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A phylotranscriptomic analysis of gene family expansion and evolution in the largest order of pleurocarpous mosses (Hypnales, Bryophyta) ☆

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ABSTRACT

The pleurocarpous mosses (i.e., Hypnanae) are a species-rich group of land plants comprising about 6,000 species that share the development of female sex organs on short lateral branches, a derived trait within mosses. Many of the families within Hypnales, the largest order of pleurocarpous mosses, trace their origin to a rapid radiation less than 100 million years ago, just after the rise of the angiosperms. As a result, the phylogenetic resolution among families of Hypnales, necessary to test evolutionary hypotheses, has proven difficult using one or few loci. We present the first phylogenetic inference from high-throughput sequence data (transcriptome sequences) for pleurocarpous mosses. To test hypotheses of gene family evolution, we built a species tree of 21 pleurocarpous and six acrocarpous mosses using over one million sites from 659 orthologous genes. We used the species tree to investigate the genomic consequences of the shift to pleurocarpy and to identify whether patterns common to other plant radiations (gene family expansion, whole genome duplication, or changes in the molecular signatures of selection) could be observed. We found that roughly six percent of all gene families have expanded in the pleurocarpous mosses, relative to acrocarpous mosses. These gene families are enriched for several gene ontology (GO) terms, including interaction with other organisms. The increase in copy number coincident with the radiation of Hypnales suggests that a process such as whole genome duplication or a burst of small-scale duplications occurred during the diversification. In over 500 gene families we found evidence of a reduction in purifying selection. These gene families are enriched for several terms in the GO hierarchy related to "tRNA metabolic process." Our results reveal candidate genes and pathways that may be associated with the transition to pleurocarpy, illustrating the utility of phylotranscriptomics for the study of molecular evolution in non-model species.

1. Introduction

The Bryophyta (mosses) are one of three phyla of non-vascular land plants, and comprise more than 13,000 species (Magill, 2014). Although it is one of the oldest groups of land plants, with fossils dating to at least the lower Permian (Smoot and Taylor, 1986), a significant amount of genus-level diversity has been generated in bursts that are coincident with the diversification of extant ferns and angiosperms in the Mesozoic (Laenen et al., 2014). Approximately 42% of moss species diversity (Crosby et al., 1999) belong to the pleurocarpous mosses or Hypnanae (Buck et al., 2005) a monophyletic "crown group" of mosses typically defined by the development of female gametangia on short, lateral branches lacking differentiated vegetative leaves (La Farge-England, 1996). This is in contrast to the ancestral growth form of mosses, acrocarpy and cladocarpy, where gametangia (and thus sporophytes) are usually terminal on upright shoots, or branches bearing well developed leaves. Recent fossil discoveries have pushed the origin of the pleurocarpous growth form into the late Permian (de Souza et al., 2012), but the major diversification of extant pleurocarpous moss lineages occurred much later; in fossil-calibrated phylogenies, the most speciose pleurocarpous moss families (i.e., Amblystegiaceae, Hypnaceae, or Brachytheciaceae) are estimated to have diversified within the last 100 million years (Laenen et al., 2014; Newton et al., 2007; Shaw et al., 2003).

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The superorder Hypnanae comprises four orders: Hypnodendrales, Ptychomniales, Hookeriales, and Hypnales, with the latter including most of the diversity, namely ±4000 species in about 430 genera and 42 families (Goffinet et al., 2009; Huttunen et al., 2013). Just eight families hold the majority of genera, 5% of the genera hold the majority of species and nearly 200 genera are monospecific. Suprageneric taxa within Hypnanae were traditionally circumscribed using morphological traits and habitat type. Given the uneven distribution of species among genera and families and the rapid tempo of diversification in Hypnales, it is not surprising that most of these morphologically-defined taxa are not monophyletic (Cox et al., 2010). Lineage-through-time plots revealed that Hypnales, unlike its sister group (Hookeriales), underwent a rapid, explosive diversification rather than a gradual diversification early in its history (Shaw et al., 2003). Many of the families within Hypnales likely diversified after the radiation of angiosperm forests (Laenen et al., 2014; Newton et al., 2007), which is hypothesized to have been a major driver of diversification in other epiphytic and understory-dwelling plants, such as leptosporangiate ferns and liverworts (Feldberg et al., 2014; Schneider et al., 2004). The diversification of leptosporangiate ferns parallels that of major families in Hypnales in both timing (late Cretaceous) and extant diversity in similar habitats (e.g., low-light, epiphytic; (Li et al., 2014)), which raises the possibility that a signature of the radiation can be found in the genomes of pleurocarpous mosses.

Recent genomic evidence has revealed that whole genome duplication (WGD) events are associated with many gene family expansions in land plants (Barker et al., 2008; Blanc and Wolfe, 2004a; Jiao and Paterson, 2014; Jiao et al., 2011; Li et al., 2015; Maere et al., 2005). Although most of the gene copies generated by WGD events are lost due to fractionation and subsequent "red iploidization" or nonfunctionalization (Jiao et al., 2011), in *Arabidopsis* some of the duplications led to neo- and/or sub-functionalization (Moore and Purugganan, 2005), resulting in the evolution of parallel gene networks (Blanc and Wolfe, 2004a) with copy-specific gene regulation (Spangler et al., 2012). Paleopolyploidy events in plants may provide opportunities for positive selection (or, at the very least reduced purifying selection) on retained duplicated copies of genes. Gene duplications in some gene families have been associated with key innovations in angiosperms; for example, the expansion of the CYCLOIDEA gene family is linked to the development of zygomorphic flowers. Members of the CYCLOIDEA gene family that have been implicated in symmetry contain conserved domains with sites under positive selection following duplication (e.g. Chapman et al., 2008).

The identification of genomic signatures, such as WGD, associated with radiations in non-model organisms has benefited from the emergence of sequencing techniques that reduce genomic complexity, such as transcriptome sequencing (Cannon et al., 2015; Yang et al., 2015). These methods allow researchers to generate large, comparative data sets without having to invest considerable resources in sequencing entire genomes, which can vary enormously in size. By using only the coding portion of the genome in a phylotranscriptomic approach, it is possible to provide an evolutionary context to inferred paleopolyploidy, gene family expansion, and shifts in selective regimes of duplicated genes, without having to sequence the extensive non-coding portion of a genome. To date, all phylogenetic analyses involving pleurocarpous mosses have sampled few discrete nuclear coding loci and focused on dense taxon sampling (Cox et al., 2010; Huttunen et al., 2012; Shaw et al., 2003). In contrast, transcriptome sequencing generates enough data to leverage methods that have the potential to identify genomic signatures, such as evidence of WGD, associated with the radiation of Hypnales, as has been observed in other land plant radiations.

Here, we identify homologous and orthologous genes from sequenced transcriptomes using a phylogeny-based approach (Yang and Smith, 2014). We construct a species tree from over 650 nuclear, protein-coding loci for 21 pleurocarpous mosses, five acrocarpous mosses, and the well-annotated proteome of the model moss *Physcomitrella patens*. Our sampling includes *Aulacomnium palustre*, an exemplar of the Aulacomniales resolved by Bell et al. (2007) as the sister-group to the Hypnanae. We use the species tree to infer the history of gene duplication events, gene family expansions, signatures of whole genome duplications, and shifts in rates of selection. Functional analysis of gene families that underwent expansion during the diversification of Hypnales will inform future studies on the genetic basis of the radiation of this most speciose lineage of pleurocarpous mosses.

2. Materials and methods

2.1. RNA extraction and sequencing

We generated 25 transcriptomes for this study from 21 pleurocarpous and four acrocarpous moss species (Table 1). Two of our samples are from the same species, *Aulacomnium palustre*, which was sequenced twice due to its critical phylogenetic position. All samples were wild-collected and then placed in clear plastic containers within a growth chamber for at least one week prior to RNA extraction. Tissue was sampled from young, green shoots and flash-frozen in liquid nitrogen, then ground to a powder with a mortar and pestle. Total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma–Aldrich, St. Louis, MO, USA) with no modification to the standard protocol. RNA was quantified using a Qubit Fluorometer (Life Technologies, Grand Island, NY, USA). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA) at the Northwestern University Center for Genetic Medicine (Chicago, IL, USA). RNA-Seq libraries for Illumina sequencing were prepared at BGI (Shenzhen, China) using the TruSeq RNA Sample Preparation Kit v2 (Illumina Inc., San Diego CA, USA). Three of the libraries (NW_1, NW_2, and NW_3, see Table 1) were multiplexed and sequenced on one lane of Illumina HiSeq2000 (2×100 Paired End) in February, 2014. A second batch of ten samples (accession numbers NW_45 through NW_62, see Table 1) was multiplexed and sequenced across two lanes in July, 2014, and the remaining fourteen samples were multiplexed and sequenced across two lanes in November, 2014. All sequencing was carried out at BGI (Shenzhen, China). All unedited sequence reads were deposited in the NCBI Sequence Reads Archive (BioProject PRJNA296787).

2.2. Transcriptome assembly and filtering

Raw reads were demultiplexed and adapters were removed by BGI (Shenzhen, China) prior to delivering the data. We further trimmed the sequences with Trimmomatic (Bolger et al., 2014) using the following parameters: LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN: 36. We assembled each transcriptome from the filtered reads with the Trinity pipeline (Grabherr et al., 2011; Haas et al., 2013) using default parameters. We applied a hierarchal filtering approach in order to reduce the complexity of downstream analyses, and to reduce the possibility of contamination of our datasets by transcripts from associated organisms. First, the transcripts were translated using the Transdecoder (version 20140704, http://transdecoder.github.io) tool included with Trinity. Transdecoder chooses the most valid open reading frames from each transcript using a likelihood approach that incorporates domain similarity matches to the Pfam database (Finn et al., 2014) using the hmmscan function in HMMER (Johnson et al.,

Table 1
Transcriptome assembly statistics and herbarium voucher information. Buck–NY, Goffinet, Quandt–CONN, Shaw–DUKE.

ID	Species	Voucher collection	Paired reads (Millions)	Trinity transcripts (thousands)	Percent of transcripts passing filter (BLAST and Transdecoder) (%)	Masked transcripts kept	Homologous gene family trees	BUSCOs
NW-1	*Climacium americanum*	Goffinet 11684	58.86	129.4	33.7	20,018	12,295	390
NW-2	*Thuidium delicatulum*	Goffinet 11686	67.17	137.6	46.1	20,289	12,501	391
NW-3	*Hypnum cupressiforme*	Goffinet 11687	72.38	235.6	31.7	22,035	12,735	392
NW-45	*Pleurozium schreberi*	Goffinet 11700	23.11	108.1	37.3	18,098	11,965	388
NW-51	*Aulacomnium palustre*	Goffinet 11701	38.23	135.4	40.9	19,894	12,313	390
NW-53	*Bryoandersonia illecebra*	Buck 63016	34.75	111.8	41.8	20,891	12,702	392
NW-55	*Rhytidiadelphus subpinnatus.*	Goffinet 11708	34.76	82.4	48.0	18,490	12,068	391
NW-56	*Kindbergia praelonga*	Goffinet 11707	31.73	132.8	34.5	21,146	12,528	387
NW-57	*Callicladium haldanianum*	Goffinet 11688	25.38	169.4	33.6	19,332	11,991	381
NW-59	*Hylocomium brevirostre*	Goffinet 11699	30.56	211.8	31.1	25,976	13,205	386
NW-60	*Hylocomium splendens*	Goffinet 11696	26.69	147.6	35.3	20,902	12,375	391
NW-61	*Calliergon cordifolium*	Goffinet 11693	29.07	300.3	27.0	21,494	12,417	389
NW-62	*Aulacomnium palustre*	Goffinet 11702	23.79	147.0	39.3	20,489	12,335	390
NW-65	*Anomodon rostratus*	Goffinet 11711	12.88	115.3	35.8	19,720	12,253	390
NW-66	*Thelia asprella*	Goffinet 11712	22.01	135.6	37.6	19,994	12,421	391
NW-69	*Pilotrichella flexillis*	Quandt DR158/ WP281	22.91	128.0	37.5	20,181	12,535	391
NW-71	*Antitrichia curtipendula*	Shaw 17555	21.87	101.5	44.2	20,736	12,481	385
NW-72	*Meteoridium remotifolium*	Quandt DR152/ WP281	22.78	102.5	42.0	18,649	12,066	387
NW-74	*Rhytidiopsis robusta*	Shaw 17554	19.18	113.1	37.8	20,312	12,453	386
NW-76	*Forrstroemia trichomitria*	Shaw 17557	21.27	113.8	40.9	19,161	12,365	389
NW-77	*Rhodobryum ontariense*	Goffinet 11803	13.34	53.3	60.6	11,296	9470	382
NW-79	*Dicranum scoparium*	Goffinet 11775	20.08	120.7	37.1	13,577	9843	383
NW-84	*Platyhypnidium riparioides*	Goffinet 11802	19.28	100.4	39.1	18,969	12,011	385
NW-85	*Plagiothecium laetum*	Goffinet 11851	17.19	144.7	29.5	20,620	12,560	382
NW-86	*Leucobryum glaucum*	Goffinet 11773	20.64	171.5	30.3	12,566	9450	386

2010). All transcripts with a valid translation were searched against a custom BLAST database containing protein sequences from 22 land plant nuclear genomes, including the moss *Physcomitrella patens*, downloaded from Phytozome (phytozome.jgi.doe.gov). We accepted protein matches in blastp (version 2.2.29) with an e-value below 10^{-10}. For the next stages of analysis, we included all transcripts that had a significant hit to the proteome database, as well as all Trinity-annotated isoforms of that same transcript.

2.3. Clustering transcripts into homologous gene families

In order to cluster transcripts from all species, remove redundant isoforms, and construct a species tree from low-copy genes, we employed the phylogenetic clustering method described by Yang and Smith (2014). In this pipeline (hereafter, the Yang/Smith Pipeline), all transcripts that passed the above filtering procedures were first grouped using an all-vs-all BLAST search of nucleotide sequences from every species. Significant hits (e-value < 10^{-5}) were clustered, using the software MCL (Enright et al., 2002), into gene families using a hit-fraction of 0.3 and an inflation parameter of 2.0. All clusters containing sequences from at least four species (of 26 total, including the *Physcomitrella* proteome) were aligned using MAFFT (Katoh and Standley, 2013) and nucleotide gene trees were reconstructed from peptide sequences with RAxML (Stamatakis, 2014) under the CAT model.

At this stage in the pipeline, the gene clusters may include isoforms as inferred by Trinity. To account for the possibility that some of these may actually be paralogs, rather than alternative splice forms, the Yang/Smith Pipeline extracts monophyletic or paraphyletic groupings of transcripts from a single taxon. These clades are reduced to contain only the sequence with the longest

unambiguous alignment. We also trimmed the gene family trees to remove terminal branches whose length exceeded an absolute (0.3 substitutions/site) or relative (more than 10× longer than its sister branch) cutoff. These terminal branches represent transcripts with potentially spurious homology to the other transcripts in the gene family tree, and were removed from further analysis. This approach retains multiple isoforms from the same Trinity component if they are not part of the same clade on the gene tree; however, recent lineage-specific paralogs will be lost. Since the goal of this project is to identify genomic changes prior to, or coincident with the diversification of Hypnales, rather than species-specific changes, the masking of lineage-specific duplications does not impact our conclusions. We refer to the subset of transcripts that remain following this phylogenetic transcript clustering as the **masked** dataset.

When using transcriptomes for gene discovery, rather than quantifying relative expression, it is important to assess whether or not it is likely that sequences for all possible transcripts were recovered. One method to approximate whether or not we sequenced all possible transcripts that were present in the tissues we sequenced is to determine how well we have recovered a core set of genes for our taxonomic group. Although no such set is known for mosses (or even land plants), a set of core genes is defined for all eukaryotes. The Basic Universal Single Copy Orthologs (BUSCOs) are curated from all metazoan and fungal genomes, and maintained as a set of profile Hidden Markov Models (HMMs), and an inferred ancestral amino acid sequence for each orthogroup is provided (Simão et al., 2015). To determine whether our masked dataset contained BUSCOs, we first searched the translated amino acids from our masked dataset against the ancestral sequences using BLASTP. Sequences with hits were then searched against the 429 BUSCO profile HMMs using HMMER. In order to accept a match, the hmmsearch score had to exceed a minimum score threshold defined for each BUSCO.

2.4. Species tree reconstruction

Many methods of species tree reconstruction rely on the identification of orthologous sequences, that is, sequences that arose by speciation rather than duplication. The Yang/Smith Pipeline identifies sets of orthologous genes (orthogroups) by decomposing the unrooted homologous gene family trees into subtrees where a monophyletic outgroup (here, acrocarpous mosses) is sister to a monophyletic ingroup (pleurocarpous mosses). The extracted subtrees represent inferred orthologous gene families, i.e. gene families for which the most recent common ancestor (the ancestral node) underwent a speciation event and not a duplication event. More than one of these orthologous subtrees may appear within a homologous gene tree if, for example, a gene duplication occurred prior to the divergence of the ingroup and outgroup.

We further filtered the orthologous gene trees by requiring that each species be represented by exactly one transcript, and refer to this subset as the **one-to-one orthologs.** The final matrix for species tree reconstruction represented 659 orthogroups where all 26 species are represented, with a total alignment length of 361,745 amino acid residues. Transdecoder produces an amino acid file and a coding domain sequence (CDS; nucleotides) for each putative protein. We aligned the proteins from each orthogroup with MAFFT, and back-translated the sequences using the corresponding nucleotide sequences using TrimAl (Capella-Gutierrez et al., 2009). We concatenated all of the coding regions into a supermatrix using phyutility (Smith and Dunn, 2008). This matrix comprised 659 genes and 1,062,897 nucleotides, with all 26 species represented for each gene. We reconstructed the species tree in RAxML using the GTRGAMMA model using two partitions per gene (one partition for the first and second codon positions, and another partition for the third). We evaluated nodal support using 200 bootstrap replicates.

We also reconstructed the Maximum Quartet Support Species Tree (MQSST) using ASTRAL (Mirarab et al., 2014). Individual gene trees were reconstructed using RAxML with the GTRGAMMA model, including a single maximum likelihood tree as well as 200 "fast bootstrap" trees. We evaluated support on the ASTRAL trees with a "gene-wise jackknife method." We generated 200 pseudoreplicates of the dataset by sampling 10% of the maximum likelihood gene trees without replacement and calculated a MQSST tree using ASTRAL on each subset.

We also repeated both the supermatrix and MQSST reconstruction methods using the corresponding amino acid alignment. The PROTGAMMA models were used for the supermatrix and individual gene tree reconstructions in RAxML.

2.5. Gene family expansion in Hypnales

We generated a table of gene family occupancy by counting the number of transcripts present for each species in each of the 27,299 homolog gene family trees generated by the Yang/Smith Pipeline. Unlike the one-to-one ortholog set of gene trees used for phylogenetic reconstruction, each gene homolog gene family can contain many transcripts from each species. To track the phylogenetic history of these gene families and identify expansions, we used the program Count (Csurös, 2010) to reconstruct ancestral states. Count uses Wagner parsimony (with a 20% penalty for gains) to identify nodes where: (a) a gene family has more than one member and (b) the gene family has exactly one member at the immediately ancestral node.

To identify gene family expansions associated with the diversification of Hypnales (the largest order of pleurocarpous mosses), we were interested in gene family expansions reconstructed at the following nodes (Fig. 1): (A) the common ancestor of all Hypnales minus *Plagiothecium* (a genus revealed to be sister to the rest of Hypnales), (B) the common ancestor of all Hypnales (including *Plagiothecium*), (C) the common ancestor of Hypnales and *Aulacomnium*, and (D) the common ancestor of the Bryidae (includes Hypnales, *Aulacomnium*, and *Rhodobryum*). In order to assign GO annotations to transcripts of non-model organisms, we used the annotation pipeline Trinotate (trinotate.github.io). Briefly, the pipeline searches transcripts (and their protein translations) against curated functional annotation databases, including Pfam and SwissProt. We assigned GO annotations to each homologous gene family cluster by recording all GO annotations from Trinotate made to each transcript in the cluster.

We performed a gene ontology enrichment analysis using the orthogroups with inferred expansions on one of the four nodes of interest, using the Python package goatools (version 0.5.4, github.com/tanghaibao/goatools). All GO categories annotated for all 15,459 homologous gene family clusters was used as the baseline, and we controlled for multiple testing using the False Discovery Rate method (Benjamini and Hochberg, 1995).

2.6. Evidence of paleopolyploidy

Whole genome duplication (WGD) events can be detected from transcriptome data by finding pairs of paralogous sequences within the transcriptome (Barker et al., 2009; Blanc and Wolfe, 2004b; Yang et al., 2015). The synonymous substitution rate (Ks) is calculated from each pair of paralogs. In principle, the distribution of Ks values should approximate an exponential distribution, reflecting the age distribution of gene duplication events—many pairs of genes with low Ks values, and fewer pairs with larger Ks values. This distribution could arise from many, ongoing small-scale duplications occurring throughout the history of the lineage,

followed by the nonfunctionalization of one duplicate. However, a WGD event would result in a very large number of paralogous pairs all having the same age. If a histogram of Ks values among pairs of parologs in a transcriptome has multiple peaks at intermediate values of Ks, it may be evidence of a paleopolyploidy event.

We could not analyze the transcriptome data for the presence of recent paralogs using the masked dataset, which we used for phylogenetic analysis and ortholog detection. Masking removes recent paralogs, which would bias the estimation of Ks from paralog pairs. We also could not use the raw output from Trinity, which produces transcripts with names such as "c1250_g1_i1." The first field refers to a "component" of the DeBruijn graph, the second field to a "gene" identifier, and the third field to a putative "isoforms" for the transcript. However, these isoforms may not correspond to real alternative splice variants, but may also contain parologous gene sequences. Therefore, rather than keeping only the longest isoform, or the isoform with the highest coverage for each gene component, we used CD-Hit-EST (Fu et al., 2012) to cluster the transcripts with a high percent identity threshold (−c option, 98%) and high alignment overlap threshold (−aS option, 90%) to reduce the isoforms to a set of non-redundant transcripts for each species.

To detect ancient parology, we began with the protein sequences that matched the non-redundant transcript set from CD-Hit-EST cluster for each species separately. We clustered these protein sequences for each species again with CD-Hit, but with much lower thresholds for percent identity (−c 0.4) and alignment overlap (−aS 0.75) to maximize cluster inclusiveness. For each cluster that was not a singleton, we constructed pairwise amino acid alignments among all proteins in the cluster using MAFFT. The corresponding nucleotide transcript sequences were forced into the amino acid alignments using pal2nal (Suyama et al., 2006), and all gap regions and internal stop codons were removed.

For each pair of paralogous nucleotide sequences, we calculated the synonymous substitution rate (Ks) with KaKs_Calculator (Zhang et al., 2006), using the "GY" method (Goldman and Yang, 1994), also known as F3x4. We investigated the presence of multiple normal distributions of Ks values using mixture models, implemented in the R package mclust (Fraley et al., 2012). We evaluated mixture models with between one and ten components, and the best fit model was chosen using the Bayesian Information Criterion (BIC).

2.7. Detecting changes in selection

We investigated the effect of the Hypnales radiation on signatures of molecular selection using the codeml package implemented in PAML (version 4.7; (Yang, 2007). For this analysis we used a subset of orthogroups where (1) all six acrocarpous mosses were present and (2) at least ten pleurocarpous mosses were present. We aligned protein sequences with MAFFT and back-translated using the corresponding CDS sequences using trimAL (version 1.4.rev15 Capella-Gutierrez et al., 2009), which also removed codons in the sequence matrix if they were present in fewer than five sequences. We calculated a tree for each orthogroup using FastTree (Price et al., 2010). The common ancestor of pleurocarpous moss sequences in each orthogroup was determined using the Python package ETE2 (Huerta-Cepas et al., 2010), which also assisted in running codeml. All branches descending from the common ancestor of pleurocarp sequences were marked as "foreground" for branch-model analysis. We estimated the ratio of non-synonymous to synonymous nucleotide substitution rate (dN/dS or omega) under two models: in the "M0" model, all branches are assumed to have the same omega, but in the "bfree" model, separate omegas are estimated for the "foreground" and "background" branches. Because the M0 model is nested within the bfree model, the significance of the bfree model can be determined with a Likelihood Ratio Test (LRT) with one degree of freedom. We tested the significance of the LRT against a chi-squared distribution, and accounted for multiple tests by accepting p-values less than 0.0001.

For genes with evidence of different rates of evolution in pleurocarps and acrocarps, we conducted a GO Enrichment analysis using the same procedure as above. We summarized the GO Enrichment results using ReviGO (Supek et al., 2011), which produces a visualization of the semantic similarity among GO categories.

A conceptual diagram illustrating our entire analysis can be found in Supplemental Fig. 1.

3. Results and discussion

3.1. Transcriptome assembly and orthology detection

Across our 25 assembled transcriptomes, we recovered between 53,000 and 301,000 transcripts (Table 1). After applying our filtering steps, assuring that the transcript contained a valid protein using Transdecoder and that the protein had BLAST hits against known land plant proteomes, we retained between 32,349 and 81,018 protein coding sequences per species. The Yang/Smith Pipeline then clustered these transcripts from all 25 transcriptomes and the *Physcomitrella patens* proteome into 27,299 homologous gene families that contained transcripts from at least four different species. We then produced the "masked" dataset by removing sequences if transcripts from the same species form monophyletic or paraphyletic groups on the multispecies gene family trees. On average, 19,393 translated proteins were retained (Table 1), and each transcriptome had at least one sequence in an average of 12,054 homologous gene families. Following the filtering and clustering steps, we retained between 12,566 and 25,976 transcripts within 27,299 homolog family trees (Table 1).

To approximate whether we sampled the pool of all possible transcripts as deeply as possible, we searched for the 429 BUSCOs (universal orthologous genes) defined for all eukaryotes (Simão et al., 2015). We were able to detect between 382 and 391 BUSCOs in our 25 transcriptomes. For comparison, we could find 391 BUSCOs in the *Physcomitrella* proteome, suggesting that this is the maximum number for mosses, and that the remaining 38 orthogroups are not universal to all eukaryotes if plants are included. The authors of the BUSCOs pipeline have begun development of a more plant-specific set of universal orthologs (buscos.ezlab.org). We therefore accept 391 as the maximum number of eukaryote BUSCOs that can be found in mosses.

Because we used different multiplexing schemes, we also tested whether the number of reads correlated with our assessment metrics, but the number of reads per sample did not correlate with the number of Trinity transcripts ($F_{23} = 3.2$, $r^2 = 0.08$, $p = 0.09$) or the number of proteins retained in the masked dataset ($F_{23} = 3.1$, $r^2 = 0.08$, $p = 0.09$). The correlation between the number of reads and the number of BUSCOs recovered was significant ($F_{23} = 8.0$, $r^2 = 0.23$, $p = 0.01$). On average, we recovered three additional BUSCOs from the eight samples with more than 30 million reads, compared to the seventeen samples with fewer than 30 million reads. Overall, this suggests that additional sequencing of each species would not change the inferences made here.

We also assessed the completeness of our transcriptomes by comparison to the well-curated proteome of *Physcomitrella patens*. Although 21,312 proteins from *Physcomitrella* clustered with transcripts from at least four of our transcriptomes, only 7778 gene families in the masked data set contain a protein from the model moss proteome. This is likely due to the masking step of the Yang/Smith pipeline, which removes sequences if they form a

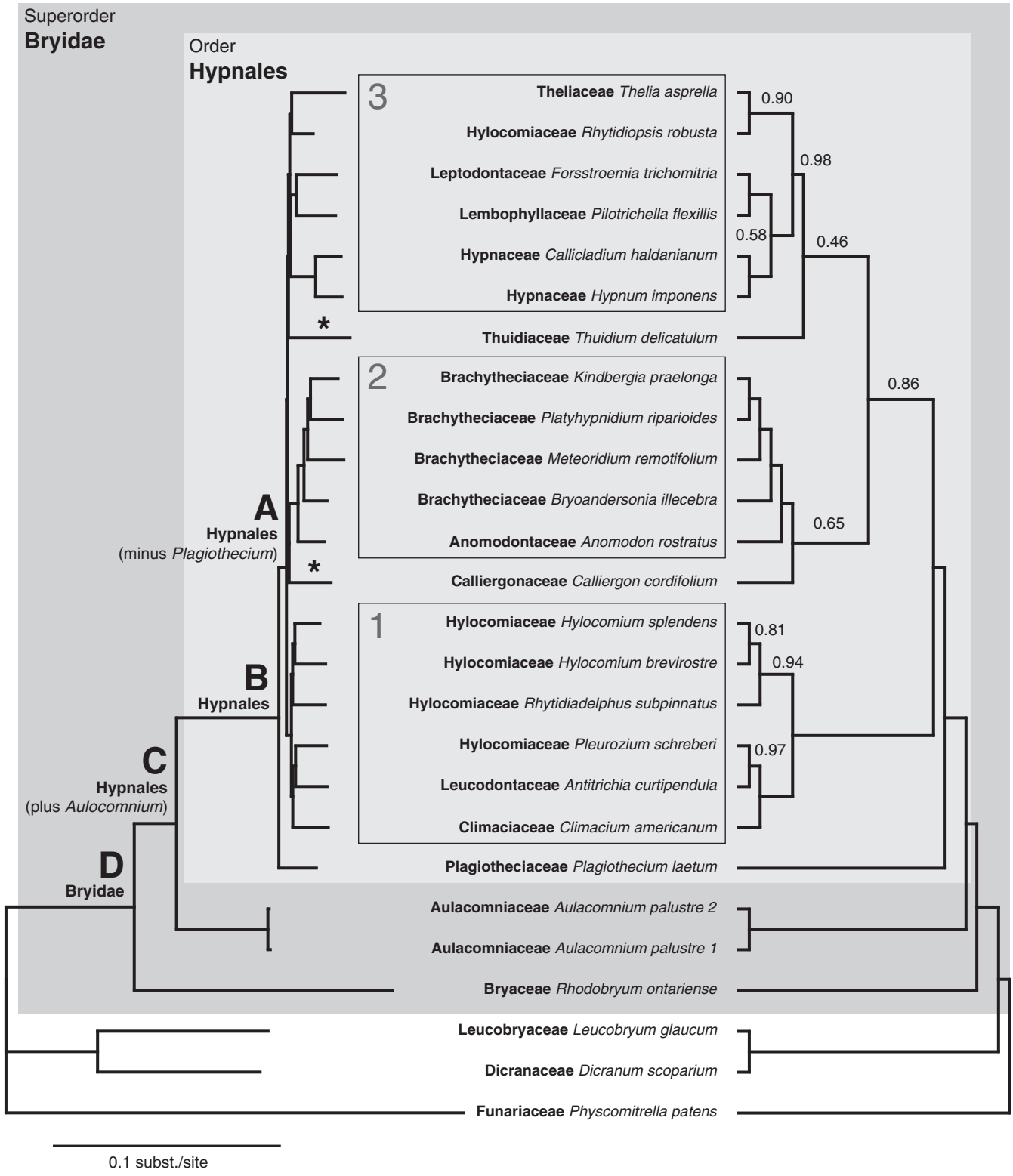

Fig. 1. Species trees constructed from 659 orthogroups in 21 pleurocarpous and five acrocarpous mosses, and the number of homologous gene family expansions at key nodes on the tree. Left: Maximum-likelihood nucleotide tree from RAxML. All nodes are supported at 100% bootstrap support (200 pseudoreplicates) except for the nodes indicated by stars. The letters indicate four key nodes at which gene family expansions were calculated: A: Hypnales minus *Plagiothecium*, B: Hypnales, C: Hypnales mosses plus *Aulacomnium*, D: Superorder Bryidae. Right: Maximum Quartet Support Species Tree from ASTRAL, reconstructed from nucleotide gene trees estimated in RAxML. Nodal support is 100% except where indicated by genewise-jackknife.

monophyletic group that includes only sequences from the same taxon. As a result of the whole genome triplication event that occurred during the diversification of Funariaceae (Rensing et al., 2007), many *Physcomitrella* paralogs clustered together in our gene family trees at a 3–1 ratio relative to other mosses. On the initial gene family trees, the *Physcomitrella* paralogs would share a common ancestor within *Physcomitrella*, and the masking step would retain only one of these paralogs per gene family.

There are also a large percentage of homolog gene families (over 19,000) that do not contain a representative from *Physcomitrella*, but do contain transcripts from at least four different transcriptomes. The transcriptome sequences could only progress to this point in our pipeline if they had a significant (*e*-value < 10^{-5}) BLAST hit to a land plant proteome. Therefore, some of these gene families may be ancestral land plant families that have since been lost in the lineage leading to *Physcomitrella*. Alternatively, because Yang/Smith Pipeline is intended primarily as a way of identifying low-copy orthologs for phylogenetic inference, gene families may be circumscribed narrowly. Higher-order clustering may reveal homology between gene families. Additional genome sequences from other bryophytes would assist in this effort.

3.2. Species tree reconstruction

We constructed species trees from 659 orthogroups that had a one-to-one orthology between acrocarpous and pleurocarpous mosses, as well as a representative sequence from all 26 taxa. Both the Maximum Likelihood (ML) Tree and the (Maximum Quartet Support Species Tree (MQSST) have strong backbone support, reflecting relationships among acrocarpous mosses, and their relationships to Hypnales (Fig. 1). When rooted using *Physcomitrella patens*, *Aulacomnium palustre* (both accessions) is sister to the pleurocarpous mosses (Hypnales), as expected (Bell et al., 2007; Cox et al., 2010). Sister to this clade is *Rhodobryum*; together with *Aulacomnium* and Hypnales these species represent the subclass Bryidae (Goffinet et al., 2009; Stech and Frey, 2008). Previous phylogenetic evidence supports Bryidae as sister to Dicranidae (Chang and Graham, 2011; Cox et al., 2010), which in our dataset is represented by *Leucobryum* and *Dicranum*.

Within the pleurocarpous mosses, *Plagiothecium* is resolved as sister to the rest of Hypnales with maximum support in both tree reconstruction approaches (Fig. 1), consistent with previous efforts using one or a few genes (Cox et al., 2010; Huttunen et al., 2012; Merget and Wolf, 2010). Two other multi-species relationships are maximally supported using all methods. Clade 1 contains four of our five samples from the Hylocomiaceae plus *Climacium* (Climaciaceae) and *Antitrichia* (Leucodontaceae), and is resolved as sister to the remaining Hypnales (minus *Plagiothecium*) with maximal support in both the RAXML and ASTRAL trees. Clade 2 contains four Brachytheciaceae, which compose a monophyletic sister lineage to *Anomodon* (Anomodontaceae), and relationships within this clade are fully resolved in both trees. Clade 3 contains the remaining species of Hylocomiaceae (Rhytidiopsis) along with two species of Hypnaceae, Forsstroemia (Leptodontaceae), Pilotrichella (Lembophyllaceae), and Thelia (Theliaceae). Rhytidiopsis has been accommodated in the Hylocomiaceae (Buck and Vitt, 1986) but affinities to Thelia had first been proposed by Chiang and Schaal (2000), and then by Huttunen et al. (2012). These three well-resolved clades are consistent with those recovered from inferences from discrete loci from all genomic compartments (Huttunen et al., 2012).

Two branches on the maximum likelihood tree did not receive 100% support. Inspection of bootstrap trees reveals that full phylogenetic resolution within Hypnales is impeded by the positions of two species: *Thuidium delicatulum* and *Calliergon cordifolium* (Supplemental Fig. 2). Lineage movement analysis of the bootstrap replicates revealed the two species are as likely to be sister-species (25%) as they are in their maximum likelihood arrangement (26%). Likewise, the placement of *Thuidium* sister to Clade 3 (46%) and *Calliergon* as sister to Clade 2 (65%) have the lowest gene-wise jackknife values of any relationship on the ASTRAL tree. We expect that denser taxon sampling of transcriptomes within Hypnales and the less species-rich orders of pleurocarpous mosses would allow us to more confidently reconstruct the affinities of *Thuidium* and *Calliergon*. However, while we are unable to completely resolve relationships in these clades, the focus of this study is to reconstruct the history of gene family evolution at higher phylogenetic levels.

The backbone of the phylogeny was reconstructed with equal confidence using amino acid characters (Supplemental Fig. 3). The resolution of clades within Hypnales was less certain; Clades 1 and 2 were fully supported using both RAxML and ASTRAL, but the support for Clade 3 was similarly reduced by the placement of *Thuidium* and *Calliergon*. The relationships among the three major clades within Hypnales were unresolved with both methods using the amino acid matrix.

Our results suggest that intra-genomic phylogenetic conflict complicates the resolution of family-level relationships within Hypnales. Earlier studies, which focused effort on taxon sampling, with few genes, had similar difficulty resolving the same relationships (Cox et al., 2010; Huttunen et al., 2012). Although we have employed a phylotranscriptomic approach, it is likely that the relationships within Hypnales may not be resolved without a broader taxonomic sampling. Specifically, several major families of Hypnales were not sampled in our phylogeny, and the addition of transcriptomes from the other orders of pleurocarpous mosses (e.g. Hookeriales) would root the Hypnales phylogeny more accurately. Though not suitable for taxonomic revision, our data do present a large step forward in the genomic sampling effort, increasing the number of genes sequenced in mosses by two orders of magnitude over previous studies. We anticipate that the phylogenetic framework presented here, particularly with respect to the identification of orthologous gene families, will provide a foundation for more taxon-dense phylogenetic studies in the future.

3.3. Gene family expansion analysis

Despite some instances of low phylogenetic resolution within Hypnales, the strong support among backbone clades of the mosses enables us to infer patterns of gene family evolution. Because we are using transcriptomes, gene family membership may be reduced due to incomplete expression of the proteome. However, we can treat the number of distinct transcripts per species (an approximation of gene copy number, not relative expression) as a discrete trait that evolves along the species tree. By reconstructing the "gene family occupancy" for each gene family at each node on the species tree, we minimize the noise associated with gene loss and under-expression in terminal taxa. Specifically, we identified expansions in homologous gene families at four robustly supported (100% in all methods), nested nodes, marked in Fig. 1: (A) Hypnales minus *Plagiothecium*, (B) Hypnales, (C) Hypnales plus *Aulacomnium*, and (D) Bryidae.

We reconstructed gene family occupancy using a Wagner parsimony approach in the software Count (Csurös, 2010). Expansions were identified by two criteria at each of the four target nodes: the gene family had to be reconstructed as (1) containing multiple paralogs at the target node and (2) exactly one paralog at the immediately ancestral node. Gene family contractions were identified by inverse criteria. Our analysis revealed that homologous gene family expansions at the four target nodes were extremely enhanced compared to homologous gene family contractions (Fig. 2A, Supplemental Table 2). The highest discrepancy between expansions and contractions occurred at the Hypnales + *Aulacomnium* (C) node, with 799 expansions and only 11 contractions.

Across all four target nodes, 1712 homologous gene families exhibited low ancestral occupancy and high occupancy in Hypnales. Sixty-one GO categories (Fig. 2B, Supplemental Table 1) were enriched among the homologous gene families that had expanded in Hypnales. The enriched categories included "post-embryonic morphogenesis" (GO:0009986) and "developmental process

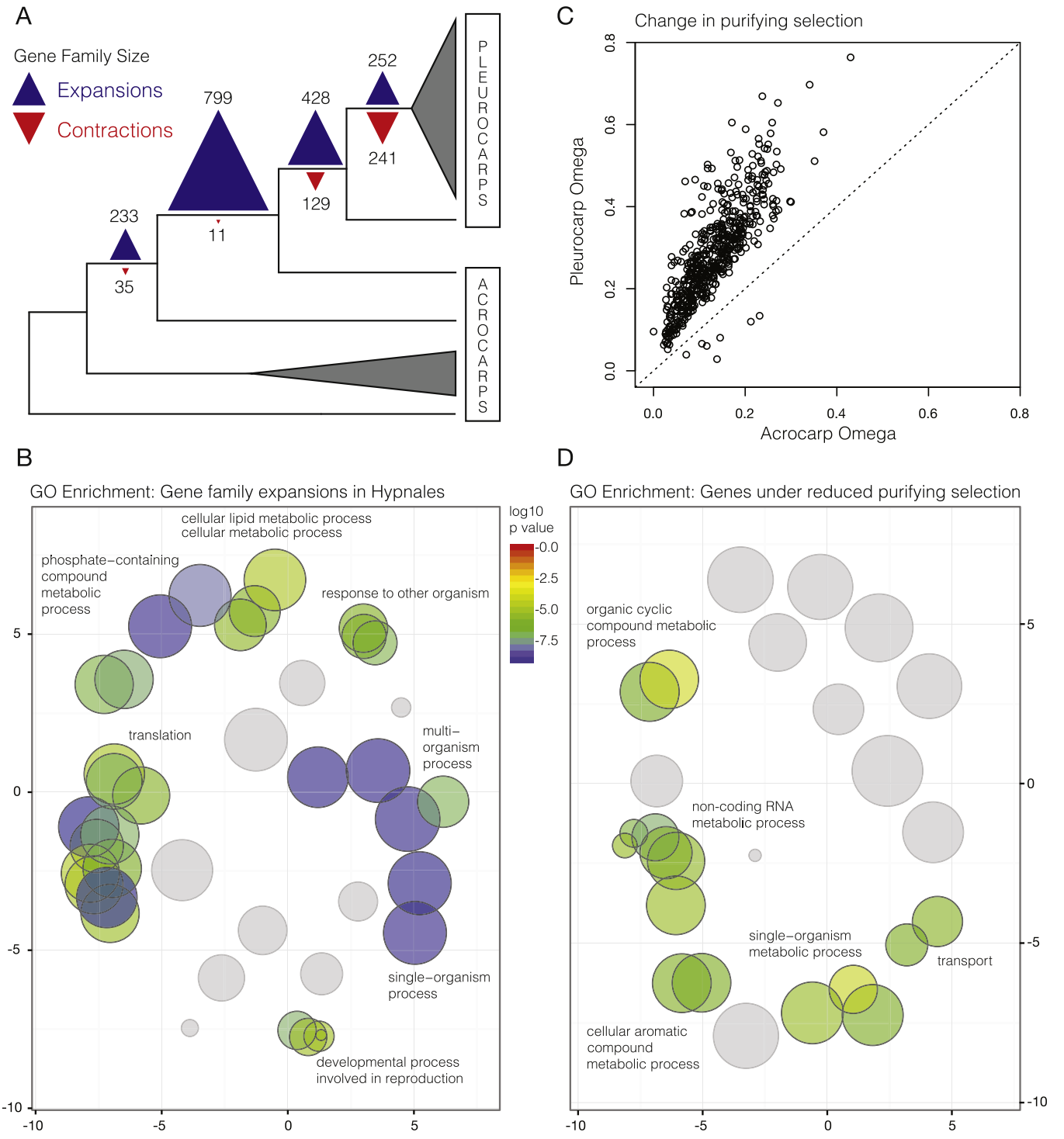

Fig. 2. Gene family expansion in pleurocarpous mosses is associated with enriched gene ontology (GO) categories and reduced purifying selection. (A) Expansions and contractions in homologous gene families reconstructed by Wagner parsimony at four nested nodes that represent common ancestors of the pleurocarpous mosses. (B) Multidimensional scaling of semantic similarity among GO categories for gene families that have expanded in pleurocarpous mosses. Two GO terms (circles) have similar semantic similarity if they are siblings in the GO heirarchy or are related by inheritance. Overlapping circles of terms share similar characteristics and are labeled in the figure with one representative GO term. (C) Comparison of omega values (ratio of non-synonymous to synonymous substitution rate) in 526 orthogroups where a two-omega model was preferred to a single omega for the tree. The remaining 2220 gene family trees for which the two-omega model was not preferred are not shown. Each point represents a gene tree, where the horizontal axis is the omega inferred from the "background" branches (acrocarpous mosses), and the vertical axis is the omega inferred from the "foreground" branches (pleurocarpous mosses). The dotted line represents equivalent omegas in the two sets of branches; only seven genes have a lower inferred omega in pleurocarpous mosses, compared to acrocarpous mosses. An omega over 1.0 represents evidence of positive selection, but an increased omega in the foreground branches may represent reduced purifying selection. (D) Semantic similarity plot of GO terms that are enriched in the set of gene families shown to have reduced purifying selection. Overlapping sets of GO categories are represented by one GO term. For a full list of all enriched categories, see the supplemental information.

involved in reproduction" (GO:0003006). Of particular interest were several categories involved in interactions among organisms, such as "response to external biotic stimulus" (GO:0043207) and "defense response to other organism" (GO:0098542).

These functional annotations are intriguing because the shared derived growth form of pleurocarpous mosses (a shift in reproductive structure from terminal to lateral, and a generally more prostrate and branching growth form) may be associated with traits that later facilitated the rapid radiation of Hypnales. Because most of the major families in Hypnales diversified concurrent with the diversification of other plants (Laenen et al., 2014), the opportunity for new biotic interactions may have arisen. For example, the diversification of angiosperms may have presented mosses with a novel substrate, driving neofunctionalization of genes responsible for external stimuli and defense responses. It is clear from our results that many gene families have expanded coincident with the radiation of the pleurocarpous mosses. However, it is unclear when the gene families expanded during the evolution of pleurocarpous mosses, because our sampling was limited to Hypnales. Future studies could identify whether many of the gene families instead expanded in the shared ancestor of Hypnales and Hookeriales (the other major order of pleurocarpous mosses), or perhaps prior to the divergence of earlier pleurocarpous lineages, namely the Hypnodendrales and the Ptychomniales (Bell et al., 2007). Detailed studies involving gene knockouts are also needed to confirm that these gene families have significant impact on life history traits in pleurocarpous mosses.

3.4. Evaluation of paleopolyploidy in pleurocarpous mosses

The increase in gene family occupancy that we observe can be explain either by (1) a whole genome duplication (WGD) event, or (2) many small-scale duplications (SSDs). If the large increase in gene family occupancy in the pleurocarpous mosses indicates a whole genome duplication, it does not appear to have been accompanied by an increase in the base chromosome number. Most of the species in our transcriptome dataset have a base chromosome count of 10, 11, or 12 (Fritsch, 1991), below a threshold that has been used previously for inferring polyploidy in mosses (Crawford et al., 2009).

We observe a consistent pattern of gene family occupancy ratios (2:1 or 3:1 pleurocarp:acrocarp) in about 6% of the gene family trees in the masked dataset. Many gene family duplications localize to specific branches on our species tree (Figs. 1 and 2A), particularly the common ancestor of Hypnales and *Aulacomnium*, suggesting that the gene family expansions share a common age, indicative of WGD. However, Hypnales shares a more recent common ancestor with other groups of pleurocarpous mosses (such as Hookeriales) that were not sampled as part of this study. The apparent increase in gene family occupancy at the common ancestor of Hypnales and *Aulacomnium* may be the result of SSDs that occurred along the long branch that separates these groups (Fig. 1), but could not be reconstructed to more specific nodes due to our taxon sampling. Data from additional groups of mosses, including the other orders of pleurocarpous and protopleurocarpous mosses, would be necessary to pinpoint the age of gene duplications and better distinguish WGD from SSD using the gene family occupancy reconstruction method.

We were also unable to detect a clear signal of WGD in our 25 transcriptomes using a Ks-based method. Although our pipeline reliably recovered the WGD previously described from *Physcomitrella* (Rensing et al., 2007), none of our transcriptomes showed an obvious intermediate "peak" of Ks values between 0.5 and 2.0 (Fig. 3, Supplemental Fig. 1). Using the mclust method to fit Gaussian distributions to the Ks values, the best fit (evaluated by BIC score) was typically between 6 and 9 components (Supplemental Table 3), which would suggest evidence of several WGD events. However, in most cases the BIC values for several values of "g" (the number of components) were very similar. When only the model with the best BIC value was considered for each transcriptome, the means of distributions for each species did not show consistent overlap (Supplemental Fig. 4). We therefore consider any signal of WGD with this method to be weak. If an ancient WGD event occurred, the intermediate peak of Ks values may be obscured due to the age of the duplication event, as seen in the *Amborella* proteome (Amborella Genome Project et al., 2013). Additionally, we may not be able to observe consistent peaks across Hypnales if the substitution rates are too variable (Barker et al., 2009).

3.5. Molecular signatures of selection

When a gene is duplicated, one or both copies may take on a new function (neofunctionalization), and these innovations result from positive, or reduced purifying selection acting on one or both copies (Blanc and Wolfe, 2004a; Freeling, 2009). We investigated the signature of selection in all orthologous gene trees found by the Yang/Smith pipeline that contained sequences from all acrocarpous mosses and at least ten pleurocarpous mosses (2746 orthogroups). We reconstructed gene trees for each orthogroup from back-translated nucleotide sequences and estimated branch-wise models of molecular evolution using CodeML. In the "M0" model, a single ratio of synonymous to nonsynonymous substitution rates (dN/dS, or omega) was inferred for the entire tree. In the "two-omega" model, separate omegas are estimated for the "pleurocarpous" and "acrocarpous" branches, and we determined whether this was a significantly better fit to the data using a Likelihood Ratio Test ($p < 0.0001$ to correct for multiple tests). Of the 2746 orthogroups tested, the two-omega model was preferred in 526 orthogroups, and for 519 of these, omega was greater in the pleurocarpous moss lineages (Fig. 2C). None of the inferred omega values were greater than one (which would suggest positive selection), but the large number of genes with elevated omegas in pleurocarpous lineages relative to acrocarpous lineages supports a hypothesis of reduced purifying selection.

We performed a GO enrichment analysis of the functionally annotated *Physcomitrella* proteins in the 519 orthogroups with reduced purifying selection, and revealed 19 enriched GO categories (Fig. 2D, Supplemental Table 4). Many of these GO categories belong to a single "family" of GO categories (Supplemental Fig. 5), the most specific of which is "metabolic process" (GO: 006399). To determine whether this was related to codon usage bias, we analyzed all transcriptomes and the *Physcomitrella* coding domain sequences using four metrics of codon usage calculated by the program codonW (http://codonw.sourceforge.net/). However, none of the metrics showed significant differences in codon usage between acrocarps and pleurocarps (Supplemental Table 5).

For seven orthogroups for which the two-omega model was preferred, omega was greater in the background (acrocarp) branches (Table 2). The *Physcomitrella* proteins in these orthogroups have been studied in controlled differential expression experiment, and several pairs of these seven genes are known to have correlated co-expression (see phytozome.jgi.gov). *Physcomitrella* genes Phpat.011G004300.1 (Membrane-associated hemotopoietic protein) and Phpat.011G069500.1 (Ankyrin repeat and protein kinase domain-containing protein) are known to be significantly co-expressed (Pearson's coefficient 0.82). In contrast, Phpat.001G030000.1 (Putative RNA Polymerase II regulator) and Phpat.024G039100.1 (histone H-3) are known to be significantly inversely expressed (Pearson's coefficient −0.33). If the co-expression of these genes is maintained in Hypnales, it would suggest that entire gene networks have shifted regimes of molecular

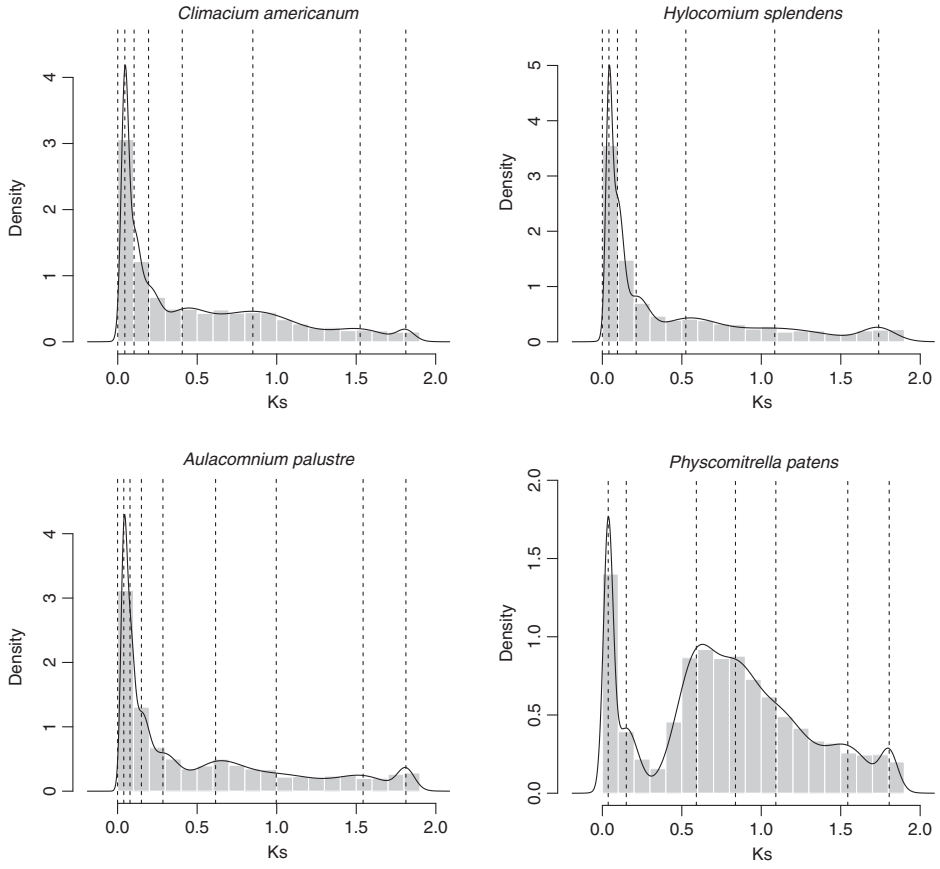

Fig. 3. Distribution of synonymous substitution rates (Ks) among pairs of paralogous genes within selected species. The dotted vertical lines represent the mean values of component distributions inferred by mclust under the model with the highest BIC score. The solid curved line is the inferred density distribution for the mixture model by mclust. For plots of all species, see Supplemental Fig. 2. *Climacium americanum* and *Hylocomium splendens* are pleurocarpous mosses, *Aulacomnium palustre* is a "protopleurocarpous" moss generally considered to be acrocarpous, and *Physcomitrella patens* is acrocarpous.

Table 2
Description of gene families with increased purifying selection in Hypnales, relative to the acrocarpous mosses.

Cluster ID	Acrocarp omega	Pleurocarp omega	*Physcomitrella* Gene ID	*Physcomitrella* gene annotation
cluster7933	0.145	0.051	Phpat.001G030000.1	Putative RNA Polymerase II regulator
cluster4410	0.212	0.12	Phpat.011G069500.1	Ankyrin repeat and protein kinase domain-containing protein
cluster5502	0.071	0.039	Phpat.011G004300.1	Membrane-associated hemotopoietic protein
cluster6993	0.106	0.066	Phpat.024G039100.1	Histone-H3
cluster10685	0.139	0.028	Phpat.007G045900.1	U2 small nuclear ribonucleoprotein B
cluster8238	0.116	0.061	Phpat.001G146700.1	ATP Binding/DNA Binding/Helicase
cluster3968	0.232	0.134	Phpat.012G062300.1	Hypothetical protein F4|10.140

selection coincident with the radiation of Hypnales. A controlled-condition differential expression study is needed to determine if the genes that have shifted regimes of selection have retained correlated expression in Hypnales pleurocarpous mosses.

It is possible that selection may act differently upon gene copies that result from small-scale gene duplications (SSDs), compared to genes that result from WGD. For example, a WGD generates gene copies in roughly equal proportions throughout enzymatic pathways, while SSDs may cause dosage imbalances and result in poor pathway flux (Lynch and Conery, 2000). As such, an alternative explanation to WGD is that the excess of gene family occupancy in Bryidae (and in Hypnales) is the result of several SSDs. In an investigation of the fate of gene copies resulting from small-scale duplications in land plants, Carretero-Paulet and Fares (2012) found that gene copies resulting from SSDs had reduced purifying selection in three angiosperm species, unlike gene copies resulting from WGD events. However, they did not observe this effect in *Physcomitrella patens*. Because we see a similar pattern (reduced purifying selection) in gene families that have expanded in Hypnales, small-scale duplications may be more plausible than a WGD. Additional taxon sampling and genome-wide analyses of synteny, particularly of other lineages within Bryidae and among other orders of the pleurocarpous mosses, are required to further distinguish between whole genome and small-scale duplications as sources of expanded gene families in pleurocarpous mosses.

4. Conclusions

This study is the first to investigate the genomic signatures associated with a rapid radiation in the largest order of pleurocarpous mosses, the lineage that accounts for the largest proportion of extant moss diversity. We describe here a set of 659 orthologous gene families and demonstrate their utility for phylogenetic

reconstruction in pleurocarpous mosses. These genes and analyses will likely form the foundation for future analyses of pleurocarp diversity, and our phylogenetic hypothesis provides a starting point to ask whether genomic features common to other rapid radiations in land plants occurred in pleurocarps. Our results suggest that both gene family expansion and a relaxation of purifying selection on many genes are significant features of the radiation of Hypnales and provide a set of candidate genes that, with further refinement, may be used in functional studies of pleurocarp development. The utility of transcriptome data for the phylogenetic analysis of molecular evolution depends on careful curation of datasets, including removal of contaminants, detection of homologous and orthologous sequences, and data analysis that allows the presence of missing data. Future phylotranscriptomic work, in this group and others, will rely on the continued development of bioinformatics pipelines to handle the challenges of working with transcriptome data. However, we have shown here that the use of transcriptomes to discover fundamental evolutionary processes that underlay the radiation of pleurocarpous mosses yields significant results and shows great promise for testing evolutionary hypotheses more broadly in mosses.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.01.008.

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Genetic diversity, sexual condition, and microhabitat preference determine mating patterns in *Sphagnum* (Sphagnaceae) peat-mosses

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In bryophytes, the possibility of intragametophytic selfing creates complex mating patterns that are not possible in seed plants, although relatively little is known about patterns of inbreeding in natural populations. In the peat-moss genus *Sphagnum*, taxa are generally bisexual (gametophytes produce both sperm and egg) or unisexual (gametes produced by separate male and female plants). We sampled populations of 14 species, aiming to assess inbreeding variation and inbreeding depression in sporophytes, and to evaluate correlations between sexual expression, mating systems, and microhabitat preferences. We sampled maternal gametophytes and their attached sporophytes at 12–19 microsatellite loci. Bisexual species exhibited higher levels of inbreeding than unisexual species but did generally engage in some outcrossing. Inbreeding depression did not appear to be common in either unisexual or bisexual species. Genetic diversity was higher in populations of unisexual species compared to populations of bisexual species. We found a significant association between species microhabitat preference and population genetic diversity: species preferring hummocks (high above water table) had populations with lower diversity than species inhabiting hollows (at the water table). We also found a significant interaction between sexual condition, microhabitat preference, and inbreeding coefficients, suggesting a vital role for species ecology in determining mating patterns in *Sphagnum* populations. *Biological Journal of the Linnean Society*, 2015, **115**, 96–113.

ADDITIONAL KEYWORDS: bryophytes – haploid – inbreeding depression – mating systems.

INTRODUCTION

A central issue in plant reproductive biology is intra- and interspecific variation in inbreeding, and the avoidance of inbreeding depression. In angiosperms, self-incompatibility systems and dioecy both reduce the probability of mating between gametes produced by the same sporophyte (i.e. intergametophytic selfing). The most common explanation for this avoidance of self-fertilization is the accumulation of slightly deleterious recessive alleles, especially in outcrossing populations (Nei, Maruyama & Chakraborty, 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, Kalisz & Eckert, 2005). General surveys confirm a wide variety of outcrossing rates in angiosperms, from obligate outcrossing to obligate selfing and every rate inbetween (Barrett, 2003).

Homosporous spore-producing plants, such as ferns and bryophytes, are capable of an additional type of selfing not possible in seed plants. Intragametophytic selfing (i.e. the union of genetically identical sperm and egg) produces a completely homozygous offspring in a single round of mating. In ferns, the rate of intragametophytic selfing is generally low (Soltis & Soltis, 1992) and, in some species, is prevented by the production of chemicals (antheridiogens) that block production of sperm in bisexual gametophytes (i.e. those producing both antheridia and archegonia, Chiou & Farrar, 1997). Therefore, there may also be

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selective pressure to avoid intragametophytic selfing, even though exposure of deleterious alleles in homozygous sporophytes would likely result in these alleles being quickly purged from the population (Hedrick, 1987).

This is especially true for bryophytes because of their haploid-dominant life cycle. Genes co-expressed in both the haploid and diploid stages are likely to experience less genetic load than genes expressed in just the diploid stage (Shaw & Beer, 1997; Joseph & Kirkpatrick, 2004). However, compared to seed plants, relatively few studies have tested theoretical predictions about the cost of inbreeding in natural populations of bryophytes. A few have made indirect inferences about mating patterns from genetic diversity (Stoneburner, Wyatt & Odrzykoski, 1991; Shaw, 2009), although only one (Eppley, Taylor & Jesson, 2007) compared levels of inbreeding in moss species with uni- versus bisexual gametophytes in natural populations. They documented significantly higher inbreeding coefficients in bisexual species.

There is also a paucity of data regarding inbreeding depression in bryophytes. Taylor, Eppley & Jesson (2007) found sporophytic inbreeding depression in a species with unisexual gametophytes (i.e. producing archegonia or antheridia but not both) 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* (Hedw.) P. Beauv., a species that produces both unisexual and bisexual gametophytes. Szövényi, Ricca & Shaw (2009) found a significant association between sporophyte size [correlated with fitness (spore number)] and heterozygosity in *Sphagnum lescurii* Sull., a unisexual species.

Even if inbreeding depression is a significant selective pressure in bryophytes, their mating patterns may also be influenced by abiotic environmental factors. Similar to all seed-free plants, bryophyte sperm must swim to the egg to effect fertilization. Sperm dispersal distance is generally quite limited (Wyatt, 1977; Bisang, Ehrlén & Hedenäs, 2004) and strongly dependent upon water availability (Shortlidge, Rosenstiel & Eppley, 2012). Species that prefer dryer habitats are more likely to experience self-fertilization as a result of limited sperm dispersal. Therefore, fertilization success may depend on species ecology, especially for unisexual species, where intragametophytic selfing is not possible.

Sphagnum L. (peatmosses) presents an excellent case study for investigating the influences of sexual condition and ecology on mating behaviour in a group of closely-related species because they vary in gametophyte sexuality (uni- versus bisexual) and microhabitat. An early diverging lineage of the mosses (Bryophyta), *Sphagnum* includes approximately 250–400 species worldwide, organized in six monophyletic groups that have been classified as subgenera (Shaw *et al*., 2010). *Sphagnum* is best known for its prominence in Northern Hemisphere peatlands that have huge impacts on biogeochemistry and global climate (Wieder & Vitt, 2006; Tuba, Slack & Stark, 2011). Twenty or more species can co-occur in wetland communities and many species are ecologically differentiated in relation to microenvironmental variation within those peatlands (Rydin & Jeglum, 2013).

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, and to infer the paternal haploid genotype by subtraction (Szövényi *et al*., 2009). Previous studies have demonstrated a high correlation across multiple species between the diameter of a *Sphagnum* sporophyte capsule (sporangium) and the number of spores that it contains (Sundberg & Rydin, 1998), presenting an easily measured proxy for fitness in *Sphagnum* sporophytes. Based on studies of seed plants, we can hypothesize that bisexual species are more inbred than unisexual species, that unisexual but not bisexual species might exhibit inbreeding depression, and (in bryophytes) that water availability impacts outcrossing rates.

Of the 91 species of *Sphagnum* 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). Studies of mating patterns in *Sphagnum* are facilitated by a set of microsatellite markers that amplify in almost every species in the genus without evidence of ascertainment bias (Shaw *et al*., 2008a; Shaw, Terracciano & Shaw, 2009; Karlin *et al*., 2010, 2011). Microhabitat preference (in the sense of very narrow, species-specific, realized niches) likely plays a role in determining mating patterns in *Sphagnum*. Species are generally differentiated with respect to their 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). The hydrological gradient is of particular interest because *Sphagnum* species that generate hummocks high above the water table are likely to experience limited water availability for the dispersal of sperm.

In the present study, we investigated 18 populations across 14 species of *Sphagnum* aiming to address three questions. (1) What is the diversity of mating patterns in *Sphagnum*? We tested this using standard statistics to measure genetic diversity and inbreeding. (2) Is inbreeding depression common in populations of unisexual species and absent in populations of bisexual species, as predicted by population

genetics theory? (3) Are there connections between mating patterns and sexual condition or microhabitat preference?

MATERIAL AND METHODS

Bryophyte life cycle

Most moss species (approximately 60%) have separate sexes, comprising unisexual gametophytes that produce either sperm or eggs but not both (Wyatt & Anderson, 1984). Many species (approximately 30%) have bisexual gametophytes that can produce both archegonia (containing eggs) and antheridia (containing sperm). Sexual system (uni- versus bisexual gametophytes) is generally a species trait, although a minority of species can be polymorphic for gametophyte sexuality: gametophytes may be unisexual or bisexual. Haploid plants germinate from spores and form leafy gametophytes at maturity; many species also accomplish various forms of asexual reproduction via branching, fragmentation or the production of specialized vegetative propagules. At sexual maturity, males (Fig. 1A) produce sperm via mitosis in antheridia and females (Fig. 1B) produce eggs in archegonia, also via mitosis. Thus, all sperm produced by a single male gametophyte are genetically identical to each other and to the male gametophyte that produced them, with a comparable pattern for females. In mosses with bisexual gametophytes, all eggs and sperm are genetically identical to one another, as well as to the parental gametophyte.

In all species, water is required for the sperm to reach an egg-containing archegonium and effect fertilization. The resulting sporophyte begins development within the archegonium and remains attached to the maternal gametophyte for its entire life cycle. At maturity, the sporophyte forms haploid spores via meiosis within a single sporangium. Individual gametophytes often bear multiple sporophytes (Fig. 1B).

Sexual conditions in *Sphagnum*

When considering mating patterns, the possibility of intragametophytic selfing is arguably the most important distinction for homosporous plants. For the present study, intragametophytic selfing is defined as fertilization between genetically identical sperm and egg, producing offspring that are completely homozygous at every locus. Thus, a large vegetative clone may produce many ramets that have identical genotypes, although fertilization within genets is always intragametophytic. The critical distinction among species is the classification of sexual condition, and depends on whether a gametophyte is capable of producing both kinds of gametes ('bisexual') or only one type of gamete ('unisexual'). Although a few bryophyte species have variable sexual systems (Jesson *et al*., 2012), in *Sphagnum*, gametophytic sex expression is generally a fixed species trait (Szövényi *et al*., 2009; Ricca *et al*., 2011). Therefore, in the present study, we describe a species that produces male gametes and female gametes from the same plant as a 'bisexual species'. A species that produces gametes in separate male and female gametophytes is a 'unisexual species'.

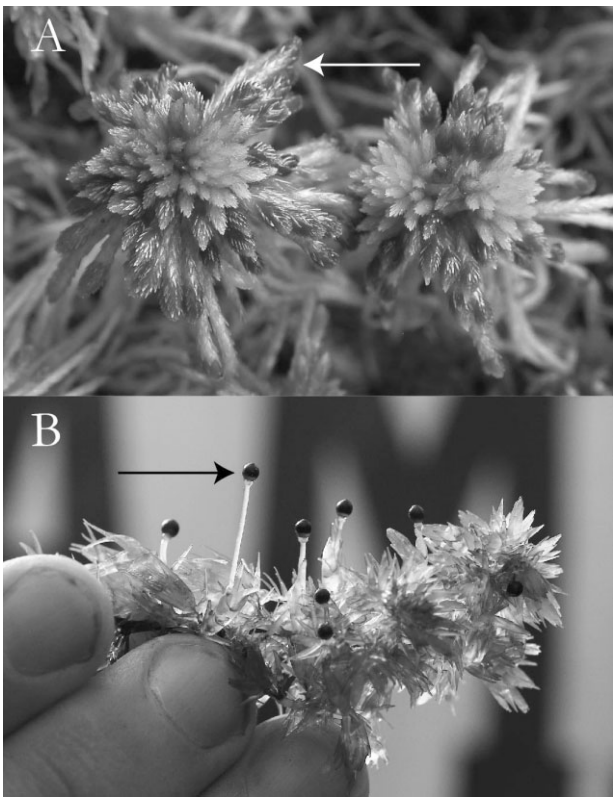

Figure 1. Unisexual gametophytes in *Sphagnum*. A, male gametophyte of *Sphagnum capillifolium* produces sperm in antheridia on specialized branches (white arrow). B, female gametophyte of *Sphagnum macrophyllum*, bearing a 'brood' of sporophytes (at least eight sporophytes are visible). The sporophyte is a single spheroid capsule (sporangium, black arrow). Photos courtesy of Blanka Shaw.

We classified each species in the present study as 'unisexual' or 'bisexual' based on personal observation of sexual conditions in the field, including thousands of *Sphagnum* specimens personally collected on five continents spanning more than a decade. Our classifications generally agree with two major monographs of *Sphagnum* (Crum, 1984; McQueen & Andrus, 2009); one notable exception is discussed below.

Species and population sampling

In general, our sampling of sporophyte-producing populations of *Sphagnum* was opportunistic. Plants

with sporophytes were collected when encountered during field work, and so we include multiple population samples for some species but only one population for others. We chose to maximize numbers of species rather than infraspecific populations, although additional data be will be valuable in the future to assess mating pattern variation among populations within species.

When a population with sporophytes was identified, our sampling strategy depended on the nature of the population. In populations where the sporophytes were spread across a large area, we sampled gametophytes and sporophytes along transects. In other cases, when the populations consisted of one or a few patches only, we sampled from within each patch. Because we had no information a priori about the genetic structure of these populations, our sampling strategy aimed at collecting a minimum number of maternal ramets. Populations are primarily from sites in Maine, Alaska, British Columbia, and the south-eastern USA. Collection and DNA voucher information are provided in the Appendix. All vouchered specimens were deposited in the Duke University Herbarium, and are identified as vouchers in the herbarium database (http://plantdb.biology.duke.edu:8080/BryoFullSearch/advanced.jsp).

We sampled populations from species in each of the major subgenera of *Sphagnum*, including both bisexual species and unisexual species. Although allopolyploidy is a common feature in *Sphagnum*, all fourteen species sampled have haploid gametophytes (no fixed heterozygosity in gametophytes). We sampled no more than two populations of each species; most species are represented by a single population. Because of this, our results are not intended to be extrapolated to represent the 'mating system' of particular species. Many previous studies have demonstrated that mating systems can vary within species (Goodwillie *et al*., 2005). Instead, our results are intended to show, across species, the relationship between sexual condition and mating patterns.

Several decades of peatland ecology research show that individual *Sphagnum* species have narrow microhabitat ranges and, in particular, are differentiated along a hydrological gradient (Vitt & Slack, 1984; Andrus, 1986; Gignac, 1992). This differentiation can be generally described as species preferring either a 'hollow' habitat (i.e. living right at the water table or even aquatically) or a 'hummock' habitat (i.e. forming high mounds or cushions well above the water table) (Rydin and Jeglum, 2013).

We assigned each species in the present study to a microhabitat group based on data collected from peatland ecological surveys that measured species preferences on the hummock–hollow gradient (Johnson *et al*., 2015b). From these data, we designated five 'hummock' species (mean height above water table > 10.0 cm): *Sphagnum angustifolium* (Warnst.) C.E.O. Jensen, *Sphagnum austinii* Sull., *Sphagnum fallax* H. Klinggr., *Sphagnum magellanicum* Brid., *Sphagnum squarrosum* Crome and four 'hollow' species: *Sphagnum compactum* Lam. & DC., *Sphagnum cuspidatum* Ehrh. ex Hoffm., *Sphagnum pulchrum* (Lindb.) Warnst., and *Sphagnum tenellum* (Brid.) Brid. The remaining species included in the present study did not have data in the ecological surveys; however, *Sphagnum macrophyllum* Bernh. ex Brid. and *Sphagnum cribrosum* Lindb. are both aquatic species that grow in open water (Anderson *et al*., 2009), and are classified here as 'hollow', whereas *Sphagnum molle* Sull. and *Sphagnum strictum* Sull. both form cushions, and are classified here as 'hummock' species.

Genotyping

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

Plants were genotyped at nineteen microsatellite loci using previously described protocols (Shaw *et al*., 2008a). 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; and Set 5: p9, p14, p20, p56. Locus designations follow Shaw *et al*. (2008a). These microsatellite loci have been shown to be variable in a multitude of *Sphagnum* species from every subgenus.

In the present study, 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. Loci were also discarded if multiple individuals had more than one allele in the haploid stage or more than two in the diploid stage (a total of three loci across all populations were discarded). Missing data for within-species matrices ranged from 2.7% to 15.9%. Microsatellite allele tables were submitted to the Dryad data repository.

To assess paternity, we first inferred the haploid paternal genotype from every sporophyte by subtracting the haploid genotype of the maternal gametophyte. A custom PYTHON script that accounted for missing data was used to assign multilocus paternal microsatellite genotypes to genets. The script began by sorting samples with no missing data into multilocus genotypes. If a sample could be unambigu-

ously assigned to just one multilocus genotype despite missing data, it was retained; otherwise, it was discarded.

Mating patterns: statistics

To characterize mating patterns in *Sphagnum*, we focused on two main properties of the sporophyte generation in each species: genetic diversity and inbreeding. Sporophytes attached to the same maternal gametophyte necessarily share half of their multilocus genotype, and are not therefore independent draws from mating events in the population. Particularly fecund maternal gametophytes (those with many sporophytes) will introduce a bias when estimating allele frequencies.

To partially address this bias, we generated 1000 'subsets' of each population. Each subset contained a random draw of one sporophyte per maternal brood. For example, imagine a population with three maternal gametophytes (A, B, and C), each of which bears four sporophytes (numbered 1–4). A subset population would contain one sporophyte randomly drawn from each mother, such as: [A1, B2, C3]. We calculated diversity and inbreeding statistics on this subset population. We then repeated this procedure by randomly drawing another sporophyte (with replacement) from each mother and recalculating each statistic on this new subset, such as: [A3, B2, C4]. This was carried out a total of 1000 times, resulting in a range of values for each population statistic.

We calculated three measures of sporophyte genetic diversity at each locus. The included 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), 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 used sporophyte diameter as a proxy for sporophyte fitness. Sundberg & Rydin (1998) demonstrated a correlation between the diameter of a *Sphagnum* sporophyte and the number of spores it contains. Sundberg and Rydin found that this correlation holds for a phylogenetically diverse set of eight *Sphagnum* species, including three sampled in the present study. This phenotypic measurement allows for a simple estimate of potential reproductive output for the sporophyte without damaging the tissue necessary for genotyping the sporophyte. Other studies have also used the height of the sporophyte as a proxy for fitness in bryophytes (Eppley *et al*., 2007; Taylor *et al*., 2007; Jesson *et al*., 2012) because sporangial height may correlate with dispersal ability. However, each of those studies focused on mosses in the class Bryopsida, which have sporangia exerted on seta derived from sporophytic tissue. By contrast, *Sphagnum* sporangia are raised on a pseudopodium derived from haploid maternal gametophyte tissue, and thus height would be an inappropriate proxy for fitness of the diploid phase in *Sphagnum*. Sporophyte diameter was also used by Szövényi *et al*. (2009) to assess the presence of inbreeding depression in *S. lescurii*.

For each maternal haploid gametophyte sampled, we removed all attached sporophytes and photographed the sporophytes using a calibrated digital microscope camera (Olympus BX41; Olympus Imaging America, Inc.) under a dissecting microscope at × 10 power. We measured the diameter of each sporophyte from the image using MICROSUITE, version 5 (Olympus Imaging America, Inc.). Measurements were accurate to 0.1 μm.

We estimated the extent of sporophytic inbreeding depression using the linear regression of heterozygosity (percentage of loci with two alleles) on fitness (sporophyte size) using two methods. Our main approach was to test the significance of the correlation including all sporophytes in a population. However, to consider the possibility that sporophytes may not be independent draws from the population, we also used a pseudoreplication approach as above and determined the significance of the linear relationship in 1000 pseudoreplicates. The pseudoreplicate approach will have less power to detect significant relationships because they will be based on the sample size of mothers in the population (rather than of sporophytes).

RESULTS

Microsatellite diversity

Out of our panel of 19 microsatellite loci, we genotyped at least 12 loci in each population (mean loci scored: 13.5; range 12–15) (Table 1). The loci with successful amplification differed among species and, in one case (*S. strictum*), successful amplification of two loci (17 and 22) were specific to populations within species (Table 1). By using a standard panel of loci, we ensured amplification of sufficient loci necessary to assess genetic variability and mating patterns within populations. The allelic diversity of loci varied among species and subgenera of *Sphagnum*: for example, locus 1 was diverse in the subgenera *Cuspdiata* and *Acutifolia* but was generally invariable in the other subgenera. Across loci, allelic diversity was highest in subgenus *Subsecunda* (4.5–6.0 alleles/locus) (Table 1), the group that includes the species for which the microsatellite loci were originally designed *S. lescurii* (Shaw *et al*., 2008a). Allelic diversity was also high in *Cuspidata* (2.2–5.9 alleles/locus) and *Acutifolia* (1.8–3.3 alleles/locus), although

Table 1. Allelic diversity of sporophytes at 19 microsatellite markers across *Sphagnum* populations

Species	Population	*Subgenus*	1	4	7	9	10	12	14	17	18	19	20	22	29	30	56	65	68	78	93	Average unique
Sphagnum angustifolium	ME-S	*Acut*	3	NA	4	4	5	1	7	4	1	NA	4	3	NA	3	2	3	2	NA	NA	3.3
*Sphagnum molle**	SC-36	*Acut*	1	1	1	NA	3	1	3	2	3	NA	2	1	1	2	3	NA	1	NA	NA	1.8
*Sphagnum tenellum**	ME-W	*Cusp*	5	NA	6	2	4	NA	2	2	1	7	2	NA	NA	NA	2	1	1	2	NA	2.8
Sphagnum warnstorfii	AK-M	*Acut*	2	NA	1	5	4	2	6	NA	4	4	2	3	1	2	NA	NA	NA	NA	4	3.1
Sphagnum cuspidatum	GA-28	*Cusp*	6	3	6	12	12	1	15	7	2	NA	3	NA	NA	4	8	NA	3	1	NA	5.9
Sphagnum cupidatum	NC-JL	*Cusp*	2	3	5	5	5	1	6	4	1	NA	2	NA	NA	2	5	NA	1	1	NA	3.1
Sphagnum fallax	ME-S	*Cusp*	3	NA	5	NA	5	2	7	3	1	NA	4	2	3	2	5	3	1	NA	NA	3.3
Sphagnum fallax	ME-W	*Cusp*	5	4	11	NA	8	2	8	4	1	NA	4	3	5	4	NA	5	1	NA	NA	4.6
Sphagnum pulchrum	ME-C	*Cusp*	3	2	1	2	5	1	7	3	1	NA	2	1	NA	1	NA	1	NA	1	NA	2.2
Sphagnum compactum	AK-PC	*Rig*	1	NA	NA	1	1	NA	4	NA	1	1	6	NA	NA	1	5	1	1	1	1	1.9
Sphagnum compactum	AK-PH	*Rig*	1	NA	NA	1	1	NA	3	NA	1	1	3	NA	NA	1	3	1	1	1	1	1.5
*Sphagnum strictum**	SC-36	*Rig*	1	NA	1	1	1	1	2	NA	2	2	1	1	NA	2	NA	NA	1	1	NA	1.3
*Sphagnum strictum**	NC-LL	*Rig*	1	NA	1	1	1	1	1	1	1	1	1	NA	NA	1	NA	NA	1	1	NA	1.0
Sphagnum cribrosum	GA-32	*Subs*	NA	5	4	6	10	NA	8	4	3	NA	4	NA	2	NA	NA	2	3	3	NA	4.5
Sphagnum macrophyllum	SC-39	*Subs*	1	4	3	13	12	NA	11	5	9	6	5	NA	3	NA	NA	2	3	6	7	6.0
Sphagnum macrophyllum	SC-36	*Subs*	NA	4	5	8	12	NA	9	2	4	NA	6	NA	2	NA	NA	2	1	5	NA	5.0
Sphagnum austinii	VI	*Spha*	1	NA	2	2	3	2	1	2	2	1	1	1	1	3	NA	NA	NA	NA	1	1.6
Sphagnum magellanicum	NC-JL	*Spha*	2	1	2	2	2	1	2	1	2	NA	2	2	2	2	NA	2	2	NA	NA	1.8
*Sphagnum squarrosum**	AK-W	*Squa*	1	NA	1	2	2	NA	5	1	4	2	2	NA	NA	2	5	NA	3	1	3	2.4
		Average unique	2.3	3.0	3.5	4.2	5.1	1.3	5.6	3.0	2.3	2.8	2.9	1.9	2.2	2.1	4.2	2.1	1.6	2.0	2.8	

NA, locus did not amplify in the population. The mean number of unique alleles across populations (columns) or within populations (rows) is also indicated. Asterisks indicate bisexual species. Subgenus abbreviations: Acut, *Acutifolia*; Cusp, *Cuspidata*; Rig, *Rigida*; Subs, *Subsecunda*; Spha, *Sphagnum*; Squa, *Squarrosa*. Information on each population is ptovided in the Appendix.

it was much lower in populations of species in subgenera *Squarrosa* (2.4 alleles/locus), *Sphagnum* (1.6–1.8 alleles/locus), and *Rigida* (1.0–1.9 alleles/locus). Overall, there was a significant subgenus effect on within-population allelic diversity (analysis of variance: $F_5 = 7.1$, $P < 0.01$). However, as shown below, the subgenus effect is confounded by the effects of sexual condition and species ecology.

Bisexual populations

All seven populations of five bisexual *Sphagnum* species showed high levels of intragametophytic selfing and low genetic diversity (I) (Table 2). Inbreeding was lowest [$F_{IS} = 0.35$; 95% confidence interval (CI) = 0.24–0.47] in the AK-PH population of *S. compactum*, in which 46% of sporophytes were completely homozygous despite moderate genetic diversity in the population ($I = 0.63$, 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 and there was zero genetic diversity ($I = 0$) in this population. The other population of *S. strictum* we sampled, from South Carolina, had a low, but nonzero genetic diversity ($I = 0.19$, 95% CI = 0.12–0.30), and also had several sporophytes that were heterozygous at exactly one locus (locus p19). The two populations, which were approximately 80 miles apart, had fixed differences at several loci (results not shown). We also observed high intragametophyte selfing rates in the population of *S. molle*, despite a comparatively larger amount of genetic diversity in that species. Although the one population of *S. squarrosum* that we sampled had the highest genetic diversity among populations of bisexual species ($I = 0.81$, 95% CI = 0.75–0.88), it also had a very high inbreeding coefficient ($F_{IS} = 0.71$, 95% CI = 0.65–0.88). In most bisexual populations, homozygous sporophytes exhibit a large range in fitness that is equivalent to the ranges observed for heterozygous sporophytes (Fig. 2).

The linear regression of heterozygosity on sporophyte fitness was significant for only two populations, both of *S. compactum* (Fig. 1). In one population (AK-PH), 23 of 39 sporophytes (59%) were completely homozygous. These sporophytes were, on average, larger than heterozygous sporophytes in the population ($b = -25.1$, d.f. = 21, $r^2 = 0.12$, uncorrected $P < 0.05$), suggesting outbreeding depression. The pseudoreplication approach indicated significant linear relationships between sporophyte size and percent heterozygosity in 231 of 1000 pseudoreplicates of the AK-PH population (see Supporting information, Table S1).

By contrast, the other population of *S. compactum* (AK-PC) showed a strong positive association between heterozygosity and fitness (inbreeding depression, Fig. 2); homozygous sporophytes are significantly

Table 2. Genetic diversity and mating patterns in bisexual *Sphagnum* species

Species	*Sphagnum compactum*	*Sphagnum compactum*	*Sphagnum molle*	*Sphagnum squarrosum*	*Sphagnum strictum*	*Sphagnum strictum*	*Sphagnum tenellum*
Subgenus	Rig	Rig	Acut	Squ	Rig	Rig	Cusp
Population	AK-PH	AK-PC	SC-36	AK-W	SC-36	NC-LL	ME-W
Ecology	hol	hol	hum	hum	hum	hum	hol
Female gametophytes	16	10	10	23	8	6	15
Average sporophytes per brood	2.4	2.9	3.2	2.5	4.5	4.5	2.5
Sporophytes	39	29	32	58	36	48	37
Maternal genotypes	5	6	4	11	2	1	10
Inferred paternal genotypes	8	17	7	15	4	1	23
Ambiguous paternity	0	10	2	7	1	0	16
Homozygous sporophytes	23	14	28	42	32	48	23
Effective alleles (N_E)	1.54	1.9	1.52	2.22	1.23	1	1.78
Shannon's Diversity (I)	0.51	0.59	0.34	0.81	0.19	0	0.63
Inbreeding coefficient (F_{IS})	0.35	0.42	0.86	0.71	0.6	NA	0.41
Heterozygosity versus size	OUT	IN	NO	NO	NO	NA	NO

NA, there are no heterozygous sporophytes to calculate the value.
Significant relationships between sporophyte heterozygosity and size indicate sporophytic inbreeding depression (IN, positive correlation) or outbreeding depression (OUT, negative correlation). Only sporophytes that could be unambiguously assigned an inferred paternal genotype were included in the analysis; sporophytes that had to be discarded due to missing data are indicated for each population. Key to subgenera: Acut, *Acutifolia*; Cusp, *Cuspidata*; Sph, *Sphagnum*; Rig, *Rigida*. Key to species microhabitat preference: Hum = hummock, Hol = hollow. More information on the populations is provided in the Appendix.

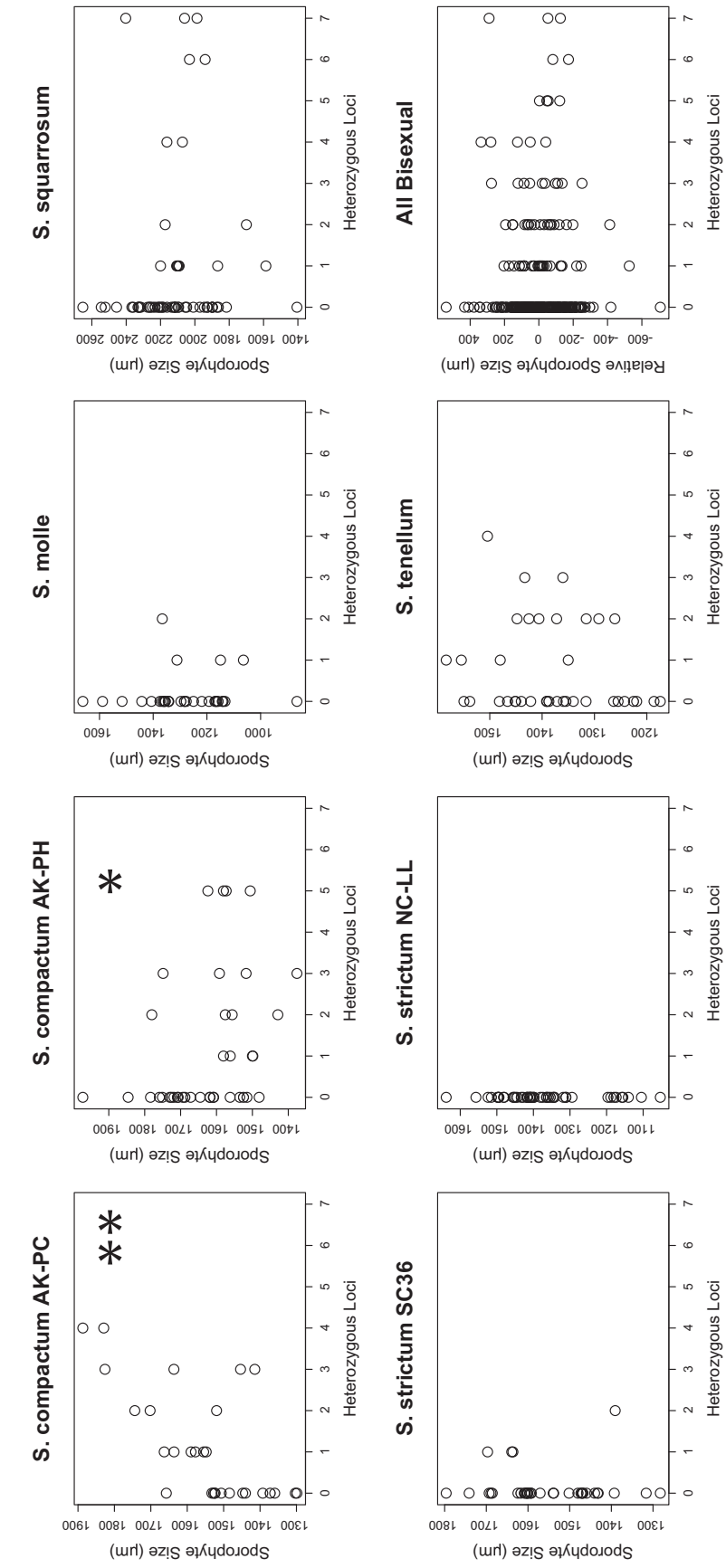

Figure 2. Relationship between heterozygosity and sporophyte size in seven populations of bisexual *Sphagnum* species. A significant positive correlation indicates inbreeding depression, whereas a significant negative correlation indicates outbreeding depression. Bottom right: sporophyte sizes have been standardized within populations (mean of zero) to allow comparison of all bisexual populations. Asterisks indicate significance of the correlations (*$P < 0.05$; **$P < 0.001$).

smaller than heterozygous sporophytes ($b = 74.1$, d.f. = 26, $r^2 = 0.43$, uncorrected $P < 0.001$). The pseudoreplication approach also identified a large percentage of pseudoreplicates with significant relationships (480; see Supporting information, Table S1). In this population, the 15 heterozygous sporophytes were sired by 15 unique inferred paternal genotypes. Six unique maternal genotypes were present, and five of these mothers raised sporophytes that were self-fertilized.

Unisexual populations

We observed a greater variance in genetic diversity and mating patterns among populations of *Sphagnum* species with separate sexes (Table 3). 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*, showed no genetic variation at any locus among maternal gametophytes. Additional alleles appeared at two loci in the sporophytes but 42 of 48 sporophytes were completely homozygous. Excepting this population, genetic diversity was lowest in our population of *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).

Populations varied from a mixed mating in the GA28 *S. cuspidatum* population ($F_{IS} = 0.56$, 95% CI = 0.50–0.63), to the population of *S. magellanicum* ($F_{IS} = -0.98$, 95% CI = −0.98 to −1.00) in which almost all sporophytes were heterozygous at all variable loci (Fig. 3). Seven of the eleven unisexual populations have significantly negative inbreeding coefficients. Excepting our population of *S. austinii*, the only unisexual population with any completely homozygous sporophytes was the GA28 population of *S. cuspidatum*; three sporophytes, all attached to the same maternal ramet, had zero heterozygosity.

There was a strong positive, significant relationship between genetic diversity (I) and inbreeding coefficient in unisexual populations (d.f. = 10, $r^2 = 0.44$, $P < 0.05$) (Fig. 4). The relationship was even stronger when considering the effective number of alleles (d.f. = 10, $r^2 = 0.53$, $P < 0.01$).

Inbreeding depression appeared to be absent in all but two unisexual populations, both of *S. macrophyllum* – the SC36 population has a stronger association between heterozygosity and fitness ($b = 30.5$, d.f. = 44, $r^2 = 0.15$, uncorrected $P < 0.01$) than the SC39 population ($b = 13.5$, d.f. = 273, $r^2 = 0.02$, uncorrected $P < 0.05$). Using the pseudoreplication approach, neither population showed strong assotiations between heterozygosity and size (see Supporting information, Table S1). A highly significant *negative* relationship (outbreeding depression) between heterozygosity and size was found in the population of *S. cribrosum* ($b = -64.7$, d.f. = 44, $r^2 = 0.31$, uncorrected $P < 0.0001$). The pseudoreplication approach revealed 533 out of 1000 pseudoreplicates had significant linear relationships (see Supporting information, Table S1).

In two populations, genetic diversity among the inferred paternal genotypes was low or absent. For the population of *S. magellanicum*, only two unique paternal genotypes were inferred; one of these was found in only two sporophytes, and differed from the other genotype at only one locus. In the population of *S. austinii*, almost all sporophytes were genetically identical and homozygous, and only three different parental genotypes could be inferred.

Effect of ecology and sexual condition on mating patterns

In addition to assignment based on sexual condition (bisexual versus unisexual), species were assigned to ecological groups based on microhabitat preference (hummock versus hollow). We investigated the relationship between each of the genetic diversity and mating pattern statistics, and found four associations related to ecology and sexual condition. First, as mentioned above, there was a significant correlation between genetic diversity and inbreeding coefficient, but only for unisexual populations (Fig. 4).

Genetic diversity (Shannon's Diversity) was significantly associated with sexual condition (analysis of variance: type III SS $F_1 = 9.83$, $P < 0.01$) (Fig. 4) and species ecology ($F_1 = 5.89$, $P < 0.05$), although there was no interaction effect ($F_1 = 0.11$, $P > 0.5$). A similar pattern was found for effective number of alleles per locus (results not shown). For inbreeding coefficient, there was a significant interaction effect between ecology and sexual condition ($F_1 = 6.5$, $P < 0.05$) (Fig. 4). F_{IS} tends to be higher in bisexual hummock populations than bisexual hollow populations, although the reverse is true for unisexual populations.

DISCUSSION

Inbreeding depression

Only three studies on bryophytes have previously investigated the relationship between inbreeding and sporophyte fitness. Taylor *et al*. (2007) found that heterozygosity was associated with reduced sporophyte height but not reduced spore output in a unisexual moss. In a population of *S. lescurii*, an aquatic unisexual species, Szövényi *et al*. (2009) found a significant increase in sporophyte size with increased heterozygosity (with a stronger linear relationship

Table 3. Genetic diversity and mating patterns in unisexual *Sphagnum* species

Species	*Sphagnum angustifolium*	*Sphagnum austinii*	*Sphagnum cribrosum*	*Sphagnum cuspidatum*	*Sphagnum cuspidatum*	*Sphagnum fallax*	*Sphagnum fallax*	*Sphagnum macrophyllum*	*Sphagnum macrophyllum*	*Sphagnum magellanicum*	*Sphagnum pulchrum*	*Sphagnum warnstorfii*
Subgenus	Cusp	Sph	Subs	Susp	Cusp	Cusp	Cusp	Subs	Subs	Sph	Cusp	Acut
Population	ME-S	VI	GA-32	GA-28	NC-JL	ME-S	ME-W	SC-36	SC-39	NC-JL	ME-C	AK-M
Ecology	hum	hum	hol	hol	hol	hum	hum	hol	hol	hum	hol	hum
Female gametophytes	7	24	9	18	8	7	10	12	41	6	8	18
Sporophytes	36	48	46	68	39	31	36	46	275	32	35	43
Average sporophytes per brood	5.1	2	5.1	3.7	4.9	4.4	3.6	6.7	5.3	5.3	4.4	2.4
Maternal genotypes	2	1	8	17	2	3	1	10	11	1	7	8
Inferred paternal genotypes	14	3	25	43	12	8	27	91	33	2	13	22
Ambiguous paternity	6	0	2	0	0	2	6	0	46	0	3	11
Homozygous sporophytes	0	42	0	3	0	0	0	0	0	0	0	0
Effective alleles (N_E)	1.78	1.51	2.73	3.78	2.13	1.96	1.90	3.18	2.65	2.01	1.77	2.35
Shannon's diversity (I)	0.58	0.48	1.08	1.29	0.79	0.63	0.74	1.17	1.04	0.67	0.49	0.85
Inbreeding coefficient (F_{IS})	−0.46	0.12	0.09	0.56	−0.14	−0.23	−0.32	0.12	−0.13	−0.98	−0.09	−0.06
Heterozygosity versus size	NO	NO	**OUT**	NO	NO	NO	NO	**IN**	**IN**	NO	NO	NO

Significant relationships between sporophyte heterozygosity and size indicate sporophytic inbreeding depression (IN, positive correlation), outbreeding depression (OUT, negative correlation) or no significant correlation. Only sporophytes that could be unambiguously assigned an inferred paternal genotype were included in the analysis; sporophytes that had to be discarded as a result of missing data are indicated for each population Key to subgenera: Acut, *Acutifolia*; Cusp, *Cuspidata*; Sph, *Sphagnum*; Subs, *Subsecunda*. Key to species microhabitat preference: Hum, hummock; Hol, hollow. More information on the populations is provided in the Appendix.

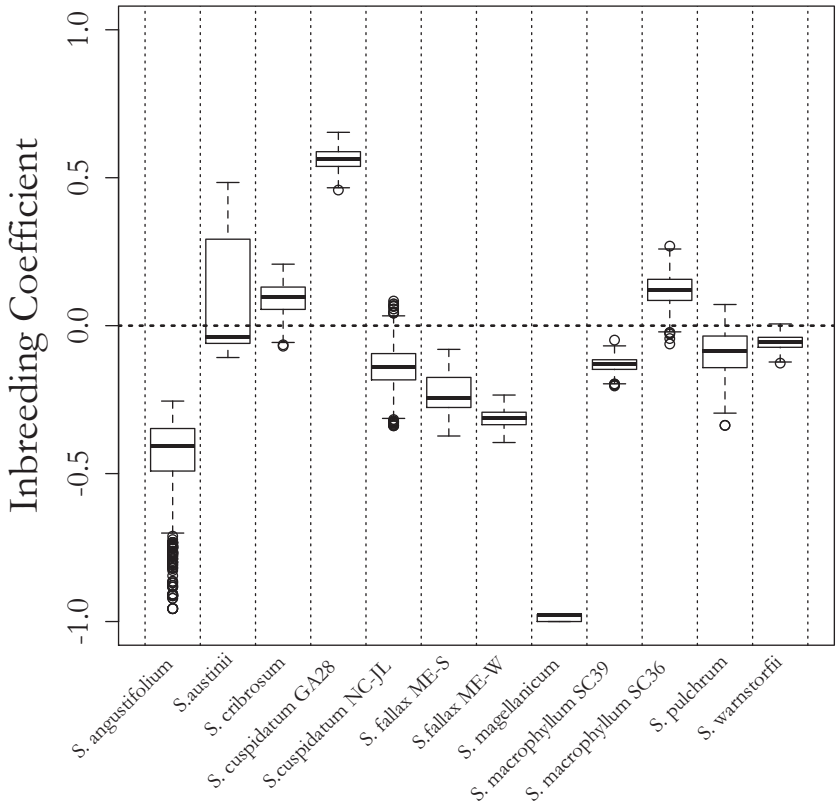

Figure 3. Variation in inbreeding coefficients (F_{IS}) among unisexual *Sphagnum* populations. Inbreeding coefficients were calculated by randomly sampling one sporophyte from each maternal gametophyte. The boxplot shows means and standard variations for 1000 subsamples from each population.

than is found in any population in our study, $b = 35.7$). Our data suggest that these studies cannot be extrapolated to a general conclusion that unisexual moss populations show inbreeding depression. Only two of our unisexual populations, both in the aquatic species *S. macrophyllum*, showed a significant relationship between sporophyte size and heterozygosity. In both, the inbreeding depression effect was smaller than found in *S. lescurii*. In addition, if we correct our *P*-values for multiple comparisons (Bonferonni correction), the significance of the relationship between heterozygosity and size is not maintained for any population. We would therefore caution against interpreting our findings as evidence for significant inbreeding depression in any population. More intense sampling may be necessary to detect weak inbreeding depression in unisexual *Sphagnum* species. Sporophytic inbreeding depression may be masked in some populations as a result of reproductive compensation or early sporophyte abortion; these possibilities require further study.

Most theoretical predictions suggest a small role for inbreeding depression in species capable of intragametophytic selfing (Hedrick, 1987; Stenøien & Såstad, 2001), and this is supported by our findings. If our results are typical for bisexual *Sphagnum* species, it is difficult to imagine a scenario in which inbreeding depression can be maintained in a population where almost 50% of the sporophytes are completely homozygous in each round of mating. For the lone bisexual population with evidence of inbreeding depression, it is possible that the effect we observed resulted from the specific combination of parental genotypes, rather than from heterozygosity per se, although this would require additional study with a larger sample size.

Several studies have suggested that inbreeding depression may not be a universal feature in outcrossing populations when there is substantial overlap in gene expression between the haploid gametophyte and diploid sporophyte generations (Shaw & Beer, 1997; Joseph & Kirkpatrick, 2004). Indeed, approximately 70% of genes expressed in the diploid stage of the bisexual moss *Funaria hygrometrica* are also expressed in the haploid stage, compared to just 3% in *Arabidopsis* (Szövényi *et al*., 2011). If the same is true for *Sphagnum*, then deleterious alleles could be purged from the population during the

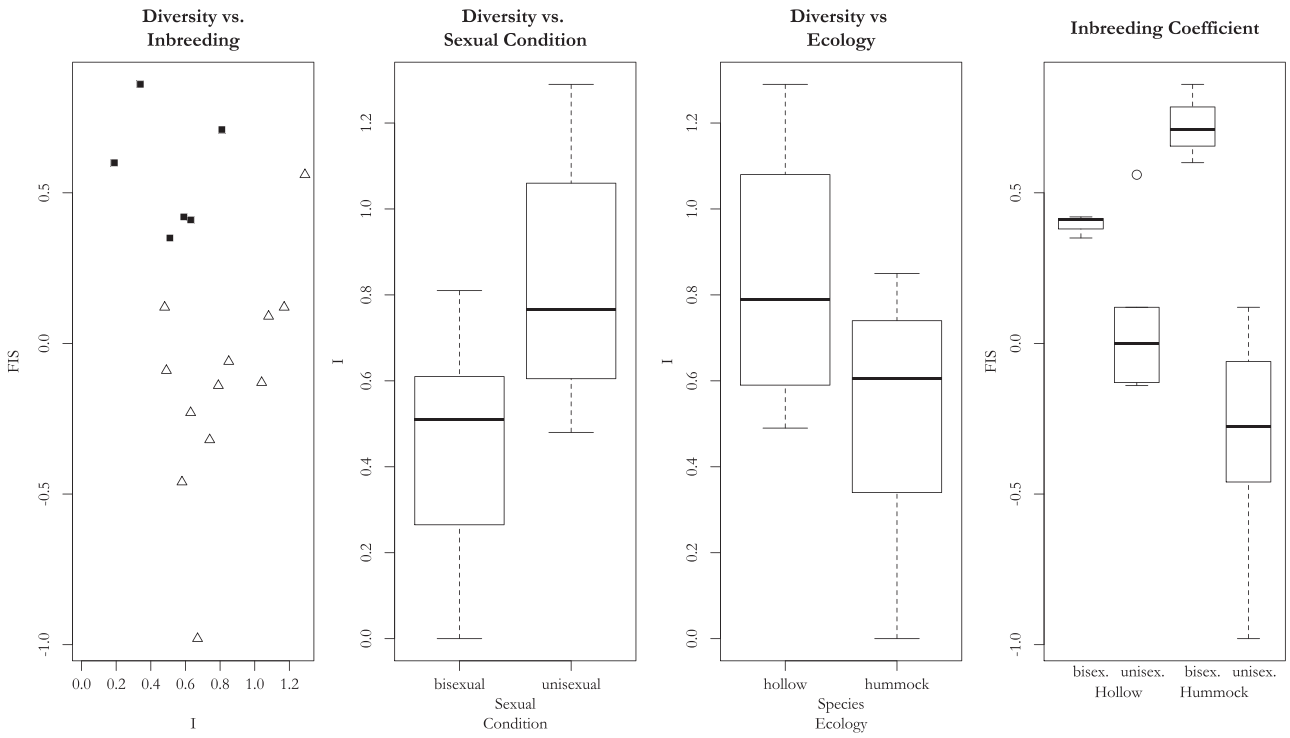

Figure 4. Effect of sexual condition and microhabitat preferences on mating pattern and genetic diversity. Far left: relationship between genetic diversity (*I*, Shannon's Allelic Diversity) and inbreeding coefficient (F_{IS}) for bisexual (circles) and unisexual (triangles) populations. Left centre: relationship between sexual condition (uni- versus bisexual) and genetic diversity. Right centre: relationship between species ecology (hummock versus hollow) and genetic diversity. Far right: interaction effect between sexual condition and ecology (hummock – hollow) versus F_{IS}.

perennial haploid stage, where there is no 'shielding' by dominance, and many unisexual mosses might therefore show no inbreeding depression.

Outbreeding depression (a negative relationship between heterozygosity and fitness) was observed in the AK-PH population of bisexual *S. compactum*, and in the unisexual species, *S. cribrosum*. Outbreeding depression is feasible in populations of predominantly selfing plants, because many generations of local adaptation may result in strong linkage disequilibrium, which would be broken up by hybridization between inbred lines (Fischer & Matthies, 1997; Edmands, 2006). If the intragametophytic selfing rate observed in our *S. compactum* population is typical, this would explain why heterozygous sporophytes are less fit, although 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, Willis & Shaw, 2008). Genetic incompatibilities developed in isolation have been suggested as a type of postzygotic isolation important in speciation (Dobzhansky, 1950; Lynch, 1991). By contrast, we observed outbreeding depression in crosses that naturally occurred within a single population. Interestingly, the *S. cribrosum* population investigated in the present study is known to have the highest genetic diversity of any population in the species (Johnson *et al.*, 2012). In that previous study, we suggested that the GA32 population has been a source of genetic diversity for other populations of *S. cribrosum* in the eastern USA. 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, Keller & Arcese, 2002) and among genetically diverse individuals in a population of *Ipomopsis aggregate* (Pursh) V.E. Grant (Waser, Price & Shaw, 2000).

Overall, we find a lack of association between heterozygosity and fitness in many natural populations of *Sphagnum*. This is unexpected, given the high incidence of unisexual species in *Sphagnum* and other mosses. This suggests that, in contrast to flowering plants, selection for inbreeding avoidance mechanisms might be weak. Bryophytes have perennial haploid gametophytes that are exposed to

environmental selection pressures for a much longer proportion of the life cycle than the haploid gametophytes of flowering plants. This, coupled with relatively high co-expression of genes in the haploid and diploid moss generations (Szövényi *et al*. 2011), may generate weaker selection for inbreeding avoidance. Additional work on a diversity of moss species is needed to determine whether there is a consistent difference in mosses relative to flowering plants in the occurrence of inbreeding depression. Other metrics, such as germination rate and survival of spores from inbred sporophytes, would be useful to further characterize the effect of inbreeding on bryophyte fitness.

Sexual condition, mating patterns, and sex determination

We find sexual condition to be the major factor affecting mating patterns in *Sphagnum*. Most of the unisexual populations had inbreeding coefficients near or below zero, whereas the bisexual populations had significantly higher inbreeding coefficients. In unisexual populations, there was a strong correlation between genetic diversity and inbreeding, as predicted by theory (Charlesworth, Morgan & Charlesworth, 1993).This correlation was, however, absent in bisexual populations.

F_{IS} values indicate a high level of intragametophytic selfing in populations of bisexual *Sphagnum* species, consistent with values found in a limited survey of several other moss species (Eppley *et al*., 2007). Mixed mating (F_{IS} near 0.50) is a common feature in bisexual *Sphagnum* populations. This is in contrast to the theoretical predictions for angiosperms, in which outcrossing (F_{IS} near zero) and inbreeding (F_{IS} near 1) are the theoretical preferred stable states (Lande & Schemske, 1985). However, Lande & Schemske (1985) also postulate that reproductive compensation may lead to stable mixed mating when the threat of inbreeding depression is low. In these cases, inbreeding is advantageous for local populations but outcrossing is preferable for colonization of new areas with potentially different selective pressures (Lande & Schemske, 1985). More recent surveys of natural populations of angiosperms have revealed a wide variety of mating regimes, frequently variable within species (Barrett, 2003).

For species with separate sexes, a low effective population size may generate excesses of heterozygosity because of binomial sampling error (Rasmussen, 1979). A female *Sphagnum* plant in a species with unisexual gametophytes must, by necessity, mate with a different genetic individual. If the overall genetic diversity of the population is low, it is possible that many matings occur between two very different genotypes, generating highly heterozygous offspring. In spore-producing bryophytes characterized by highly effective dispersal, individual populations of gametophytes may be highly diverse, representing genotypes from across the continental range of the species (Ramaiya *et al*., 2010; Johnson *et al*., 2012), and clonal propagation rather than spore-mediated reproduction within populations may be most important for population maintenance (Sundberg, Hansson & Rydin, 2006). In this scenario, the binomial sampling issue may be accentuated, and the inbreeding coefficient (F_{IS}) may be strongly negative.

Traditionally, F_{IS} is often interpreted as the 'probability of identity by descent', although this definition complicates interpretation of negative values of F_{IS}. Instead, it is more meaningful to think in terms of Wright's original definition of the inbreeding coefficient as the 'correlation between uniting gametes' (Wright, 1922, 1965). A high correlation results in inbred offspring, whereas a low correlation results in highly heterozygous offspring. This may be the more appropriate interpretation for bryophytes, especially given low levels of genetic diversity, coupled with extensive clonal reproduction within populations.

Of the unisexual species studied here, two populations contained homozygous sporophytes: the GA28 population of *S. cuspidatum*, and (the only population we sampled of) *S. austinii*. These outliers could be explained by either incorrect assignments of their sexuality, or 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 was only one allele detected at each locus and all sporophytes were 100% homozygous. A broader survey of *S. austinii* in North America has revealed very low diversity compared to other members of subgenus *Sphagnum* (M. Kyrkjeeide, pers. comm.), and a survey of *S. austinii* in Europe with enzyme markers also revealed low genetic diversity (Thingsgaard, 2002). There are no known bisexual species within subgenus *Sphagnum*, making misdiagnosis of sexual condition unlikely, and so we conclude that the higher F_{IS} value for *S. austinii* likely reflects very low levels of genetic diversity rather than misdiagnosis of its sexual condition.

Many of these same microsatellites were used in other studies of species across all subgenera of *Sphagnum* (Ricca *et al*., 2008; Shaw *et al*., 2008b, 2009; Szövényi *et al*., 2009; Karlin *et al*., 2010; Stenøien *et al*., 2011; Johnson *et al*., 2012). In one study, 15 of these loci were used in a global study of the bisexual species *Sphagnum subnitens* Russ. & Warnst. (Karlin *et al*., 2011). Karlin *et al*. (2011) found high genetic diversity in European populations but a single multilocus haploid genotype in western North America.

Their conclusion was that the microsatellites were sufficiently variable to distinguish genetic variability in that species, and that the single genotype was indeed a very large clone. Taken together, these studies suggest no evidence of ascertainment bias, and that the microsatellite loci contain sufficient polymorphism to assess genetic diversity with populations.

By contrast, the GA28 population of *S. cuspidatum* exhibited a high degree of genetic variability. Despite this, the population inbreeding coefficient was more similar to bisexual populations than to other unisexual populations. In addition, three sporophytes, all attached to the same maternal ramet, were 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 appears to be unlikely. An alternative explanation is that *S. cuspidatum* is not always unisexual. There has been some disagreement about the sexual condition of this species. McQueen & Andrus (2009) state that *S. cuspidatum* is dioicous (unisexual gametophytes), whereas Crum (1984) described the species as 'monoicous, and apparently also dioicous'.

Although sexual condition is generally fixed within species, a few species have been recorded as having both unisexual and bisexual gametophytes ('polyoicous', c.f. Cronberg, 1991). Although none of the species identified as polyoicous were included in the present study, it is safe to say that the determination of sexual condition in *Sphagnum* is poorly understood, and it may be polymorphic within some species or populations. Gene expression analysis, along with careful tracking of individual ramets within populations, would be very beneficial for understanding genetic differences between male and female gametophytes, and to assess whether a single gametophyte can vary temporally in sexual expression. Our results suggest that *S. cuspidatum* would be a very good choice for this type of study.

Microhabitat preference affects mating patterns

Sphagnum species are known to prefer narrow ranges of microhabitats within peatlands (Vitt & Slack, 1984; Andrus, 1986), including the tendency of each species to prefer either 'hollow' conditions (aquatic microhabitats at the water table) or 'hummock' conditions (cushions forming high above the water table). Water availability (necessary for fertilization in bryophytes) is reduced in hummock habitats, and we predicted that this would reduce fertilization probabilities, resulting in lower genetic diversity and higher inbreeding rates. We confirmed this prediction, finding that populations of species preferring hummock habitats have significantly reduced genetic diversity compared to populations of species preferring hollow habitats.

We found the inbreeding coefficient to be significantly correlated with species microhabitat distribution along the hummock–hollow gradient (Fig. 4). Rather than direct effects, there is an interaction with sexual condition. Inbreeding coefficients were higher in bisexual hummock-preferring populations than bisexual hollow populations. The limited water availability atop hummocks means that sperm, which must swim to effect fertilization, may have fewer opportunities for intergametophytic mating, especially if genetic diversity within hummocks is low. Our data support this because the same inferred paternal genotypes were rarely found in sporophytes attached to mothers from different parts of the population in hummock-preferring species.

We found the opposite pattern in unisexual populations: hummock-preferring populations had lower inbreeding coefficients than hollow populations (Fig. 4). Intriguingly, this pattern could be explained by the same phenomenon of limited water availability. As discussed earlier, an overabundance of one type of gamete in the mating pool could increase observed heterozygosity above expected, generating negative values of F_{IS}. By definition, a unisexual gametophyte must mate with a different genet to produce sporophytes; if these opportunities are limited to local genotypes, it could explain the extremely negative values found in unisexual hummock populations.

Bisexual species tend to be colonizers (Sundberg *et al.*, 2006); a disproportionate percentage of hummock preferences among bisexual species (such as *S. molle* and *S. strictum* in the present study) may thus explain the interaction between habitat preference, sexual condition, and mating patterns. Microhabitat preference along the hummock-hollow gradient is phylogenetically conserved within *Sphagnum*. (Johnson *et al.*, 2015a) Therefore, our findings about the connection between species ecology and population mating patterns suggest that related species are expected to retain mating system characteristics along with microhabitat preferences at macroevolutionary time scales.

Conclusions

We find that sexual condition, genetic diversity, and microhabitat preference all correlate with mating patterns in *Sphagnum* populations. Sexual condition is the most prevalent; the effect of microhabitat is detectable but subtler and more complex. 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 studies investigating the genetic components of sexual determination in *Sphagnum*. We have greatly expanded the knowledge of inbreeding depression in natural populations of mosses, and have revealed that it is not a universal phenomenon in either unisexual or bisexual populations.

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SHARED DATA

Data deposited in the Dryad digital repository (Johnson & Shaw, 2015b).

APPENDIX

We describe the twenty populations sampled for the survey of mating patterns in *Sphagnum*.

Sphagnum angustifolium Population: ME-S. Washington County, Maine, USA. Locality: near Steuben, E side of East Side Rd, 0.8 miles N of US1, 44.5221°N, 67.9500°W, elevation 35 m. Description: poor fen,

approximately 100 m from open water. Fruiting plants forming several hummocks in an area of approximately 100 m^2. 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. 48.8155°N, 125.1275°W. 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 approximately 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 miles W of jct Parks Hwy (AK2), N 62.3674° W 150.7121°, elevation 375 m. Description: Plants collected along an approximately 25-m 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, approximately 30 miles S of Cantwell at 180 Mi Lake. 63.0822°N, 149.5252°W, elevation 560 m. Description: Fen on S side of Rd, with rich areas (*Tomenthypnum*, *Paludella*, etc.), and poorer depressions (with *Sphagnum lindbergii*, *Sphagnum balticum* and *Sphagnum* cf. *orientale*) plus higher hummocks (with *Sphagnum fuscum*, *Sphagnum lenense, Sphagnum capillifolium*). Plants collected in an approximately 100-m 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 miles NE of US-25 in Ludowici. 31.7232°N, 81.7270°W. Description: Wet trenches running parallel and perpendicular to W side of road. Growing intermixed in an area of approximately 25 m^2 area with *S. macrophyllum*. 8 May 2009.

Sphagnum cuspidatum. Population GA28. Ware County, Georgia, USA. Locality: W side of GA 177, 0.5 miles S of US 1, entrance to Okeefenokee Swamp. 31.1225°N, 82.2723°W. 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 an approximately 200-m transect, floating in the water. Duke Herbarium collections: Matt Johnson 101-113. 6 February 2009.

Sphagnum cuspidatum. Population NC-JL. Bladen County, North Carolina, USA. Locality: Jones Lake State Park, along Cedar Loop Trail on E shore. 34.6884°N, 78.5960°W, elevation 21 m. 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 miles N of US1, 44.5221°N, 67.9500°W, elevation 35 m. Description: poor fen, approximately 50 m from open water. Fruiting plants forming several hummocks in an approximately 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 miles N of Preserve parking lot. 44.5221°N, 67.9500°W, 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 miles E of US 601 on SC 462, near Coosawatchie, SC. 32.6193°N, 81.0653°W. Description: Disturbed pond cypress dome S of highway, plants collected on an approximately 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 miles NE of FR 138. 33.218°N, 79.712°W. Description: Open canopy of *Taxodium distichum* surrounding approximately 2000 m^2 of open water approximately 1 m deep. Understory of *Lyonia lucida*, *Nyssa biflora*, and *Vaccinium formosum*. In the water, plants form almost 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. 34.6884°N, 78.5960°W, elevation 21 m. Description: Wet, partically shaded depression in old growth bay forest, with poison ivy. Plants formed one single hummock approximately 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 miles E of US 601 on SC 462, near Coosawatchie, SC. 32.6193°N, 81.0653°W. Description: Disturbed pond cypress dome S of highway. Plants collected in several isolated

hummocks in approximately 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). 44.4056°N, 67.9812°W, 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 an approximately 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. 65.1004°N, 147.7464°W, elevation 180 m. 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 an approximately 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 miles E of US 601 on SC 462, near Coosawatchie, SC. 32.6193°N, 81.0653°W. Description: Disturbed pond cypress dome S of highway. Plants collected in several isolated hummocks in an approximately 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, approximately 4 miles WNW of Zebulon near intersection of Marshburn Rd. and Riley Hill Rd. 35.8447°N, 78.3731°W, 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 miles N of Preserve parking lot. 44.5221°N, 67.9500°W, elevation 14 m. Description: poor fen near trail, many fruiting plants in an approximately 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 miles W of McCarthy foot bridge. 61.3861°N, 143.1821°W. 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.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Assessment of relationship between percent heterozygosity and sporophyte size in *Sphagnum* using a pseudoreplication approach. One sporophyte per maternal shoot was chosen at random. The significance of the linear correlation and the r^2 value were recorded for each simulation. The number of pseudoreplicates with $P < 0.05$ and the mean r^2 across all pseudoreplicates for a population are shown.