0.73 (0.86)	1.74 (2.17)	0.99 (1.16)
Sphagnum squarrosum	5.13 (0.92)	60.86 (28.09)	17.97 (19.38)	1.7 (1.39)	3.57 (5.97)	0.28 (0.31)
Sphagnum subfulvum	5.88 (0.86)	21.99 (4.14)	6.22 (7.14)	3.1 (3.33)	2.47 (1.53)	1.22 (0.8)
Sphagnum subnitens	5.7 (0.33)	16.34 (3.78)	1.56 (1.56)	0.83 (0.22)	1.48 (1.16)	0.06 (0.06)
Sphagnum subsecundum	5.27 (0.8)	42.5 (33.76)	2.17 (3.69)	0.86 (0.49)	2.75 (1.76)	0.96 (1.1)
Sphagnum tenellum	4.48 (0.78)	28.9 (29.6)	0.55 (0.37)	0.43 (0.23)	2.26 (1.16)	0.69 (0.73)
Sphagnum teres	4.58 (0.97)	99.41 (67.49)	4.62 (9.34)	1.13 (2.07)	2.82 (2.42)	2.33 (1.54)
Sphagnum warnstorfii	5.75 (0.96)	122.5 (112.97)	14.09 (17.54)	3.89 (4.63)	3.45 (3.25)	3.62 (5.96)
Sphagnum wulfianum	5.54 (0.23)	59.13 (7.02)	6.11 (4.03)	0.07 (n/a)	1.39 (n/a)	1.15 (n/a)

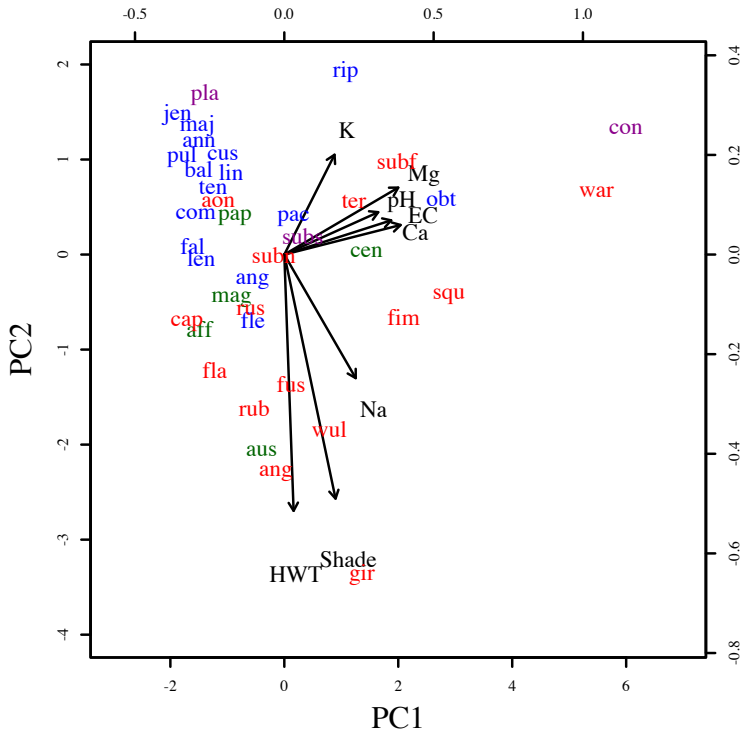

Figure 11 Biplot of Principal Components Analysis (PCA) for eight microhabitat preferences in 39 species of *Sphagnum*. Each species is plotted in Euclidian space for the first two principal components, which cumulatively represent 65.0% of the total variance. Loadings upon each axis are indicated by arrows and lines—PC1 is a "pH - ionic gradient" while PC2 is predominantly a "hummock-hollow" gradient. Species are colored by subgenus: *Sphagnum* (green), *Subsecunda* (purple), *Acutifolia* (red), *Cuspidata* (blue). Key to species abbreviations: aff- *S. affine*, anf- *S. angustifolium*, ang- *S. angermanicum,* ann- *S. annulatum*, aon- *S. aongstroemii*, aus- *S. austinii*, bal- *S. balticum*, cap- *S. capillifolium*, cen- *S. centrale*, com- *S. compactum*, con- *S. contortum*, cus- *S. cuspidatum*, fal- *S. fallax,* fim- *S. fimbriatum*, fla- *S. flavicomans*, fle- *S. flexuosum*, fus- *S. fuscum*, gir- *S. girghensonii,* jen- *S. jensenii*, len- *S. lenense*, lin- *S. lindbergii,* mag- *S. magellanicum*, maj- *S. majus*, obt- *S. obtusum*, pac- *S. pacificum,*pap- *S. papillosum*, pla- *S. platyphyllum*, pul- *S. pulchrum,* rip- *S. riparium*, rub- *S. rubellum*, rus- *S. russowii*, squ- *S. squarrosum*, subf- *S. subfulvum,* subn- *S. subnitens,* subs- *S. subsecundum*, ten- *S. tenellum*, ter- *S. teres*, war- *S. warnstorfii*, wul- *S. wulfianum*

Phylogenetic Reconstruction

Each gene in the DNA sequence matrix had varying amounts of missing data, ranging from 2 sequences missing (ITS) to 29 (nad5 and nad7) while sampling for each species ranged from two genes to the full eighteen (Table 6). The maximum credibility

tree from the Bayesian inference, using BEAST, is presented in Figure 12. The amount of missing data in the alignment does not appear to deflate support for the maximum credibility tree. All major subgenera are resolved at 99% posterior probability or greater, while relationships among subgenera are less supported. This is consistent with previous reconstructions of *Sphagnum* phylogeny when both chloroplast and nuclear genomes are used (Shaw, Cox, *et al.* 2010a). Notably, among-subgenera median branch lengths are very short; therefore, comparative methods that consider only phylogenetic distance (and not topology) should be relatively unaffected by topological uncertainty.

Table 6 Gene location, length, variability, and preferred substitution models for 18 loci in phylogenetic reconstruction.

Gene	Location	BIC Model	Length (bp)	Informative Characters	Number of Sequences
nad5	Mitochon.	HKY+I	941	1	**13**
nad7	Mitochon.	K80	952	3	**13**
A15	Nuclear	K80+I	698	62	**18**
ATGc89	Nuclear	K80	611	54	**15**
ITS	Nuclear	HKY+G	826	110	**40**
LL	Nuclear	HKY+G	550	33	**22**
LS	Nuclear	HKY	394	11	**21**
rapdA	Nuclear	K80+1	1204	80	**20**
rapdB	Nuclear	HKY+G	763	44	**22**
rapdF	Nuclear	HKY+G	604	54	**22**
psbA	Chloroplast	HKY+I	984	7	**13**
psbT	Chloroplast	HKY+G	530	18	**36**
rbcL	Chloroplast	HKY+G	1364	25	**37**
rpl16	Chloroplast	HKY+I	926	19	**23**
rpoC1	Chloroplast	KHY+G	814	6	**23**
rps4	Chloroplast	HKY	1028	26	**36**
trnG	Chloroplast	HKY+G	853	48	**38**
trnL	Chloroplast	HKY	876	35	**39**

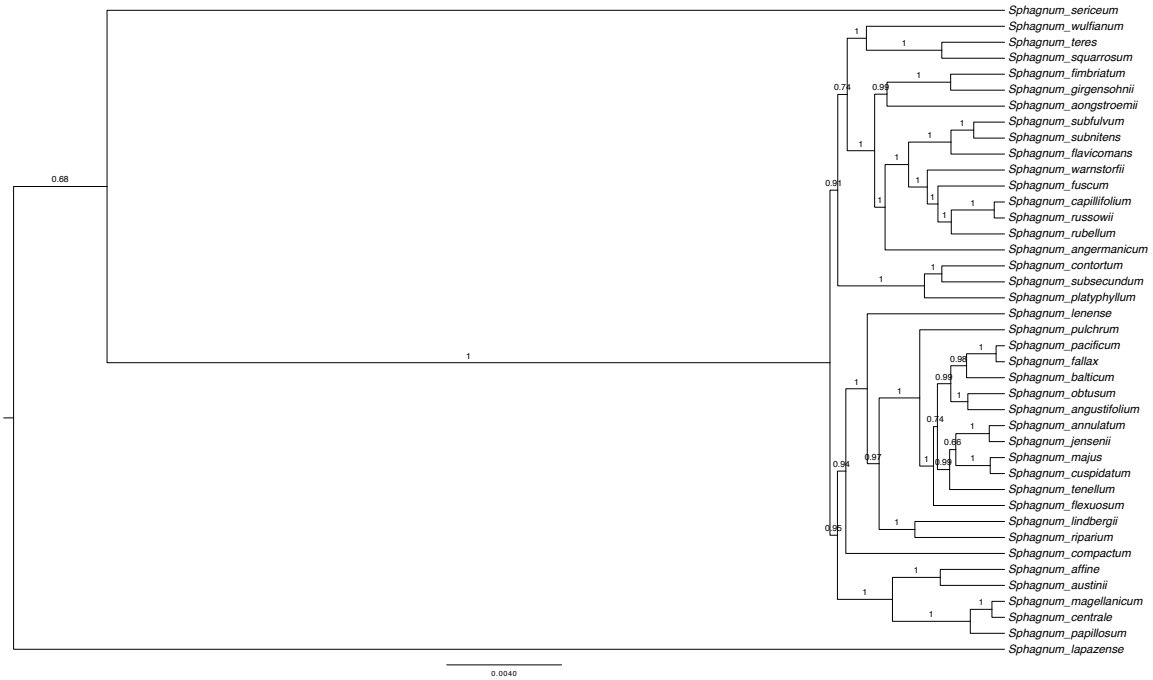

Figure 12. Maximum credibility tree from BEAST analysis, created using TreeAnnotator. Node labels are posterior probabilities, and node heights are the median heights for each clade in 18,000 posterior trees. *Eosphagnum* and *Flatbergium* were used as outgroups; monophyly of *Sphagnum* was enforced.

Full Dataset: Model Choice

For five of the six ionic descriptors (pH, Ca, Mg, Na and K), the model preferred by AICc across all 1000 trees was **WN**, indicating a lack of phylogenetic signal for these descriptors (Table 7). These descriptors contribute heavily to the first principal component (Figure 11), the evolution of which also is best fit by the white noise model (Table 7, Figure 13a). A few descriptors, such as Ca and K, show very narrow distributions in AICc score for the **lambda** model across the tree sample (Figure 13a). In these cases, the values of lambda inferred are very close to zero, providing additional evidence for lack of phylogenetic signal in these descriptors. On 79.7% of the trees, AICc chose **delta** model over **WN** for Electrical Conductivity (EC). Values of delta ranging

from 2.33 to 18.51 suggest microhabitat evolution is extremely concentrated at the tips of the trees—as the value of delta increases to infinity, the delta model collapses to the WN model.

For pH, inferred lambdas range from 0.17 to 0.40, but **lambda** never exceeded **WN** in AICc on any of the 1000 trees. Additionally, a likelihood ratio test between **lambda** and **WN** on each tree fails to achieve significance at the $p < 0.05$ level on any tree (results not shown).

In contrast, models with phylogenetic signal are unambiguously a better fit than white noise for two traits—percent cover (shade) and height above water table (Figure 13a, Table 7). The **lambda** model is the best fit for shade, with values of lambda ranging from 0.50 to 0.71. None of the other models surpass **WN** in AICc score for shade. Among the univariate traits, height above water table (HWT) shows the highest support for phylogenetic signal. The best model was **BM** with a single rate across *Sphagnum*, although all models tested have better AICc scores than **WN**. The distributions of AICc scores for shade, HWT, and PC2 (the hummock-hollow gradient) all indicate phylogenetic signal is strongly supported on all 1000 trees (Figure 13a).

Sensitivity analyses indicate that the data are generally robust to influence from individual species. In nearly all cases, the AICc score difference between a model and **WN** changes very little, and we never observe a model losing support after deletion of individual species (Figure 3). There are two exceptions: deletion of either *S. magellanicum* or *S. centrale* results in support for the **OU** model for pH, each of which showed a ∂AICc > 7, compared to **WN** (Figure 13a).

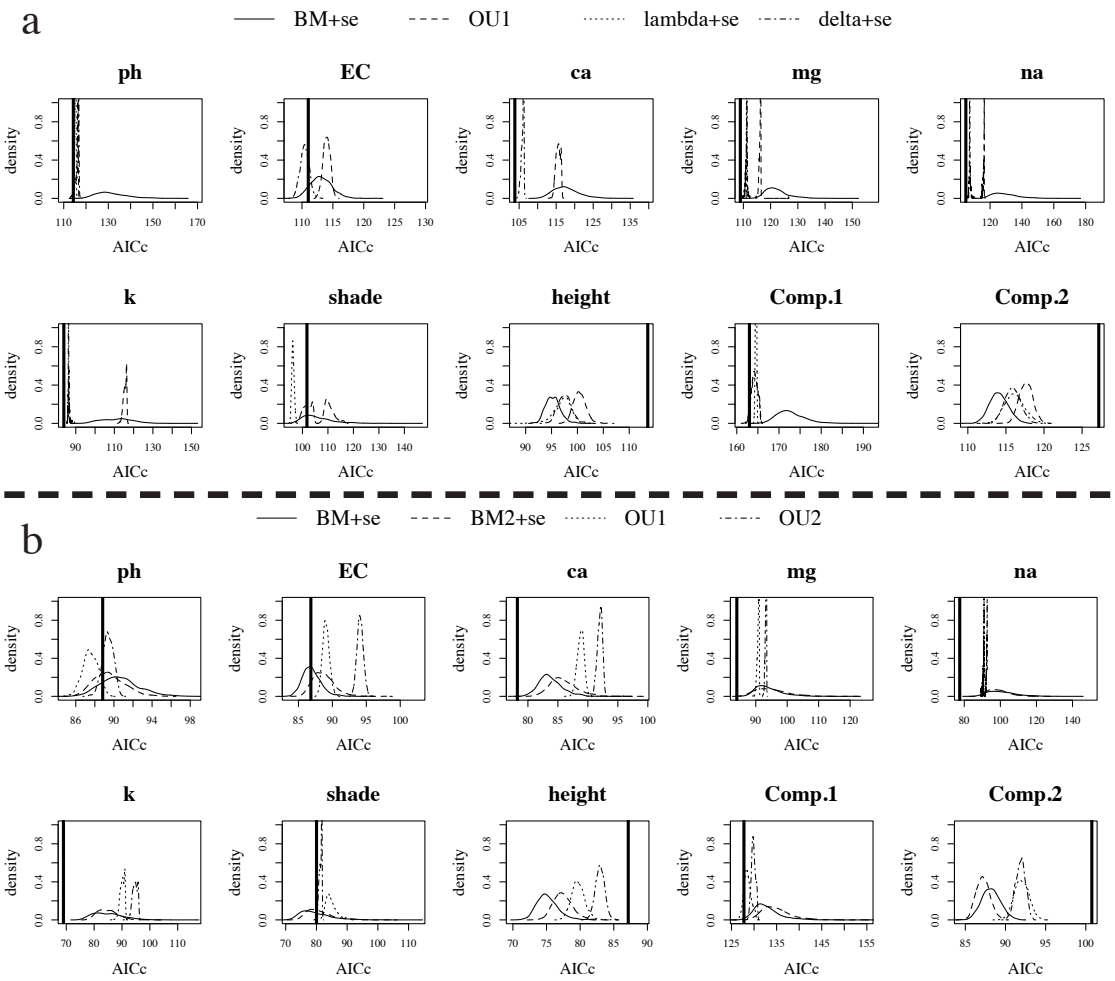

Figure 13. Model choice using AICc distributions for five models of continuous trait evolution, on two niche descriptors (shade and HWT) and the second principal component (hummock-hollow gradient) across 1000 trees. a) The full dataset (all of *Sphagnum*). For each niche descriptor, the distributions in AICc scores are shown for Brownian Motion (BM), Ornstein-Uhlenbeck stabilizing selection model (OU), and adjusted models of Brownian motion, lambda and delta. b) The reduced dataset (subgenera *Acutifolia* and *Cuspidata* only), used to detect changes in niche preference evolution within the genus. For each niche descriptor, the AICc curves for BM1 (one rate) vs BM2 (separate rates) and OU1 (one optimum) vs OU2 (separate optima) are plotted. In each plot, the thick black line for indicates the AICc score for White Noise (no phylogenetic signal). Lower AICc scores are better; descriptors with AICc distributions falling mostly or entirely to the left of the WN line are preferred.

Without phylogenetic correction, the species means for shade and HWT are significantly positively correlated ($t = 2.55$, $r^2 = 0.13$, $p = 0.015$). To assess whether this correlation is explained by phylogeny, we tested for correlated evolution between shade and height using a PGLS approach in caper, where lambda was a free parameter, using the maximum credibility tree, but this was not significant ($t = 1.92$, $r^2 = 0.07$, $p = 0.062$). Because the correlation disappears when accounting for phylogeny, the small but significant correlation observed between shade and HWT is derived from phylogenetic signal.

Table 7 Model selection for trait evolution using AICc in eight niche descriptors and two microhabitat gradients on the full dataset. Mean AICc scores from 1000 BEAST trees shown (s.d. in parentheses). The preferred model, with the lowest AICc score, is indicated in bold.

	Full Dataset				
Trait	**WN**	**BM**	**OU**	**lambda**	**delta**
pH	**114.3**	133.8 (8.4)	115.9 (0.7)	115.3 (0.2)	116.2 (0.7)
EC	**111**	117 (2.1)	114 (0.6)	113.4 (0)	110.4 (0.7)
Ca	**103.8**	123.3 (3.3)	115.5 (0.6)	106.2 (0)	105.9 (0.4)
Mg	**108.9**	128.9 (4.2)	116.2 (0.5)	111.2 (0)	111.2 (0.2)
Na	**104.8**	133.4 (9.6)	116.2 (0.3)	107.1 (0)	107.3 (0.4)
K	**83.8**	118.6 (5.6)	115.5 (0.8)	86.2 (0)	86.3 (0.2)
Shade	101.8	112.1 (4.8)	110.7 (1.9)	**96.3 (0.5)**	101.9 (1.7)
HWT	113.6	**95.5 (1.7)**	100.4 (1.2)	97.1 (1.7)	97.6 (1.3)
PC1	**163**	172.7 (3.3)	164.2 (0.7)	164.6 (0.1)	164.2 (0.7)
PC2	127.2	**114.1 (1.2)**	117.6 (0.9)	116.1 (1.3)	115.9 (1)

Rate Change Within Sphagnum

The reduced dataset used to investigate rate changes contains only species from subgenera *Acutifolia* (17 species) and *Cuspidata* (13 species). For the eight niche descriptors and PC1, neither the **OU2** model nor the **BM2** models were supported

(Figure 13b, Table 8). On PC2, however, 91% of the trees supported the **BM2** model over the **BM1** model in the reduced dataset with an average ∂AICc of 1.01 (both models were always better than **WN,** Figure 13b). The **BM2** model for PC2 inferred a mean evolutionary rate of 496.6 (range 218.4 – 1233.5) for subgenus *Acutifolia* and a mean evolutionary rate of 177.8 (range 80.6 – 516.11) for subgenus *Cuspidata*. A paired Student's t-test indicates high support for separate rates of PC2 evolution between the subgenera (mean rate difference: 318.8, $p < 0.0001$).

Although there was no support for an **OU2** model for pH, the **OU1** model was supported in the reduced dataset—953 of the 1000 trees had better AICc scores for the **OU1** model than for **WN** (Table 8). The **OU** model was not supported in the full dataset; but as noted, the **OU** model was supported when either *S. magellanicum* or *S. centrale* were deleted in the sensitivity analysis (arrow in Figure 14). Both species are in subgenus *Sphagnum*, and were therefore not included in analysis of the reduced dataset.

Table 8. Model selection for trait evolution using AICc in eight niche descriptors and two microhabitat gradients on the reduced dataset.Mean AICc scores from 1000 BEAST trees shown (s.d. in parentheses). The preferred model, with the lowest AICc score, is indicated in bold. These models test for evidence of separate rates of evolution (BM1 vs BM2) or for separate optimum niche preference values (OU1 vs OU2) between subgenus *Acutifolia* versus subgenus *Cuspidata*

	Reduced Dataset				
Trait	**WN**	**OU1**	**OU2**	**BM1**	**BM2**
pH	88.8	**87.5 (0.8)**	89.4 (0.6)	90.6 (2)	89.5 (1.6)
EC	**86.8**	89.1 (0.5)	94.1 (0.5)	87 (1.4)	88.7 (1.6)
Ca	**78.2**	88.8 (0.6)	92 (0.4)	83.8 (2)	85.9 (2.2)
Mg	**84**	90.8 (0.3)	93.3 (0.2)	94 (4.2)	95 (5.1)
Na	**77.5**	91 (0.2)	92.6 (0.2)	100.9 (7.9)	99 (5.6)
K	**69**	90.1 (0.8)	94.7 (0.9)	85 (5.4)	84.8 (3.5)
Shade	80	84.8 (1.8)	81.3 (0.5)	**79.7 (5.2)**	80.8 (4)
HWT	87.2	79.7 (1)	82.9 (0.7)	**75.1 (1.6)**	77.4 (1.4)
PC1	**127.8**	128.5 (0.8)	129.9 (0.5)	132.9 (3.1)	134.9 (3.2)
PC2	100.8	92 (0.9)	91.9 (0.6)	88.2 (1.2)	**87.2 (0.9)**

The Bayesian MCMC approach to identifying exceptional evolutionary rate changes within a phylogeny produces posterior probabilities for each node on the tree for each niche descriptor and microhabitat gradient. Only four descriptors had nodes with posterior probabilities exceeding 10%. For pH, a rate change was supported within subgenus *Sphagnum*, either on the branch leading to *S. centrale* and *S. magellanicum* (29%) or on an immediately ancestral branch including *S. papillosum* (43%) (Figure 15). Rate changes were moderately supported on the terminal branches leading to *S. contortum* for EC (33%) and the clade containing *S. fallax* and *S. pacificum* for Na (46%) Finally, there is support for a rate change in K, either on a terminal branch leading to *S. riparium* (37%) or on the immediately ancestral branch that includes *S. lindbergii* (41%). No rate change was found for PC2, either in the full dataset or in the reduced dataset.

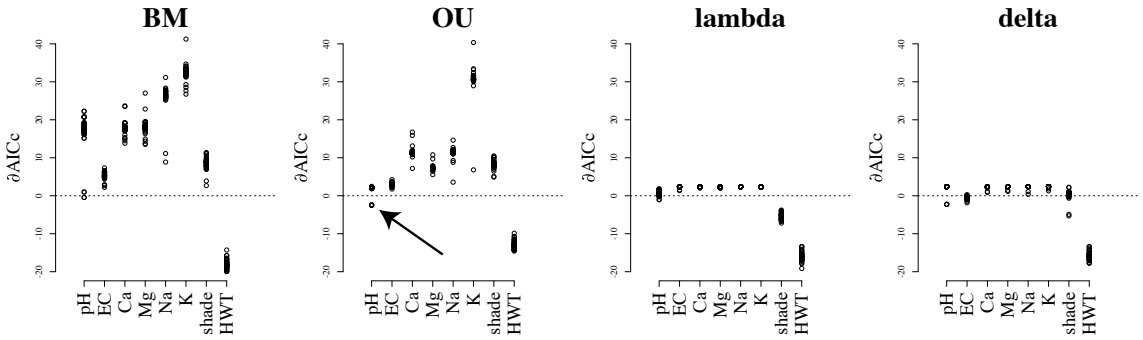

Figure 14. Sensitivity analysis for model selection in the full *Sphagnum* dataset. Each panel shows one of the four models of evolution at each of the eight niche descriptors (see Table 1 for a key to models). Each point represents the support for the model when individual species were removed from the maximum credibility tree. The y-axis is the ∂AICc score compared to WN (no phylogenetic signal). The arrow indicates two points, representing *S. magellanicum* and *S. centrale*. When either of these species is deleted, AICc supports the OU model (single optimum preference in *Sphagnum*) for pH.

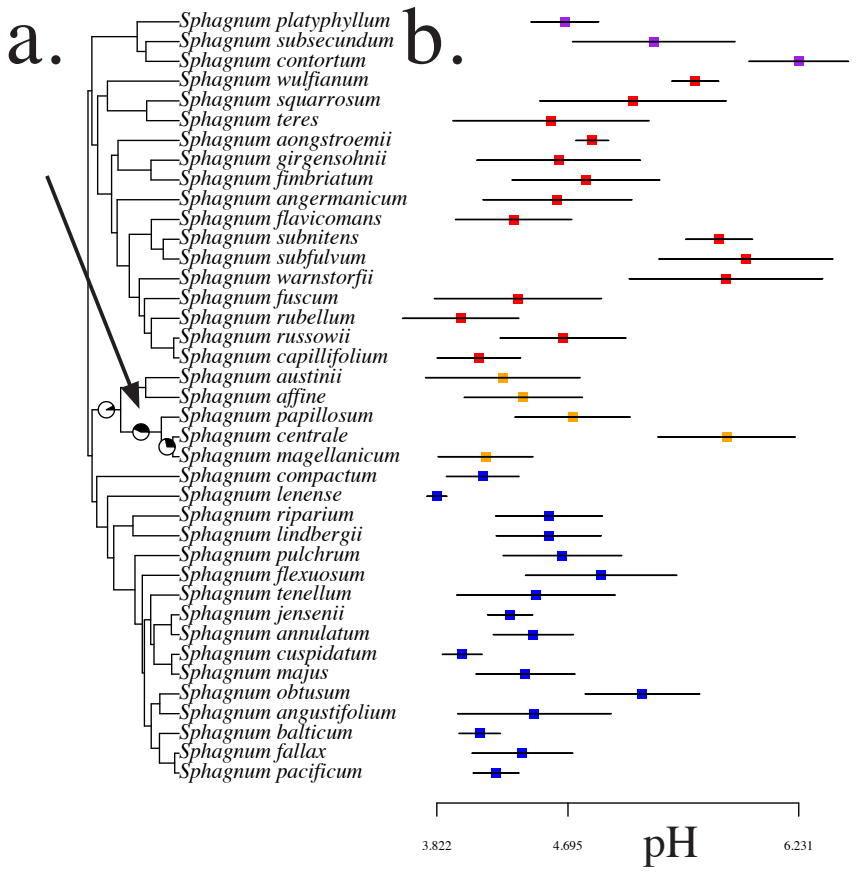

Figure 15. Evidence for exceptional rate change in evolution of pH preference in *Sphagnum*. a) Evidence of extreme pH preference shift via Bayesian MCMC (Revell *et al.* 2012)—pie charts indicate nodes receiving at least 10% posterior probability for a rate change. Black portions of each pie chart represent the support for a rate change at that node. The arrow indicates a 77% posterior probability for a rate change in subgenus *Sphagnum*. c) Phylogenetic diversity of pH preference breadth in *Sphagnum*, demonstrated by means (squares) and standard deviations (lines).

Discussion

Demonstration of phylogenetic signal in *Sphagnum* microhabitat preferences is a vital first step in elucidating the evolution of peatland ecology. Multiple comparative models of trait evolution were considered because departure from a pure Brownian motion process may be masked by several factors, such as changes in evolutionary rate. Microhabitat descriptors in *Sphagnum* fall into two general categories (Figure 11): the

abiotic electrochemical gradient (pH and ions), and the biotic "hummock-hollow" gradient (HWT and Shade). We tested the assumption of hierarchical inheritance of eight microhabitat descriptors and the two multivariate gradients along the *Sphagnum* phylogeny.

Our results clearly show the presence of phylogenetic signal in relation to the hummock/hollow gradient. Species in the major subgenera of *Sphagnum* are generally differentiated along this gradient. We find evidence for rate change in a multivariate niche gradient (encompassing shade and HWT) that suggests a higher rate of niche evolution in subgenus *Acutifolia*, which contains mostly hummock species, than in subgenus *Cuspidata*, which contains mostly hollow species. The strength of the phylogenetic signal indicates that across trees in the dataset, microhabitat preference for height is maintained within, as well as among subgenera. Shade preferences are also phylogenetically correlated, and it is possible that this is an artifact of the hummock-hollow preference—ligenous angiosperms are more likely to grow (and provide shade) in hummocks, compared to the wetter depressions (Rydin 2006). However, we did not detect a significant correlation between height and shade using PGLS model, where phylogeny is accounted for.

We find additional support for a rate shift in relation to the PC2 axis, a multivariate niche gradient encompassing shade and HWT (Figure 11). Subgenus *Acutifolia* appears to be evolving faster relative to PC2 than is subgenus *Cuspidata*. The two rates of evolution found in this analysis partially corroborate results from trait analyses related to moisture availability. Plants that invest in the capitulum relative to the stem maximize photosynthesis and growth at the individual level, but are loosely packed and grow close to the water table ((Rice *et al.* 2008), Laing et al. in review).

Conversely, plants with small capitula grow higher above the water table because they are able to tolerate water stress by growing densely packed. The driver behind this trade-off is probably related to the water flux and the need to minimize surface roughness with increasing height above the water table to decrease water loss (Price & Whittington 2010).

In contrast, we find that evidence for phylogenetic signal in "ionic" preferences is mostly absent (for ionic concentrations) or is concentrated in the tips of the phylogeny (EC). Despite the small niche breadth observed in many studies of *Sphagnum*, and that these microhabitat preferences make up much of the major axis of among-species niche variation, the lack of signal is consistent with the observation that the four species with highest PC1 scores ("ionic" niche descriptors) all represent different subgenera (Figure 11). A notable exception is pH, for which a complex pattern of stabilizing selection and rate change is suggested.

Several pieces of evidence, when taken together, suggest that the evolution of the pH niche does in fact contain phylogenetic signal in *Sphagnum*. Although the full dataset failed to support any evolutionary model better than **WN**, the sensitivity analysis (Figure 14) shows that deletion of either *S. magellanicum* or *S. centrale* provides support for an **OU** model in microhabitat pH evolution. When these species, and other members of subgenus *Sphagnum* (and subgenus *Subsecunda*) are removed in the reduced dataset, there is strong support for an **OU** model with a single optimum for the whole genus (Figure 13b). Moreover, the Bayesian analysis of exceptional rate changes showed strong support for a change in pH niche evolution within subgenus *Sphagnum* (Figure 13a). These data therefore indicate that pH niche evolution in *Sphagnum* has two phases: 1) An **OU** model, where pH niche evolution deviates from a pure Brownian motion process by

trending towards a genus-wide optimum of 5.5. Typically, support for an **OU** model is interpreted as evidence of stabilizing selection (Hansen, 1997), but may also be represented as a bounded Brownian motion process. 2) An exceptional rate change occurred within subgenus *Sphagnum,* which masks the signal of the **OU** model when considering the entire genus.

Additional descriptors show evidence of exceptional rate change using the Bayesian MCMC method (Revell *et al.* 2012), and many of the branches identified are located near the tips of the tree (e.g., *S. contortum* for EC). If the purported rate changes were masking phylogenetic signal in these other descriptors, as we suggest for pH, the sensitivity analysis should show model support when these tips are removed. However, none of the other sensitivity analyses indicate support for any model for any of the descriptors where rate changes are proposed by the Bayesian MCMC method. This lack of supporting evidence suggests it is less likely for a rate change to obscure phylogenetic signal in these descriptors, compared to pH. The lack of support for an exceptional rate change in PC2 seems to conflict with our other results, which show evidence for separate rates of PC2 evolution between subgenus *Acutifolia* and subgenus *Cuspidata*. However, the Bayesian MCMC approach was taken with the full dataset, where the rate change signal may be masked by the presence of the other two subgenera.

Several studies besides ours have found very limited infraspecific breadth of ionic niche occupancy in *Sphagnum* (Vitt & Slack 1984; Andrus 1986; Gignac 1992). It therefore seems unlikely that the lack of phylogenetic signal is explained by new species choosing their ionic microhabitats at random. An alternative interpretation is that phenotypic plasticity and among-species interactions are more important than phylogeny for these microhabitat preferences (Eterovick *et al.* 2010). Several hummock-

forming bog species have been shown to tolerate waters from rich fens (Granath *et al.* 2010), suggesting that these species may have broader fundamental niches than suggested by their realized niches.

Additional study is required to investigate the importance of among species interactions in definition of narrow microhabitat niches within peatlands. Observations and experiments involving damaged peatlands show that hummocks form several years after re-establishment of *Sphagnum* in a peatland (Pouliot *et al.* 2011), and that vigorous growth of some species (*S. magellanicum*) depends on the presence of other species (such as *S. fuscum*) (Chirino *et al.* 2009). Therefore, it is clear that interspecies interactions play some role in the formation and maintenance of species diversity in peatlands. A study designed to test the selection hypothesis could involve a damaged peatland in the process of reclamation, with observations of how microhabitat preferences change as new species recolonize the peatland.

In general, our findings are robust to uncertainty introduced by within-species measurement error and phylogenetic uncertainty. Accounting for the former improved the model fits for a few niche descriptors, but did not alter any conclusions. This is not to suggest that within-species variability is unimportant. In their current forms, the methods employed here assume that error estimation of a species mean decreases with sample size, and does not explicitly model the niche breadth of each species. Topological phylogenetic uncertainty was low in our case, but the observations of overlapping AICc distributions, for example in PC2 in the reduced dataset, indicates the necessity of including phylogenetic error in comparative methods to account for branch length uncertainty.

Conclusions

The evolution of microhabitats in *Sphagnum* has a complex relationship with phylogenetic signal. Preference for relatively narrow ranges on the ionic gradient appear to be independent of phylogeny, and further study may confirm whether phenotypic plasticity or intra-species competition play roles in eliminating phylogenetic signal. One exception is pH, for which we demonstrate a constraint on pH niche around a genus-wide optimum, though this signal is masked by an exceptional rate change in subgenus *Sphagnum*. The evolution of preferences on the hummock-hollow gradient, however, has a large component explained by phylogeny. The rate of evolution is heterogeneous; lineages classified as preferring hollow environments have lower rates of evolution and are constrained to prefer different multivariate microhabitat optima than hummock lineages.

Because our data represent the realized niches we are in fact interpreting the combined evolution of physiological tolerances and biotic interactions. Inherited ecological traits are more likely to have underlying functional traits related to *Sphagnum* peatland engineering, and are more likely to be involved in peatland community assembly. The obvious next stage would be to gather data on the basic physiological and morphological traits behind the niches to trace their evolution.

Appendix A

Here, we describe the twenty populations sampled for the survey of mating patterns in *Sphagnum* (Chapter 1).

Sphagnum angustifolium Population: ME-S. Washington County, Maine, USA. Locality: near Steuben, E side of East Side Rd, 0.8 mi N of US1, N 44.5221˚ W 67.9500˚, elevation 35 m. Description: poor fen, ca. 100 m from open water. Fruiting plants forming several hummocks in a ca. 100 m^2 area. Duke Herbarium collections: Matt Johnson 118-120. 15-June-2009.

Sphagnum austinii. Population: VI. Vancouver Island, Canada. Locality: Bamfield Area, W coast of island, on Bamfield Rd, 2.35 km S of Nuthatch Rd. N 48.8155˚ W 125.1275˚ Description: Poor-medium fen on NE side of road, around small shallow lake dominated by *Juncus*, *Carex*, and *Myrica*. Plants spread along two large hummocks ca 300 m apart. Duke Herbarium collection: Jonathan Shaw 16578, 16584, 16585, 16590, and 16591.

Sphagnum compactum. Population: AK-PC. Matanuska-Susitna Borough, Alaska, USA. Locality: Petersville Rd, 17.2 mi W of jct Parks Hwy (AK2), N 62.3674˚ W 150.7121˚, elevation 375 m. Description: Plants collected along a ca. 25m transect parallel to the road in a floating fen. Duke Herbarium Collections: Matt Johnson 143 and 144, Jonathan Shaw 16913 and 16917. 9 August 2010.

Sphagnum compactum. Population: AK-PH. Denali Borough, Alaska, USA. Locality: Parks Hwy, ca 30 mi S of Cantwell at 180 Mi Lake. . N 63.0822˚ W 149.5252˚ elevation 560m.

Description: Fen on S side of Rd, with rich areas (*Tomenthypnum*, *Paludella*, etc), and poorer depressions (with *Sphagnum lindbergii*, *S. balticum* and *S.* cf. *orientale*) plus higher hummocks (with *S. fuscum*, *S. lenense, S. capillifolium*). Plants collected in a ca. 100m transect in low depressions between huummocks. Duke Herbarium collections: Matt Johnson 146-149. 9 August 2010.

Sphagnum cribrosum. Population: GA32. Long County, Georgia, USA. Locality: US-84, 1.3 mi NE of US-25 in Ludowici. N 31.7232˚ W 81.7270˚. Description: Wet trenches running parallel and perpendicular to W side of road. Growing intermixed in a ca. 25m^2 area with *S. macrophyllum*. 8 May 2009.

Sphagnum cuspidatum. Population GA28. Ware County, Georgia, USA. Locality: W side of GA 177, 0.5 mi S of US 1, entrance to Okeefenokee Swamp. N 31.1225˚ W 82.2723˚. Description: Pondcypress depression dominated by *Taxodium ascendens, Ilex myrtifolia, Nyssa biflora and Carex striata,* on S side of road, powerline right-of-way, plants collected along a ca 200m transect, floating in the water. Duke Herbarium collections: Matt Johnson 101- 113. 6 Feb 2009.

Sphagnum cuspidatum. Population NC-JL. Bladen County, North Carolina, USA. Locality: Jones Lake State Park, along Cedar Loop Trail on E shore. N 34.6884˚ W 78.5960˚ elevation 21m. Description: Wet shaded depression in bay forest, plants collected from one large 10 m^2 patch. Duke Herbarium collections: Blanka Shaw 9746. 16 May 2009.

Sphagnum fallax. Population: ME-S. Washington County, Maine, USA. Locality: near Steuben, E side of East Side Rd, 0.8 mi N of US1, N 44.5221˚ W 67.9500˚, elevation 35 m. Description: poor fen, ca. 50 m from open water. Fruiting plants forming several hummocks in a ca. 100 m^2 area. Deeper into the fen than the S. angustifolium population, the hummocks were nearly dry. Duke Herbarium collections: Matt Johnson 121-123. 15 June 2009.

Sphagnum fallax. Population: ME-W. Washington County, Maine, USA. Locality: Great Wass Island Preserve, NE of Black Duck Cove Rd, 0.4 mi N of Preserve parking lot. N 44.5221˚ W 67.9500˚, elevation 14 m. Description: fruiting plants forming one large hummock at the edge of a moderate fen dominated by *S. tenellum*. Duke Herbarium collections: Matt Johnson 124-125. 16 June 2009.

Sphagnum macrophyllum Population SC-36. Jasper County, South Carolina, USA. Locality: 6.2 mi E of US 601 on SC 462, near Coosawatchie, SC. N 32.6193˚ W 81.0653˚. Description: Disturbed pond cypress dome S of highway, plants collected on a ca. 100 m transect arcing around the wettest areas beneath *Hypericum crux-andreae*, *Ilex myrtifolia*, and *Nyssa biflora*. Duke Herbarium collections: Matt Johnson 95-100. 5 March 2009.

Sphagnum macrophyllum. Population SC-39. Berkeley County, South Carolina, USA. Locality: Hell Hole Bay Wilderness Area in Francis Marion National Forest, FR 161 (Hell Hole Rd), 0.5 mi NE of FR 138. 33.218˚ N 79.712˚ W. Description: Open canopy of *Taxodium distichum* surrounding ca 2000 m2 of open water ca 1 m deep. Understory of *Lyonia lucida*, *Nyssa biflora*, and *Vaccinium formosum*. In the water, plants form nearly

continuous mats around *Nymphaea odorata*, *Dulichium arundinaceum*, and *Carex striata*. Collected as part of intensive survey of phenology and mating patterns (Chapter 2), April-May 2009 and December 2009-June 2010.

Sphagnum magellanicum. Population: NC-JL. Bladen County, North Carolina, USA. Locality: Jones Lake State Park, along Cedar Loop Trail on E shore. N 34.6884˚ W 78.5960˚ elevation 21m. Description: Wet, partically shaded depression in old growth bay forest, with poison ivy. Plants formed one single hummock about 0.5 m^2. Duke Herbarium collection: Blanka Shaw 9745. 16 May 2009.

Sphagnum molle. Population: SC36. Jasper County, South Carolina, USA. Locality: 6.2 mi E of US 601 on SC 462, near Coosawatchie, SC. N 32.6193˚ W 81.0653˚. Description: Disturbed pond cypress dome S of highway. Plants collected in several isolated hummocks in ca. 25 m^2 area, hidden beneath *Hypericum* and *Lyonia*. Duke Herbarium collection: Matt Johnson 115. 5 Mar 2009.

Sphagnum pulchrum. Population ME-C. Hancock County, Maine, USA. Locality: Gouldsboro Twp, E from Prospect Harbor at Corea Heath Bog (NWR). N 44.4056˚ W 67.9812˚ elevation 17 m. Description: Poor fen along edges and ombotrophic bog with raised mud flats. Plants collected near trailway at edge of fen forming many fruiting patches along a ca. 50 m^2 transect. Duke Herbarium collections: Jonathan Shaw 16085 and 16087. 11 June 2009.

Sphagnum squarrosum Population AK-W. Fairbanks North-Star Borough, Alaska, USA. Locality: Milepost 13 on Elliot Hwy (AK-2) just south of Willow Creek. N 65.1004˚ W 147.7464˚ elevation 180m. Description: Moderately rich fen with hummocks/hollows dominated by *Sphagnum teres, S. obtusum* and *Vaccinium uliginosum* and *Salix* shrubs. Plants collected from several hummocks along a ca 50 m^2 transect beneath blueberry bushes. Duke Herbarium collections: Jonathan Shaw 16849 and 16851, Matt Johnson 131-134. 4 August 2010.

Sphagnum strictum Population: SC-36. Jasper County, South Carolina, USA. Locality: 6.2 mi E of US 601 on SC 462, near Coosawatchie, SC. N 32.6193˚ W 81.0653˚. Description: Disturbed pond cypress dome S of highway. Plants collected in several isolated hummocks in ca. 25 m^2 area, hidden beneath *Hypericum* and *Lyonia*. Duke Herbarium collection: Matt Johnson 116-117. 5 Mar 2009.

Sphagnum strictum. Population NC-LL. Wake County, North Carolina, USA. Locality: Lizard Lich granitic rock outcrops, ca 4 mi WNW of Zebulon near intersection of Marshburn Rd. and Riley Hill Rd. N 35.8447˚ W 78.3731˚ elevation 80 m. Description: granitic flat outcrop, in partial shade in pine-juniper woodland. Plants collected from one cushion with a conspicuously large number of sporophytes. Duke Herbarium collection: Blanka Shaw 7202. 16 November 2008.

Sphagnum tenellum Population: ME-W. Washington County, Maine, USA. Locality: Great Wass Island Preserve, NE of Black Duck Cove Rd, 0.4 mi N of Preserve parking lot. N 44.5221˚ W 67.9500˚, elevation 14 m. Description: poor fen near trail, many fruiting

plants in a ca. 25 m^2 hollow. Duke Herbarium collections: Jonathan Shaw 16037. 9 June 2009.

Sphagnum warnstorfii Population: AK-M. Valdez-Cordova Census Area, Alaska, USA. Locality: McCarthy Rd, 9.1 mi W of McCarthy foot bridge. N 61.3861˚ W 143.1821˚. Description: Extreme rich fen with hummock-hollow structure, scattered *Salix*, *Betula*, *Picea* and *Calex*. Plants collected from several hummocks. Duke Herbarium collections: Jonathan Shaw 16714, 16715, 16717. 24 July 2010.

Appendix B

Table 9. A list of all *Sphagnum* species known from North America (McQueen & Andrus 2009), categorized by subgenus, sexual condition, and ploidy. Subgenus classification follows Shaw et al. (Shaw, Cox, *et al.* 2010a). Information on sexual conditions comes from two main sources (Crum 1984; McQueen & Andrus 2009), and disagreements are noted. Key to subgenera: Acut = *Acutifolia*, Cusp = *Cuspidata*, Ins = *Insolosa*, Rig = *Rigida Sph* = *Sphagnum,* Squ = *Squarrosa,* Subs = *Subsecunnda*. Sex. Cond. refers to whether species are Monoicous (unisexual gametophytes) or Dioicous (bisexual gametophytes). Species are classified as haploid (N) or diploid (2N) gametophytes based on several sources: chromosome counts (Fritsch 1991), flow cytometry C-values (Greilhuber *et al.* 2003), and whether gametophytes possess fixed heterozygosity at microsatellite alleles (Ricca *et al.* 2008; Karlin, Gardner, *et al.* 2010a; Karlin, Giusti, *et al.* 2010b; Ricca & Shaw 2010)

Species	Subg.	Sex. Cond.	Ploidy	Notes	Source	Chrom (Fritch 1991)	C-Value	Fixed Hetero zygosit y
affine	*Sph*	D	N		McQueen (BFNA)			no
alaskense	*Sph*	D	2N		McQueen (BFNA)			yes
andersonianum	*Acut*	D			McQueen (BFNA)			
angermanicum	*Acut*	D	N		Crum (1984)	19		
angustifolium	*Cusp*	D	N		McQueen (BFNA)	19	0.45	no
annulatum	*Cusp*	D	N		McQueen (BFNA)	19		
aongstroemii	*Ins*	D	N		Crum (1984)	19	0.47	
arcticum	*Acut*	M	2N		Flatberg (2003)		0.95	yes
atlanticum	*Cusp*	D	2N		McQueen (2007)			yes
austinii	*Sph*	D	N		McQueen (BFNA)			no
balticum	*Cusp*	D	N		Crum (1984)	19	0.45	no
bartlettianum	*Acut*	D			Crum (1984)			
brevifolium	*Cusp*	D	N		McQueen (BFNA)		0.42	no
capillifolium	*Acut*	D	N	Crum says Monoicous	McQueen (BFNA)	19	0.46	
carolinianum	*Subs*		2N				0.8	yes
centrale	*Sph*	D	2N		Crum (1984)	38		yes
compactum	*Rig*	M	N		Crum (1984)	19	0.41	
concinnum	*Acut*	M	N		Flatberg (2007)			no
contortum	*Subs*	D	N		Crum (1984)		0.4	no

cribrosum	*Subs*	D	N		Crum (1984)			no
crispum	*Subs*				Crum (1984)			
cuspidatum	*Cusp*	D	N	"monoicous and apparently also dioicous" - Crum 84	McQueen (BFNA)	19	0.41	no
cyclophyllum	*Subs*	D			Crum (1984)			
denticulatum	*Subs*		N				0.39	mixed
diplolescurii	*Subs*	D	2N				0.95	yes
diskoense	*Acut*		N					no
fallax	*Cusp*	D	N		McQueen (BFNA)	19	0.44	no
fimbriatum	*Acut*	M	N		Crum (1984)	19,38	0.46	no
fitzgeraldii	*Cusp*	M	N	Crum says Dioicous	McQueen (BFNA)			
flavicomans	*Acut*	D	N		Crum (1984)	19		
flexuosum	*Cusp*	D	N		McQueen (BFNA)	19	0.43	no
fuscum	*Acut*	D	N		Crum (1984)	19	0.49	no
girgensohnii	*Acut*	D	mixed	Crum says Monoicous	McQueen (BFNA)	19	0.47	mixed
henryense	*Sph*	D	N	"apparently dioicous, antheridia not seen"	Crum (1984)			
imbricatum	*Sph*	D	N		Crum (1984)	19	0.45	
incundum	*Acut*		N					no
isovitae	*Cusp*	D			McQueen (BFNA)			
jensenii	*Cusp*	D	2N		McQueen (BFNA)			yes
junghuhnianum	*Acut*			"Dioicous or monoicous"	Crum (1984)			
lenense	*Cusp*		N		Crum (1984)	19		
lescurii	*Subs*	D	N		McQueen (BFNA)	19		
lindbergii	*Cusp*	M	N	"but reported to be dioicous"	Crum (1984)		0.42	no
macrophyllum	*Subs*	D	N		Crum (1984)			no
magellanicum	*Sph*	D	N		Crum (1984)	19,38	0.46	
majus	*Cusp*	D	2N		Crum (1984)	38	0.81	yes

mcqueenii	*Cusp*							
mendocinum	*Cusp*	D			Crum (1984)			
microcarpum	*Subs*				McQueen (BFNA)			
mississippiensis	*Cusp*	D		?	McQueen (BFNA)			
molle	*Acut*	M	N		McQueen (BFNA)	19		
obscurum	*Acut*		2N					yes
obtusum	*Cusp*	D	N		Crum (1984)	19	0.44	
olafii	*Acut*		2N				0.92	yes
orientale	*Subs*	D		"probably"	McQueen (BFNA)			
pacificum	*Cusp*	D			McQueen (BFNA)			
palustre	*Sph*	D	2N		Crum (1984)	38	0.92	
papillosum	*Sph*	D	2N		Crum (1984)	19,38	0.93	
perfoliatum	*Subs*							
perichaetiale	*Sph*	D	N		Crum (1984)	19		
platyphyllum	*Subs*	D	N		McQueen (BFNA)	19	0.4	
portoricense	*Sph*	D	N	"apparently"	Crum (1984)			no
pulchrum	*Cusp*	D	N		McQueen (BFNA)	19		
pylaesii	*Subs*	D	N		Crum (1984)	19		
quinquefarium	*Acut*		N	"Dioicous or monoicous"	McQueen (BFNA)	19	0.47	
recurvum	*Cusp*	D	N		Crum (1984)	19		no
riparium	*Cusp*	D	N		Crum (1984)	19	0.42	
rubellum	*Acut*	D	N		McQueen (BFNA)	19	0.47	
rubiginosum	*Acut*	M			McQueen (BFNA)			
rubroflexuosum	*Cusp*		N		McQueen (BFNA)			no
rufescens	*Subs*		N		Crum (1984)			
russowii	*Acut*	D	2N	"but some specimens apparently monoicous"	McQueen (BFNA)	38	0.94	
schofieldii	*Acut*				Crum (1984)			
splendens	*Cusp*				Crum (1984)			
squarrosum	*Squ*	M	m		Crum (1984)	19,38		

steerei	*Sph*	D	N		McQueen (BFNA)			no
strictum	*Rig*	M	N	Crum says Dioicous	McQueen (BFNA)	19		
subfulvum	*Acut*	M	N		Crum (1984)	19		no
subnitens	*Acut*	M	N		Crum (1984)	19	0.45	no
subobesum	*Subs*	D			McQueen (BFNA)			
subsecundum	*Subs*	D	N		Crum (1984)	19	0.41	no
subtile	*Acut*	D			McQueen (BFNA)			
tenellum	*Cusp*	M	N	Crum says Dioicous	McQueen (BFNA)	19	0.47	
tenerum	*Acut*		N	"Dioicous or monoicous"	McQueen (BFNA)	19		
teres	*Squ*	D	N		Crum (1984)	19	0.45	no
tescorum	*Acut*	D	2N		Flatberg (2007)			yes
torreyanum	*Cusp*	D	2N		Crum (1984)	38		yes
trinitense	*Cusp*	M			McQueen (BFNA)			
tundrae	*Squ*		N		McQueen (BFNA)		0.44	no
viride	*Cusp*	D			McQueen (BFNA)			
warnstorfii	*Acut*	D	N		Crum (1984)	19,38	0.48	
wilfii	*Acut*				Crum (1984)			
wulfianum	*Acut*	D	N	"or sometimes monoicous"	Crum (1984)	19		

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Biography

Matthew G. Johnson was born on 13 July 1984 in Hardyston, New Jersey, and graduated from Wallkill Valley Regional High School in 2002. He then graduated with distinction from Duke University with B.S. in Biology in 2006. During his PhD he has been a teaching assistant (TA) for several courses, including Microbiology, Plant Diversity, and Organismal Diversity, and has guest lectured in Bioinformatics and Systematic Biology. Matthew has presented his research at two international scientific meetings: the 2011 Evolution meeting and the 2012 Botany meeting. From 2010-2012 he was a research assistant for the Bryan Alzeheimer's Research Center, and developed pipelines to standardize the comparative analysis of analyitical methods in genome wide association studies. Matthew received the E. Bayard Halsted scholarship in 2010, awarded to outstanding Duke graduate students in science, history, or journalism who also received undergraduate degrees from Duke. From 2007-2013, he tutored over sixty student-athletes in biology for the Duke University Athletics Association. He is a member of the American Society of Naturalists, the Botanical Society of America, and the American Genetic Association.

Significant Publications

1. M. G. Johnson, B. Shaw, P. Zhou, and A. J. Shaw. 2012. Genetic analysis of the peatmoss *Sphagnum cribrosum* indicates independent origins of an extreme infra-specific morphology shift. *Biological Journal of the Linnean Society*. 106(1):137-153.
2. Ramaliya, M., M.G. Johnson, J. Heinrichs, J. Hentschel, M. von Konrat, P. Davison, B. Shaw and A.J. Shaw. 2010. Morphologically Cryptic Biological Species Within the Liverwort *Frullania asagrayana American Journal of Botany*. 97:1707-1718.