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THE SPECIATION HISTORY OF THE *PHYSCOMITRIUM—PHYSCOMITRELLA* SPECIES COMPLEX

Stuart F. McDaniel,[1,2] Mark von Stackelberg,[3,4] Sandra Richardt[3,5,6] Ralph S. Quatrano,[1,7] Ralf Reski,[3,5,8] and Stefan A. Rensing[5,9]

[1]*Biology Department CB#1137, One Brookings Dr., Washington University, St. Louis, Missouri 63130*

[2]*E-mail: smcdaniel@biology2.wustl.edu*

[3]*Plant Biotechnology, Faculty of Biology, Schaenzlestr. 1, 79104 Freiburg, Germany*

[4]*E-mail: markvonstackelberg@gmx.de*

[5]*Freiburg Initiative in Systems Biology, Faculty of Biology, Hauptstr. 1, 79104 Freiburg, Germany*

[6]*E-mail: sandra.richardt@biologie.uni-freiburg.de*

[7]*E-mail: rsq@biology.wustl.edu*

[8]*E-mail: Ralf.Reski@biologie.uni-freiburg.de*

[9]*E-mail: Stefan.Rensing@biologie.uni-freiburg.de*

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A central problem in evolutionary biology is identifying factors that promote the evolution of reproductive isolation. Among mosses, biogeographic evidence indicates that the potential for migration is great, suggesting that biological factors other than geographic isolation may be critical for speciation in this group. The moss *Physcomitrella patens* (Funariaceae) has long been used as a model for interspecies hybridization and has recently emerged as an important model system for comparative genomics. We report genealogical analyses of six loci from several populations of *P. patens* and related species in the genus Physcomitrium. These results unambiguously indicate that the so-called genus Physcomitrella arose at least three times from distinct ancestors within the genus Physcomitrium. In spite of the evidence for natural hybridization in the *Physcomitrella–Physcomitrium* complex, genealogical and experimental hybridization data indicate that the taxonomically defined species are reproductively isolated. However, these analyses suggest that *Physcomitrium eurystomum* was formed from a hybridization event between two early diverging lineages in the complex, and that the ancestral population size of these lineages was much smaller than the current population sizes. We discuss these findings in the context of the inferred mating system in the *Physcomitrella–Physcomitrium* complex and patterns of speciation and diversification.

KEY WORDS: **Bryophyte, Funariaceae, genealogical conflict, hybrid speciation, reproductive isolation, self-fertile.**

Geographic isolation has long been viewed as a critical step in the process of speciation and the generation of biodiversity (Mayr 1963; Barraclough and Vogler 2000; Coyne and Orr 2004). Within many moss species, however, mounting evidence from floristics (van Zanten 1978; Miller and McDaniel 2004; Hutsemekers et al. 2008), molecular population genetics (Shaw et al. 2003; Van der Velde and Bijlsma 2003; McDaniel and Shaw 2005; Vanderpoorten and Long 2006; Heinrichs et al. 2007; Huttunen et al. 2008; Szovenyi et al. 2008), and phylogenetics (Shaw et al. 2005a) indicates that geographic barriers may not cause

a long-term impediment to migration. This observation suggests that genetic, demographic, or ecological factors, in addition to geographic isolation, cause the cessation of gene flow between nascent species. The nature of these biological factors, however, is largely unknown. The moss *Physcomitrella patens* and its relatives have long been model systems for studies of hybrid interfertility (von Wettstein 1924; Bryan 1957). *P. patens* is now emerging as a model plant for comparative genomics, and studies including *P. patens* have provided insights into the evolution of land plant gene function and genome structure (Nishiyama et al. 2003; Lang et al. 2005; Rensing et al. 2005; Stenoien 2005; Richardt et al. 2007; Rensing et al. 2008; reviewed in Quatrano et al. 2007). As a consequence, *P. patens* also holds promise as a model for understanding the process of speciation (von Stackelberg et al. 2006; Kamisugi et al. 2008).

Phylogenetic studies have shown that the monotypic genus Physcomitrella is closely related to the genus Physcomitrium, based on analyses of DNA sequences from the chloroplast (Goffinet and Cox 2000; Goffinet et al. 2007; Werner et al. 2007). The two genera are most easily distinguished by the morphology of the diploid sporophyte; the Physcomitrella sporophyte is nestled among the gametophyte leaves, rather than elevated on a seta like in Physcomitrium and most other mosses, and lacks the specialized abscission layer around the opening of the sporangium. Both genera are cosmopolitan in distribution, but Physcomitrium is more diverse than Physcomitrella, with a total of seven species found in Europe and temperate North America (Crum and Anderson 1981; Hill et al. 2006). Populations containing species of both genera are frequent in some habitats, and hybrids are widely reported (Britton 1895; Andrews 1918; Loeske 1929; Andrews 1942; Pettet 1964; Tan 1978). Therefore, the evolutionary relationships within the *Physcomitrella–Physcomitrium* complex may not be captured by a single gene or organelle, because ongoing hybridization may cause different parts of the genome to have different histories.

In spite of the evidence for hybridization, the species of Physcomitrella and Physcomitrium are phenotypically distinct, even in sympatric sites (e.g., Crum and Anderson 1981), suggesting that the evolutionary effects of hybridization may be limited. In a landmark series of experiments, von Wettstein (1924, 1928, 1932) and colleagues (Schmidt 1931; Bauer and Brosig 1959) generated artificial crosses among members of the Funariaceae, including *P. patens* and three European species of Physcomitrium (the cosmopolitan *P. pyriforme*, and the endemics *P. sphaericum* and *P. eurystomum*). In general, the recombinant haploid progeny (i.e., spores that develop into gametophytes) of most intergeneric hybrids were inviable or developmentally abnormal, making it unlikely that natural recombinants would contribute to subsequent generations (reviewed in Bryan 1957). However, occasionally a few recombinants were viable and vigorous, suggesting that the fortuitous segregation of species-difference loci (either karyotypic differences or epistatically interacting factors) may allow for the production of fertile progeny. Von Wettstein (1932) reported that the frequency of such vigorous recombinants was greatest in crosses in which *P. patens* was the maternal parent, although this frequency also depended upon the paternal species. These data have engendered a considerable amount of speculation regarding the role of introgressive hybridization in the evolution of the Funariaceae, but neither additional crossing studies nor molecular population genetics analyses of hybridization have been undertaken in the *Physcomitrella–Physcomitrium* complex.

In principle, the signature of introgressive hybridization is readily observable by comparing the genealogical patterns at multiple independent loci (Sang and Zhong 2000; McBreen and Lockhart 2006). Ongoing gene flow should cause allele sharing between distinct species and elevated levels of genetic variation within the hybridizing species, because allelic variants coalesce before the origin of either species. Historical gene flow may be indicated by conflicting genealogical relationships among loci. However, genealogical conflict due to ancient introgression can be difficult to distinguish from the retention of ancestral polymorphism (incomplete lineage sorting). This problem is particularly acute where multiple speciation events have occurred over a short period of time relative to the amount of segregating variation in the ancestral population (Maddison 1997). The coalescence times in large populations are long, increasing the probability that the branching order in a particular gene genealogy will not reflect the true order of speciation events. Moreover, because a given genealogy may arise under a broad range of demographic processes, inferences based on genealogical shape alone are generally insufficient to rule out particular demographic histories (Kuhner 2009). Recently, however, researchers have developed approaches that estimate historical demographic parameters over a range of probable genealogies, providing a means to explicitly distinguish gene flow from incomplete lineage sorting (Hey and Nielsen 2004, 2007).

To test for introgressive hybridization, we examined patterns of genetic variation in primarily European isolates of Physcomitrium and Physcomitrella, using molecular evolutionary analyses of the DNA sequence variation from one chloroplast spacer (*atp*B-*rbc*L), the nuclear ribosomal internal transcribed spacers (ITS), and intron-containing portions of four nuclear protein-coding loci (adenosine kinase, *adk*; adenosine 5′-phosphosulfate reductase, *apr*; phosphoadenosine–phosphosulphate reductase, *papr*; and heme oxygenase, *ho*). We included isolates from geographically widespread populations of four species of Physcomitrium, and four subspecies of *P. patens*, focusing in particular on the species where fertility estimates from interspecific hybrids were available in natural (Pettet 1964) and experimental crosses (von Wettstein 1924, 1928). Our results

indicate that *P. patens* has arisen at least three times from within the genus Physcomitrium, and although the species shows no history of introgression, other species within Physcomitrium show a clear signature of hybridization. We discuss these findings in the context of the patterns of interfertility in the *Physcomitrella–Physcomitrium* complex, and patterns of speciation and diversification in general.

Materials and Methods

SAMPLING AND MOLECULAR METHODS

We obtained cultures from multiple isolates of *Physcomitrella patens* (four from subsp. *patens*, two from subsp. *californica*, and one each from subsp. *magdalenae* and *readeri*) and from four species of Physcomitrium (two from *P. collenchymatum*, three from *P. eurystomum*, six from *P. pyriforme*, and three from *P. sphaericum*; Table 1, Fig. 1). This represents a large portion of the distributions of *P. patens*, *P. eurystomum*, and *P. sphaericum*, and a relatively small portion of the distribution of the cosmopolitan *P. pyriforme* (and although *P. collenchymatum* is narrowly distributed, we have only a single accession of this species). Live cultures of all samples are accessioned in Freiburg, Germany (see http://www.cosmoss.org/ecomap.content) and have been deposited in and are available from the International Moss Stock Center (http://www.moss-stock-center.org/).

Genomic DNA was extracted from axenic tissue of each haploid accession grown on standard media as described in Bierfreund et al. (2003), using modified versions of a cetyltrimethyl ammonium bromide (CTAB) extraction (following McDaniel and Shaw (2005) for *atp*B-*rbc*L spacer, *adk*, and *ho*, and von Stackelberg et al. (2006) for ITS, *apr*, and *papr*). The six loci were amplified using the primers listed in Table S1. The *atp*B-*rbc*L spacer, *adk*, and *ho* were amplified in 16-μL polymerase chain reactions (PCR) containing 8-μL GoTaq MasterMix (Promega, Madison, WI), 1 μL 10 μM forward and reverse primers, and 10–20 ng of genomic DNA. The PCR cycling conditions were 94°C denaturing temperature for 30 sec, 50°C annealing temperature for 30 sec, and 72°C extension for 1 min 30 sec for 30 cycles. The PCR products were cleaned with QIAquick columns (Qiagen, Hilden, Germany) and sequenced using 10–20 ng of product in a 10-μL cycle-sequencing reaction (BigDye Terminator Reaction, version 3.1, Applied Biosystems, Foster City, CA). The chromatograms were edited in Sequencher 4.8 (GeneCodes Corp., Ann Arbor, MI) and aligned using Se-al 2.0 software (http://tree.bio.ed.ac.uk/software/seal/).

Although we were generally able to generate clean sequences directly from the PCR products, to test for the presence of paralogs due to polyploidy, which are common in the genus (Rensing et al. 2007), we cloned sequences of *adk* and *ho* from an accession of each species and all three accessions of *P. eurystomum*. The PCR products were cloned into an *E. coli* vector using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA), and colonies were screened after 24 h of growth. Colonies with inserts present were amplified in a 12-μL PCR reaction, using M13 universal vector primers and reagents described above, under the following cycling conditions: initial 94°C for 4 min, 94°C denaturing temperature for 1 min, 55°C annealing temperature for 1 min, and 72°C extension for 2 min, for 30 cycles. The PCR products were cleaned and sequenced using the same protocols as described above. Four high-quality sequences were generated for each extraction that was cloned.

The ITS, *apr*, and *papr* regions were amplified in a 20-μL PCR mix containing 2 μL of 10 × RED-Taq-PCR buffer, 0.1 mM dATP, dCTP, dGTP, and dTTP, 5 pmol each of two primers, 0.5 Units RED-Taq-Polymerase (SIGMA-Aldrich, St. Louis, MO), and 4 ng of genomic DNA. Cycling was carried out in T1 thermal cyclers (TGradient, Biometra, Goettingen, Germany) starting with an initial DNA denaturation at 95°C for 2 min. The first cycle consisted of 30 sec denaturation at 94°C, primer annealing for 30 sec at 62°C, and elongation for 60 sec at 72°C. In each of the 13 subsequent cycles, the annealing temperature was decreased by 0.7°C. The final 24 cycles consisted of 30 sec denaturation at 94°C, 30 sec primer annealing at 52°C, and 70 sec elongation at 72°C. A final elongation was performed for 4 min at 72°C. We were unable to directly sequence ITS, *apr*, and *papr* in some accessions. We therefore cloned the amplified fragments prior to sequencing. PCR products were directly ligated using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). Vectors were transformed into an *E. coli* XL1-Blue strain and selected using ampicillin resistance and blue–white screening. The plasmids were isolated using the E.Z.N.A. Plasmid Miniprep Kit II (Peqlab, Erlangen, Germany) and the inserts were sequenced using vector-specific standard M13 primers. Sequences were quality clipped and filtered using the filtering module of the Paracel Transcript Assembler (Striking Development Inc., Los Angeles, CA) and assembled using Vector NTI Advance 9 (Invitrogen). Initial alignments were performed using Muscle 3.51 (Edgar 2004) and adjusted by eye.

STATISTICAL ANALYSES

To understand the relationships among the sampled taxa, a genealogy was constructed from the aligned sequences from each locus separately using branch-and-bound searches under the maximum parsimony criterion with PAUP* 4.0b10 (Sinauer Associates, Inc., Sunderland, MA). Characters were treated as unordered and equally weighted, and indels were coded as binary characters. Branches were collapsed if the minimum length was zero. Bootstrap analysis used 1000 pseudoreplicates subject to branch-and-bound searches. The *atp*B-*rbc*L spacer, ITS, *apr*, and *papr* genealogies were rooted with a sequence from *Funaria*

Table 1. Species and localities of isolates of the *Physcomitrella–Physcomitrium* species complex used in this study.

Species and locality		Lat.	Long.	Voucher	IMSC no.
Physcomitrella patens subsp. patens					
1	Gransden Wood, Cambridge, U.K.	52°12′N	00°11′E	Whitehouse 1962	40001
2	Reute, Baden-Württemberg, Germany	48°00′N	07°51′E	Stackelberg and Lüth 04.01.2006	40040
3	Villersexel, Haute Saône, France	47°33′N	06°26′E	Lüth 17.10.2003	40012
Physcomitrella patens subsp. californica					
4	del Valle Lake, CA	37°37″N	121°45′W	Mishler 24.10.2004	40039
5	Saitama, Honshu, Japan	36°25′N	139°30′E	Higuchi 44249	40016
Physcomitrella patens subsp. readeri					
6	Melton Reservoir, Victoria, Australia	37°43′S	144°32′E	MEL 2183013	40022
Physcomitrella patens subsp. magdalenae					
7	Mt. Bisoke, Ruhengeri, Rwanda	01°28′N	29°30′E	Buchbender RWA-VB-0107	40025
Physcomitrium sphaericum					
8	Grosshartmannsdorf, Sachsen, Germany	47°08′N	15°55′E	Frahm 4919	40018
9	Imsbach-Aue, Saarland, Germany	49°29′N	07°04′E	Saarland-Herbar Moose 6202	40047
10	Vellescot, Territore-de-Belfort, France	47°34′N	07°01′E	Teichufer, Belegnr 4283	40043
Physcomitrium eurystomum					
11	Neukirch, Baden-Württemberg, Germany	48°01′N	08°11′E	Schäfer-Verwimp 28.08.2001	40048
12	Neustadt, Thüringen, Germany	50°45′N	11°45′E	Eckstein 3139	40052
13	Schleiz, Thüringen, Germany	50°34′N	11°50′E	Eckstein 3767	40051
Physcomitrium pyriforme					
14	Bischofswerda, Sachsen, Germany	51°11′N	14°16′E	Eckstein 2655	40053
15	Nordhausen, Thüringen, Germany	51°32′N	10°39′E	Eckstein 2849	40054
16	Gera, Thüringen, Germany	50°51″N	12°11″E	Eckstein 3947	40055
17	Haardtrand, Rheinland-Pfalz, Germany	49°17′N	08°07′E	Herbar-Nr P17.236	40056
18	Oevergran, Uppland, Sweden	59°51′N	17°38′E	Lönnell 882	40057
19	Madeira, Portugal	32°50′N	17°13′E	Eckstein 472	40059
Physcomitrium collenchymatum					
20	Shaw Nature Reserve, MO	38°29′N	90°23′W	Homberg 1155	40061
21	Shaw Nature Reserve, MO (no ITS)	38°29′N	90°23′W	Homberg 1155	40062
Funaria hygrometrica					
	Germany (*apr*, ITS, *atp*B-*rbc*L)			Laboratory strain	40017

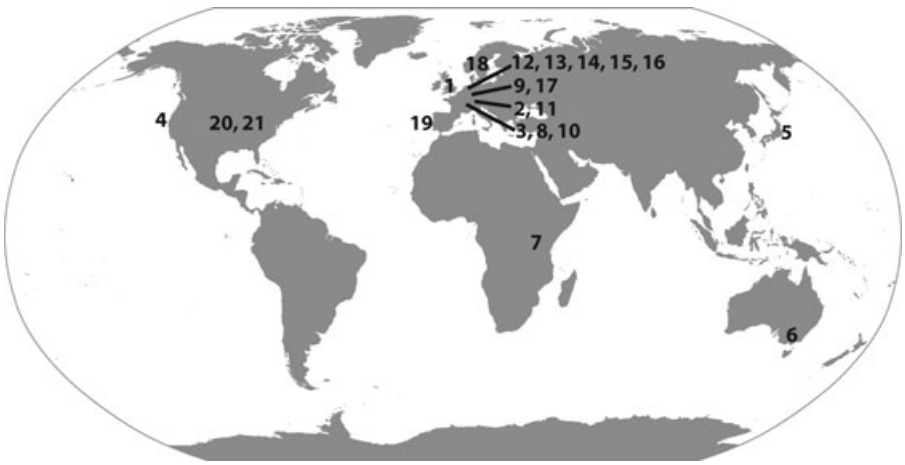

Figure 1. Geographic origins of isolates of the *Physcomitrella–Physcomitrium* complex used in this study. Numbers correspond to those in Table 1.

hygrometrica. To test the monophyly of *P. patens*, we performed searches with the species constrained to be monophyletic, and compared this to the unconstrained topology using a Shimodaira–Hasegawa test (Shimodaira and Hasegawa 1999) implemented in PAUP* assuming the HKY model of evolution.

To assess the significance of the interlocus phylogenetic conflict, we compared the phylogenetic signal among loci (including the indels) using the partition homogeneity test (Farris et al. 1994) as implemented in PAUP* under parsimony with branch-and-bound searches. We then conducted additional branch-and-bound parsimony analyses on subsets of the complete dataset, pruning isolates that were strongly supported in different topological locations across loci. We continued pruning isolates until the partition homogeneity test yielded a nonsignificant result (i.e., no statistically significant topological conflict). Because we found evidence of significant conflict when *P. eurystomum*, *P. collenchymatum*, and several isolates of *P. pyriforme* were included in the analysis, we did not estimate a genealogy from the combined data, but rather constructed a species tree based on combinable splits in the individual gene trees.

To evaluate whether the topological conflict resulted from introgressive hybridization or incomplete lineage sorting, we generated maximum likelihood estimates (MLEs) of current and ancestral population sizes, migration rates, and divergence times, using the program IMa (Hey and Nielsen 2007). This software implements a Markov Chain Monte Carlo (MCMC) search strategy to identify MLEs of demographic parameter values given in the sampled data. Flat likelihood surfaces for divergence time or migration rates between lineages, combined with large ancestral population sizes, would indicate that we could not reject lineage sorting as the source of a topological conflict. Narrow MLEs for divergence and migration, and a small estimated ancestral population size, in contrast, would suggest that incomplete lineage sorting was unlikely to cause topological conflict. This approach, however, assumes a bifurcating isolation-with-migration speciation model. *P. eurystomum* appears to violate this bifurcating model, as it contained alleles closely related to *P. sphaericum* and *P. pyriforme* at all nuclear loci, suggesting that it might be a hybrid species. We therefore indirectly evaluated the likelihood that the genetic composition of *P. eurystomum* resulted from introgressive hybridization, rather than incomplete lineage sorting, by estimating demographic parameter values for the two putative parental lineages, *sphaericum* and *pyriforme* (see the Results section).

We used IMa to perform a pairwise comparison between the inferred parental lineages of the putative hybrid species, excluding the taxa that showed evidence of hybridity (*P. eurystomum* and *P. collenchymatum*). We conducted an initial run of the program to establish parameter maxima where the MLEs reached zero probability, and an appropriate burn-in period. We then conducted three additional runs of 10 million steps, each with a burn-in of 10,000 steps, a different seed number, and narrower parameter maxima, and checked convergence among the three runs. To obtain joint-parameter estimates, we used IMa in L-mode, an approach that compares nested demographic hypotheses using likelihood ratio tests. We present the joint parameter estimates from this run.

This approach assumes free recombination between loci, no recombination within loci, an absence of selection on the surveyed loci, and random mating. The software dnaSP 4.0 (Rozas et al. 2003) was used to estimate the quantity θ ($4Ne\mu$, the genetic effective population size) for each locus, historical recombination in each locus (Hudson and Kaplan 1985; Hudson 1987), and deviations from neutral-equilibrium expectations in mutation frequency spectrum and fixation of amino acid changing substitutions. We also tested for recombination in the concatenated matrix of the four nuclear loci in the entire data matrix, and various subsets of the taxa based on lineages found in the phylogenetic analyses. The output from this analysis was visually inspected to identify sequences containing putative recombination events. In cases where evidence of recombination was found, we divided the sequences into segments that did not violate the four-gamete test, based on the output from dnaSP. It also is not clear whether the *sphaericum* and *pyriforme* lineages undergo random mating, as assumed by the IM approach, because members of the *Physcomitrium-Physcomitrella* complex are self-fertile. We conducted the IMa analyses with various partitions of our data and got similar results, leading us to suspect that any additional structure within these lineages does not substantially alter parameter estimates.

IMa generates parameter estimates expressed in units of $4Ne\mu$, $t\mu$, and m/μ, where μ is the neutral mutation rate per generation, t is the divergence time in generations, and m is the migration rate per generation. To convert these to Ne, t, and m, we assumed that Physcomitrella and Physcomitrium have an annual life cycle (one generation per year). We used a rate of 9.4×10^{-9} neutral mutations per site per generation, following Rensing et al. (2007). Given the uncertainty in estimated rates of molecular evolution, we intend these parameter estimates to be taken only as a rough guide.

Results

TESTING THE MONOPHYLY OF *P. PATENS*

To understand the evolutionary patterns in the *Physcomitrella–Physcomitrium* complex, we estimated genealogies from six loci including 21 isolates from within the complex. The individual genealogies are shown in Figure 2, and the details regarding the loci and reconstruction of these genealogies are shown in Table 2. The position of the root, determined using *F. hygrometrica* as an outgroup, is shown for the *apr*, *papr*, ITS, and *atp*B-*rbc*L genealogies, although alternate rootings could not be rejected for the latter two genealogies. The alignments for the four nuclear loci (*adk*,

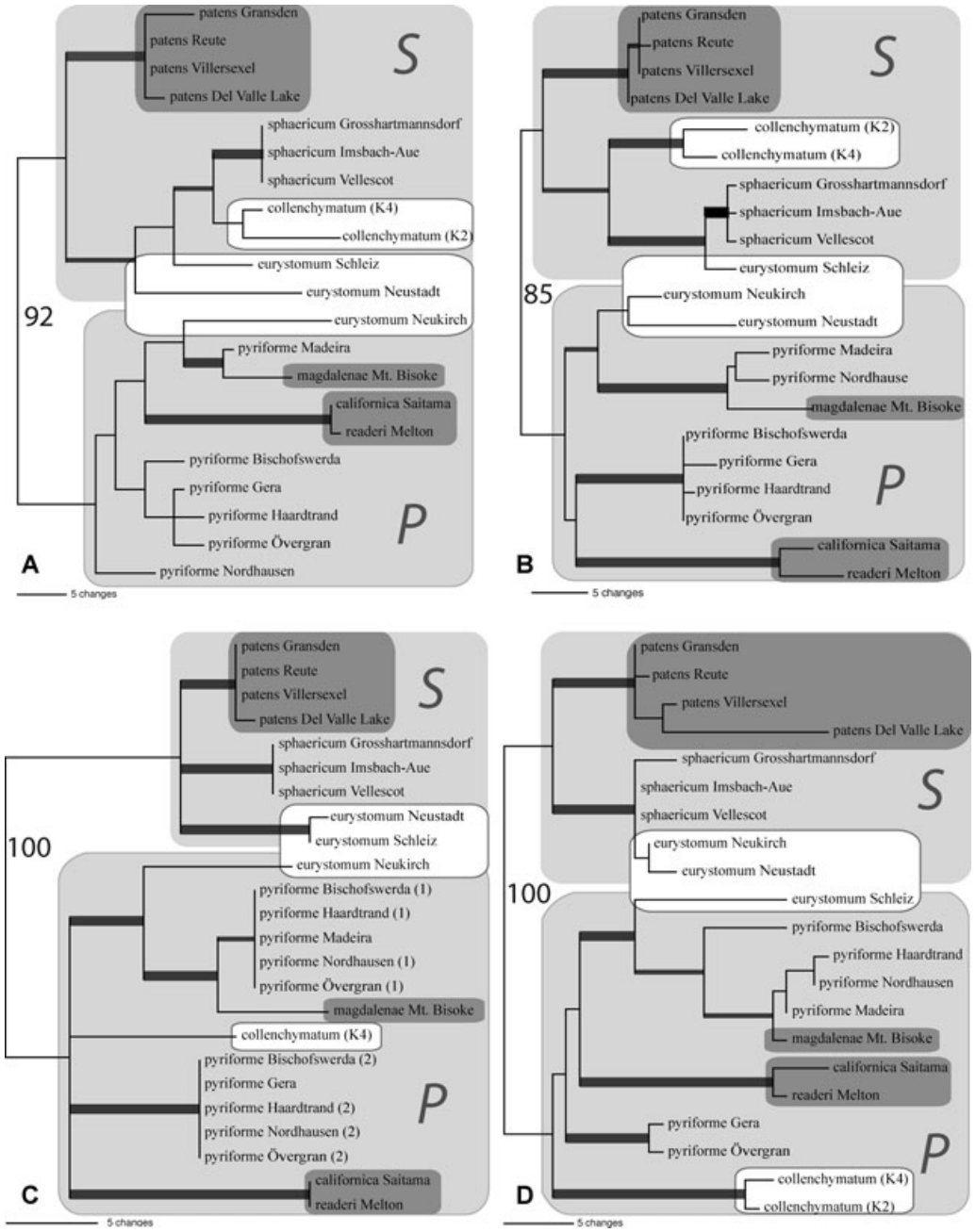

Figure 2. **Maximum parsimony genealogies of members of the *Physcomitrella–Physcomitrium* complex from (A) *adk*; (B) *apr*; (C) *papr*; (D) *ho*; (E) ITS; and (F) *atp*B-*rbc*L spacer. Thick lines indicate branches with > 70% bootstrap support, and double-thick lines indicate branches with >85% support; the light gray lines in the ITS genealogy reflect a lack of confidence in the reconstruction due to ambiguous alignment. The *apr* and *papr* genealogies were rooted with *F. hygrometrica*; the remaining genealogies are midpoint rooted. Light gray shaded areas, labeled *S* and *P*, correspond to the *sphaericum* and *pyriforme* lineages, respectively, with bootstrap support indicated on the branch between the two lineages (see the text; absent in the ITS genealogy); dark gray shaded taxa show the three origins of *P. patens*; white regions bounded by gray lines circle the putative hybrid species, *P. eurystomum* and *P. collenchymatum*. Species localities correspond with Table 1, and details regarding the sequence length and phylogenetic reconstructions are given in Table 2.**

apr, *papr*, and *ho*) were unambiguous, phylogenetic analyses of these loci resulted in well-supported relationships, and all four loci exhibited similar numbers of segregating sites (Fig. 2, Table 2). In contrast, in the ITS alignment, it was difficult to assess confidently positional homology, whereas the *atp*B-*rbc*L alignment contained too little variation to distinguish the genealogical relationships among all of the species. Therefore, we principally focus on relationships produced from analyses of the

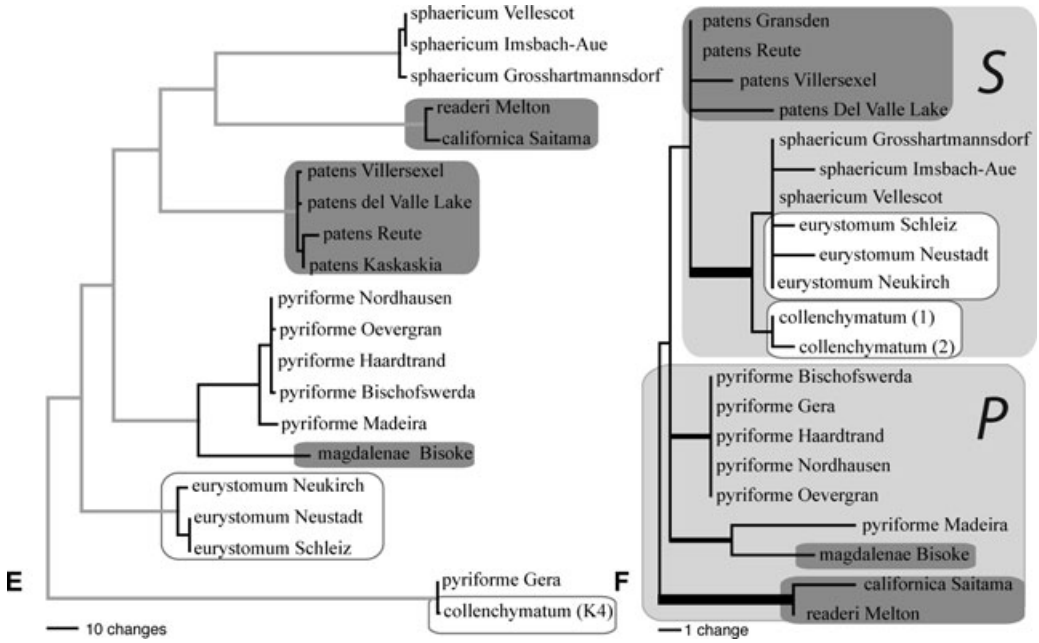

Figure 2. Continued.

nuclear protein-coding loci, referring to the ITS and the *atp*B-*rbc*L spacer genealogies where they specifically support particular relationships.

In all six loci, three distinct lineages of *P. patens* were evident (Fig. 2). To test whether these represented multiple statistically distinguishable origins of this species, we compared the most parsimonious trees for each locus separately to trees constrained to Physcomitrella monophyly using the Shimodaira–Hasegawa test. The constrained topologies were significantly longer for all loci except the *atpB-rbcL* spacer (Table 3). In the unconstrained trees, the European and North American *P. patens*, including an accession from Del Valle Lake, California, of the subspecies *californica*, were strongly supported as sister to the three *P. sphaericum* isolates. The subsp. *californica* from Saitama, Japan, was sister to the isolate of subsp. *readeri* from Australia (Melton) in all genealogies. These Austro-Asian *P. patens* isolates were either nested within *P. pyriforme* (*adk*, *papr*, Fig. 2A,C) or sister to the rest of the *Physcomitrella–Physcomitrium* species complex (*apr*, *ho*, *atp*B-*rbc*L, Fig. 2B,D,E). The subsp. *magdalenae* from Mt. Bisoke, Rwanda, was sister to an isolate of *P. pyriforme* from Madeira in all genealogies.

In genealogies from the four nuclear protein-coding loci, *P. pyriforme* was paraphyletic with respect to the *P. patens* isolate from Rwanda, and those from Japan and Australia (Fig. 2A–D). For the purpose of grouping the accessions for population genetic analyses, we refer to isolates of *P. pyriforme*, *P. patens* ssp. *magdalenae*, *P. patens* ssp. *readeri*, and *P. patens* ssp. *californica* (Japan) collectively as the *pyriforme* lineage (*P* in Fig. 2). The isolates of *P. sphaericum* and the European and North American isolates of *P. patens* were well resolved as distinct from the *pyriforme* lineage in all loci. We refer to these isolates collectively as the *sphaericum* lineage (*S* in Fig. 2). The isolates of *P. eurystomum* grouped with both the *pyriforme* and *sphaericum* lineages, whereas the *P. collenchymatum* grouped with one or the

Table 2. Tree lengths and consistency indices for parsimony reconstructions.

Locus	Aligned length	Polymorphic sites	Tree length	Informative sites	Indels[1]	Homoplasy index	MPTs
apr[2]	874	118	144	83	1	0.193	2
papr	978	108	123	76	1	0.143	4
adk	874	126	167	83	4	0.313	8
ho	714	73	83	55	3	0.049	45
ITS[2]	763	271	433	251	0	0.216	117
*atp*B-*rbc*L[2]	572	19	43	13	6	0.167	84

[1]Only two-state (0/1) indels where the rarer type was found in more than one isolate were scored.

[2]*F. hygrometrica* was not included in the analysis of tree length statistics.

Table 3. Likelihood difference between the optimal tree for each locus and trees constrained to a monophyletic *Physcomitrella* using the Shimodaira–Hasegawa test.

Locus	$-\Delta$lnL	*P*
apr	93.20	0.000
papr	78.55	0.000
adk	84.27	0.000
ho	22.23	0.023
ITS	75.62	0.000
*atp*B-*rbc*L	16.11	0.267

other, depending upon the locus. These accessions were therefore not included in demographic analyses of the *sphaericum* and *pyriforme* lineages.

GENEALOGICAL CONFLICT AMONG LOCI

Among isolated lineages, we expect that all single-locus genealogies will represent approximations of a single underlying evolutionary history. The genealogical placement of *P. eurystomum* represents a clear departure from this expectation. In genealogies of each of the nuclear loci, alleles from *P. eurystomum* were closely related to both *P. sphaericum* and *P. pyriforme*. The placement of particular isolates of *P. eurystomum* varied among the loci we sampled; in the *apr* genealogy, the isolates from Neustadt and Neukirch were nested within the *pyriforme* lineage (Fig. 2B), whereas in the *papr* genealogy these isolates were closely related to the *sphaericum* lineage (Fig. 2D). In the ITS genealogy, the *P. eurystomum* isolates were all closely related and distinct from both the *sphaericum* and *pyriforme* lineages (Fig. 2E), whereas in the *atp*B-*rbc*L genealogy, all *P. eurystomum* isolates were very closely related to chloroplast haplotypes from *P. sphaericum* (Fig. 2F).

In the *ho* genealogy (Fig. 2C), four isolates of *P. pyriforme* (Oevergran, Bischofswerda, Nordhausen, and Haardtrand) each contained two alleles, in all cases one identical to the Madeira allele and one identical to the Gera allele. This finding suggests that two divergent lineages of *P. pyriforme* hybridized and the descendants retained both copies of the *ho* gene or the surrounding genomic region. We refer to these as the "hybrid" *P. pyriforme* isolates. Because we did not find multiple alleles at any other locus in these isolates, we suspect that this is a locus-specific phenomenon, rather than an allopolyploidy event. Nevertheless, we removed the hybrid isolates from population genetic analyses involving the *ho* locus because the presence of multiple alleles complicates inferences regarding effective population size and recombination.

To assess the significance of the genealogical conflict among loci for the remaining isolates, we used the partition homogeneity test. The ITS data were excluded from this analysis due to alignment ambiguity. The test indicated that the five regions have significantly different phylogenetic signals ($P < 0.001$; Table 4). To test whether particular taxa were a significant source of genealogical conflict, we iteratively pruned individuals from the analysis and performed the test again. When the *P. eurystomum* sequences were removed, the remaining data continued to show significant discordance across the five loci ($P = 0.001$; Table 4). This finding indicated that additional taxa were strongly supported in distinct places in different genealogies. *P. collenchymatum* in particular was strongly supported as sister to *P. sphaericum* in the *adk*, *apr*, and *atp*B-*rbc*L genealogies (Fig. 2A,B,E), but sister to *P. pyriforme* in the the *papr*, *ho*, and ITS genealogies (Fig. 2C,D,F). Accordingly, a nonsignificant partition homogeneity test (i.e., no significant conflict) was achieved when *P. collenchymatum*, *P. eurystomum*, and the hybrid *P. pyriforme* isolates were pruned from the alignment (Table 4).

Table 4. Partition homogeneity tests among five loci[1] (*apr*, *papr*, *adk*, *ho*, *atp*B-*rbc*L) for pruned subsets of the *Physcomitrella*–*Physcomitrium* complex.

Included data	*P*
All taxa	0.001
P. eurystomum excluded	0.001
P. eurystomum and *P. collenchymatum* isolates excluded	0.001
P. eurystomum, *P. collenchymatum*, and "hybrid" *P. pyriforme*[2] isolates excluded	0.906

[1]ITS not included due to alignment uncertainty.

[2]*P. pyriforme* isolates from Bischofswerda, Hardtrand, Nordhausen, and Oevergran were excluded because each single isolate contained two *ho* alleles, suggesting hybridity.

ESTIMATING DEMOGRAPHIC PARAMETERS IN THE PHYSCOMITRELLA–PHYSCOMITRIUM COMPLEX

The *P. patens* isolates from Europe and North America, the Western Pacific, and Africa have different origins and were analyzed separately. The four Euro-American isolates of *P. patens* contained few segregating sites at all six loci (Table 5). Similarly, the three isolates of *P. sphaericum* contained similar or identical alleles, as did the accessions of *P. patens* from Japan and Australia, and a subset of the *P. pyriforme* isolates (Bischofswerda, Gera, Haardtrand, Oevergran) at the *atp*B-*rbc*L, ITS, *adk*, and *apr* loci. *P. pyriforme* showed greater nucleotide diversity than the Euro-American *P. patens* or *P. sphaericum*, but *P. eurystomum* consistently had the largest number of segregating sites, because it contained *sphaericum* and *pyriforme*-type alleles. We found neither a deviation from a neutral-equilibrium mutation frequency spectrum nor an elevation in nonsynonymous substitutions, and therefore no evidence of natural selection acting on any locus (data not shown).

Table 5. Nucleotide diversity in members of the *Physcomitrella–Physcomitrium* complex.

Species	*apr*	*papr*	*adk*	*ho*[1]	ITS[2]	*atp*B-*rbc*L
P. patens (Europe and North America)	0.0013	0.0097	0.0031	0	0.0045	0.0101
P. sphaericum	0.0023	0.0022	0	0	0.0022	0.0029
P. eurystomum	0.0265	0.0244	0.0388	0.0295	0.0065	0.0022
P. pyriforme	0.0174	0.0141	0.0099	–	0.0612	0.0061
Total	0.038	0.0334	0.0332	–	–	0.0127

[1]***ho* values for *P. pyriforme* not calculated due to the presence of multiple copies.**
[2]**ITS total value not calculated due to alignment uncertainty.**

We estimated the recombination parameter R and the minimum number of recombination events for each locus separately, in the *sphaericum* lineage, among the *P. pyriforme* isolates (except at the *ho* locus), in the *pyriforme* lineage, and in the entire sample (Table 6). We also estimated the minimum number of recombination events (or assortment events, as the nuclear loci are on separate *P. patens* genomic scaffolds and thus on potentially different chromosomes) for the entire concatenated matrix. In the isolates that showed evidence of a genealogical conflict, we scanned by eye for recombination breakpoints where multiple polymorphic sites support one genealogical placement in one part of a sequence, but a distinct placement in a second portion of the sequence. The alignment for the *apr* gene showed the clearest evidence of a recombination breakpoint between a *sphaericum*-type sequence and a *pyriforme*-type sequence in one of the *P. eurystomum* alleles. Indeed, a phylogenetic analysis using the 5′-side of the breakpoint resulted in this isolate being strongly supported as sister to *P. sphaericum*, whereas the sequence 3′ of the breakpoint strongly supported a placement within *P. pyriforme* (data not shown).

Table 6. Inferred intra- and intergenic recombination among nuclear loci in the *sphaericum* and *pyriforme* lineages. The top number is inferred recombination rate per site (Hudson 1987), and the bottom number is the minimum number of recombination events (Hudson and Kaplan 1985).

Species	*apr*	*papr*	*adk*	*ho*	Concatenated
sphaericum	0.0015	0.0115	0.0017	0.0026	
lineage[1]	0	2	0	0	2
pyriforme	0.0139	0.0129	0.0145	0.0092	
lineage[2]	1	2	4	0	11
P. pyriforme	0.0012	0.0074	0.0994	0.0001	
	0	2	2	0	7
All species	0.0517	0.0428	0.0351	0.0449	
	4	5	10	3	23

[1]***P. sphaericum*, *P. patens* subsp. *patens*, and *P. patens* var. *californica* (USA).**
[2]***P. pyriforme*, *P. patens* var. *magdalenae*, *P. patens* subsp. *californica* (Japan), and *P. patens* var. *readeri*.**

The IMa analyses indicated that the current *Ne* for the *pyriforme* and *sphaericum* lineages are approximately 4.92 million (HPD90 = 3.34×10^6–7×10^6) and 1.69 million (HPD = 1.05×10^6–2.73×10^6), respectively, with an ancestral population size nearly 20 times smaller (Na = 84,000, HPD90 = 19,100–1.39×10^6; Fig. 3a). The MLE of migration from *sphaericum* to *pyriforme* ($m = 0.084$, HPD90 = 0–0.474) was larger than that from *pyriforme* to *sphaericum* ($m = 0.0009$, HPD90 = 0–0.375; Fig. 3b). We estimate the divergence between the *sphaericum* and *pyriforme* lineages found ∼11.4 million years ago (MYA), with a 90% highest probability density (HPD90) including 8.37–12.8 MYA (Fig. 3c). Using a higher mutation rate would obviously decrease these divergence times and effective population sizes. The best-fit joint parameter estimates generated by IMa in L-mode matched the values above, except that the migration rate from *pyriforme* to *sphaericum* was fixed at 0 (Table 7). We could not reject additional models (at the $P = 0.05$ level) with symmetrical but either low migration rates or no migration between the *sphaericum* and *pyriforme* lineages.

Discussion

MULTIPLE ORIGINS OF PHYSCOMITRELLA

Taxonomists have long noted the gametophytic similarity between the genera Physcomitrella and Physcomitrium, although the two genera are readily distinguished based on the morphology of the diploid sporophyte (Crum and Anderson 1981; Schwartz 1994, 1997). Our results, like those from earlier molecular phylogenetic studies based on chloroplast DNA sequences (Goffinet and Cox 2000; Goffinet et al. 2007; Werner et al. 2007) support this inference. Strikingly, *P. patens* has arisen at least three times from distinct ancestors within the genus Physcomitrium, a result strongly supported by all nuclear loci ($P < 0.001$, Table 3; Fig. 4). Importantly, there was no topological conflict or evidence of recombination in the alignment that included the three lineages of *P. patens* and their immediate sister isolates (i.e., *P. sphaericum*, the Madeira and Gera isolates of *P. pyriforme*; Fig. 1, Table 4) suggesting that this arrangement is not an artifact of hybridization. The fact that members of the three *P. patens* lineages have

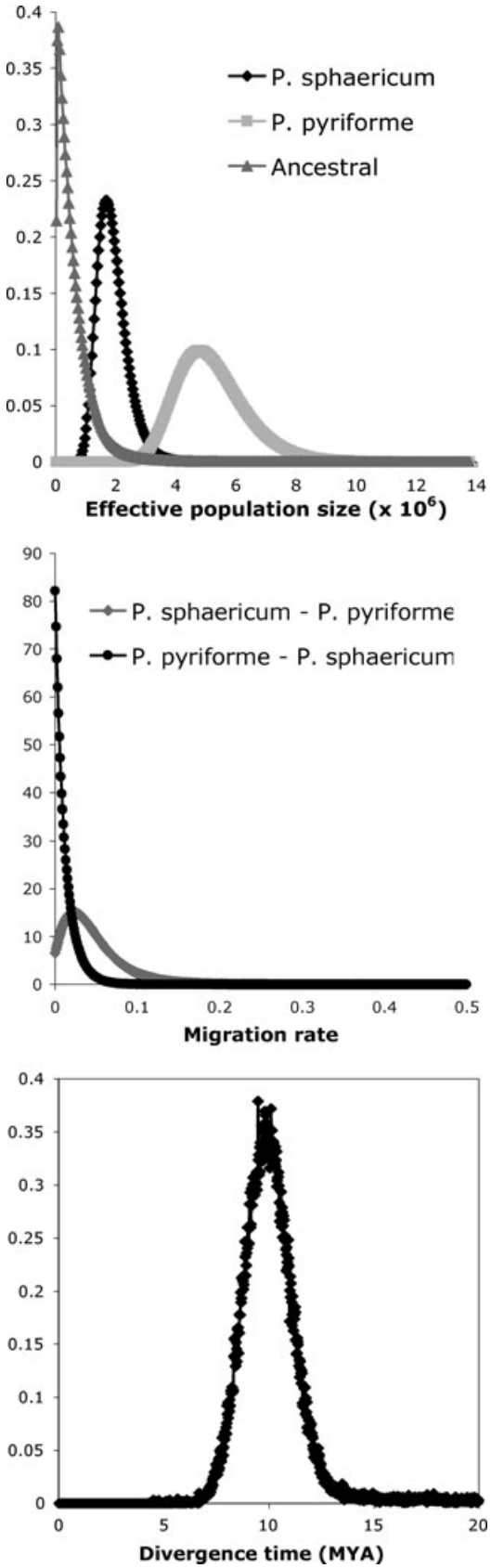

Figure 3. Marginal posterior probability distributions for IM model parameters between *sphaericum* and *pyriforme* lineages.

nonoverlapping distributions suggests that allopatry may play a role in the persistence of these taxa.

We should point out that our sampling of the genus Physcomitrium is sparse; although additional samples would not affect our inferences of multiple origins, other pygmy species in the Funariaceae, such as *Physcomitrium immersum* and *Aphanorhegma serratum*, may represent either independent cases of sporophytic reduction or diversification within *P. patens*-like lineages. Multiple origins of the *P. patens* morphology from Physcomitrium-like ancestors parallel cases of recurrent sporophytic reductions in other bryophyte families (Buck et al. 2000; Goffinet and Shaw 2002). The repeated evolution of this phenotype suggests that it may be adaptive under certain circumstances—potentially like the evolution of cleistogamy in flowering plants—but we cannot eliminate neutral explanations for the sporophytic reduction.

The extreme differences in sporophyte morphology between Physcomitrella and Physcomitrium make hybrids between these genera easy to detect, and indeed the study of hybridization in the Funariaceae has a long history relative to that in other mosses (Natcheva and Cronberg 2004). Individuals with morphologically intermediate, presumably F1, sporophytes found on otherwise pure-species gametophytes (i.e., maternal gametophytes bearing sporophytes produced with heterospecific sperm) have been reported between *P. patens* and *P. sphaericum* (Loeske 1929; Pettet 1964), *P. patens* and *P. pyriforme* (Andrews 1918, 1942; Loeske 1929; Pettet 1964; Tan 1978; Crum and Anderson 1981), and *A. serratum* and *P. pyriforme* (Britton 1895; Crum and Anderson 1981). However, we found no genealogical evidence of hybridization between species with extreme differences in sporophyte morphology. Nor do we have evidence that the species with reduced sporophytes arose following a hybridization event, as the phylogenetic placement of all three lineages of *P. patens* was consistent in all nuclear genealogies. We have repeated many intergeneric crosses produced by von Wettstein (1924, 1928, 1932), as well as generated new crosses between European and Asian isolates of *P. patens*, and confirmed that in all cases $< 1{:}1000$ spores developed beyond a few cells (S. F. McDaniel and P.-F. Perroud, unpubl. data). The nearly complete lack of recombinant progeny precludes us knowing whether the incompatibility involves nuclear–cytoplasmic interactions, as suggested by von Wettstein (1932), and whether the mutations responsible for the differences in sporophyte development have pleiotropic effects on other aspects of the reproductive biology of *P. patens*.

HYBRID ORIGINS OF *P. EURYSTOMUM* AND *P. COLLENCHYMATUM*?

The consistent genealogical placement of *P. eurystomum* alleles between the well-differentiated *sphaericum* and *pyriforme* lineages (Fig. 2), and the concomitant increase in nucleotide

Table 7. Testing nested demographic models for the *pyriforme* and *sphaericum* lineages.

Model[1]	*t*	log(*P*)	df[2]	2LLR[3]
q1, q2, qa, m1, m2	10.7358	2.7134	–	–
q1, q2, qa, m1 = m2	**10.4581**	**1.4574**	**1**	**2.512**
q1, q2, qa, m1, m2 = 0	**10.6624**	**2.7113**	**1[2]**	**0.0043**
q1, q2, qa, m1 = 0, m2	10.048	0.6419	1[2]	4.143
q1, q2, qa, m1 = m2 = 0	**10.05**	**0.6419**	**2[2]**	**4.143**
q1 = q2, qa, m1, m2	10.3944	−2.7979	1	11.0226
q1 = q2 = qa, m1, m2	10.8539	−7.3736	2	20.174
q1 = q2, qa, m1 = m2	10.601	−3.2943	2	12.0154
q1 = q2, qa, m1 = m2 = 0	10.3637	−4.6787	3[2]	14.7842
q1 = q2 = qa, m1 = m2	9.2436	−8.3774	3	22.1815
q1 = q2 = qa, m1 = m2 = 0	8.163	−10.9879	4[2]	27.4027
q1 = qa, q2, m1, m2	9.4584	−0.0985	1	5.6238
q1 = qa, q2, m1 = m2	9.1366	−1.3154	2	8.0576
q1 = qa, q2, m1 = m2 = 0	9.1271	−2.1873	3[2]	9.8014
q1, q2 = qa, m1, m2	10.7344	−3.6297	1	12.6863
q1, q2 = qa, m1 = m2	8.5912	−5.1609	2	15.7486
q1, q2 = qa, m1 = m2 = 0	8.3555	−6.9501	3[2]	19.327

[1]q1, pyriforme effective population size; q2, sphaericum Ne; qa, ancestral Ne; m1, migration rate from pyriforme into sphaericum; m2, migration rate from sphaericum into pyriforme; *t*, divergence time between pyriforme and sphaericum; *P*, model probability.

[2]Because of a parameter fixed at the boundary of the parameter space, the expected distribution is a mixture when the null model is true; 2LLR should be asymptotically distributed as a random variable that takes the value 0 with probability 0.5 and takes on a value from a χ^2 distribution with probability 0.5 (Hey and Nielsen 2007).

[3]Models under which the probability of achieving the test statistic by chance under the null model is >0.05 are shown in bold.

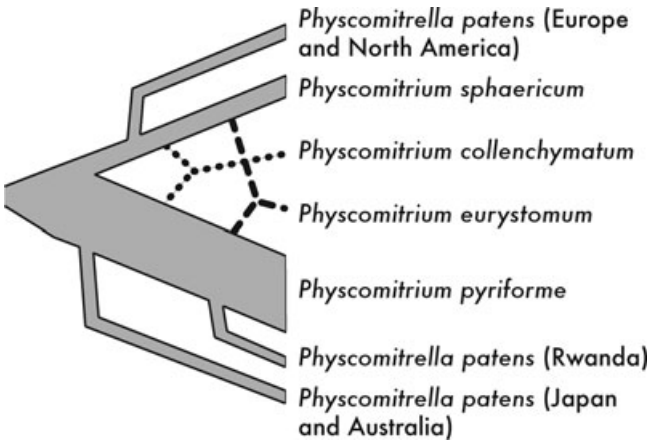

Figure 4. A model of the *Physcomitrella–Physcomitium* complex species tree. The width of the branch corresponds to the relative effective population size of the species (Table 5). The dotted and dashed lines indicate hybridization events leading to the formation of the putative hybrid species *P. collenchymatum* and *P. eurystomum*, respectively.

diversity in the species relative to other sampled taxa (Table 5), could result from taxonomic error, incomplete lineage sorting, or introgressive hybridization. We do not, however, believe that the *P. eurystomum* individuals have been misidentified. First, all three *P. eurystomum* isolates had multilocus genotypes with alleles from both the *sphaericum* and *pyriforme* lineages, which could not be explained by misidentification. Second, the ITS sequences contained obvious species–specific patterns that nearly perfectly matched the traditional taxonomic boundaries in the *Physcomitrella–Physcomitrium* complex, including the genetic similarity of the three accessions of *P. eurystomum* (but with the exception of the multiple origins of *P. patens*, Fig. 2E).

Our results also suggest that the close relationships between *P. eurystomum* and both the *pyriforme* and *sphaericum* lineages are unlikely to result from incomplete lineage sorting. Incomplete lineage sorting is most likely to cause genealogical conflict among loci where population sizes are large, divergence times are recent, and therefore coalescence times may long precede the speciation event. The highest posterior density intervals for the ancestral population size in the *Physcomitrium–Physcomitrella* complex was 20 times smaller than the current estimated population sizes (HPD90 = 19,100–1.39 × 10^6; Fig. 3A), and those for the MLEs of divergence did not include zero (HPD90 = 8.37–12.8 MYA; Fig. 3C). Thus, the most likely demographic parameters (Table 7) do not favor the retention of ancestral polymorphism (although we could not reject a model with no gene flow, which would obviously be necessary in the case of hybridization). These analyses, however, are based on a very simple demographic model that may not capture the actual demographic history of the *Physcomitrella–Physcomtrium* complex, in part because we could not include the

putative hybrid species. We found some preliminary biological confirmation of these patterns by studying the fertility of interspecies hybrids. The MLEs for migration between the *sphaericum* and *pyriforme* lineages were asymmetrical and most evident in the chloroplast *atp*B–*rbc*L gene (Fig. 3B, see also Fig. 2F); all *P. eurystomum* alleles were closely related and identical or very similar to *sphaericum* alleles. This pattern is consistent with the asymmetry in crossing success between *P. patens* and *P. pyriforme* (von Wettstein 1924, 1928) where crosses were only successful with *P. patens* as the maternal parent. The close relationships among *P. sphaericum*, *P. eurystomum*, and *P. collenchymatum* at the chloroplast *atp*B-*rbc*L locus suggest cytoplasmic introgression, a common phenomenon in angiosperms (Tsitrone et al. 2003) and potentially mosses (Shaw and Goffinet 2000; Shaw et al. 2005b; Natcheva and Cronberg 2007).

The consistent presence in *P. eurystomum* of alleles at the four nuclear loci from both the *sphaericum* lineage and *pyriforme* lineage (Fig. 2) suggests that the species may represent not a case of adaptive introgression but rather hybrid speciation. Natcheva and Cronberg (2004) cite several well-documented cases of allopolyploid hybrid species in mosses, and indeed the entire *Physcomitrella–Physcomitrium* species complex may have originated from an Eocene allopolyploidy event (Rensing et al. 2007). The few reported chromosome counts from *P. eurystomum* are variable, including $n = 9$, 26, 52, and 54 (Fritsch 1991; Kapila and Kumar 1997). Polyploid series are frequent in mosses—chromosome numbers from $n = 9$ to $n = 72$ have been reported for *P. pyriforme*—and preliminary flow-cytometric data suggest that the genome size of *P. eurystomum* is about twice that of *P. patens* or *P. sphaericum* (S. A. Rensing, unpubl. data). If *P. eurystomum* were an allopolyploid, we would presumably be able to amplify two parental homeologous alleles of the nuclear loci in this species. However, we found no evidence of multiple paralogs (i.e., multiple peaks in the direct sequencing reactions, or distinct sequences in the cloned PCR products as we found for the four "hybrid" isolates of *P. pyriforme* at the *ho* locus), suggesting either that the isolates we sampled have undergone a complex pattern of gene loss and divergence (Ku et al. 2000; Vanderpoorten et al. 2004; Lukens et al. 2006; Town et al. 2006) or that *P. eurystomum* represents a homoploid hybrid species with a large genome (Ungerer et al. 2006).

Ploidy issues aside, the fertility of natural and experimental hybrids between *P. eurystomum* and either *P. patens*, *P. pyriforme*, and even the more distant *F. hygrometrica*, was generally similar to that found in other crosses (von Wettstein 1924, 1928). We have found that the viability of recombinant spores from F1 hybrids between *P. patens* and *P. eurystomum* was similar to that of spores from *P. patens* and *P. sphaericum* crosses, where the genome size is similar between the two species—many recombinants showed signs of early viability, but none survived past the two-cell stage (S. F. McDaniel and P.-F. Perroud, unpubl. data). This similarity in viability suggests that karyotypic differences are not the only causes of hybrid sterility or inviability in this group.

In addition to the topological conflict caused by *P. eurystomum*, the phylogenetic position of the North American species, *P. collenchymatum*, also varied among loci (Fig. 2, Table 4). In the original description of the species, Gier (1955) suggested that *P. collenchymatum* may be a hybrid between *P. pyriforme* and *A. serratum*. We have no experimental crossing data involving *P. collenchymatum*, and our sample size for this species is limited to a single locality, so this inference requires further validation. We are now actively engaged in genetic analyses to more rigorously test whether the genomic structure of the putative hybrid species is similar to recombinants from either *P. patens* or *P. sphaericum* $\times$ *P. pyriforme* F1 hybrids in karyotype and allelic composition.

MATING SYSTEM AND SPECIATION IN THE *PHYSCOMITRELLA–PHYSCOMITRIUM* COMPLEX

Our data suggest that the mating system in the *Physcomitrella–Physcomitrium* complex is mixed. Members of the Funariaceae have long been known to be self-fertile under laboratory conditions (von Wettstein 1924). The limited genetic variation within species (e.g., *P. sphaericum*, or the Euro-American isolates of *P. patens*) and the small inferred ancestral population size together could result from frequent selfing, consistent with expectations based on the proximity of the gametangia and with previous allozyme studies (Eppley et al. 2006). However, among the accessions of the *pyriforme* lineage, we find evidence of inter- and intragenic recombination. In *P. pyriforme*, recombination is evident in the fixed heterozygosity at the *ho* locus and the distinct genealogical patterns among the other loci (Table 6). In *P. eurystomum*, the Neustadt isolate in the *apr* genealogy appears to be a natural recombinant between a *sphaericum*-type and a *pyriforme*-type sequence, with the 5′-end of the sequence *sphaericum*-like, and the 3′-end *pyriforme*-like. In the aggregate, the available data indicate that outcrossing occurs in self-fertile mosses, even among partially reproductively isolated relatives (Stenoien and Sastad 2001; Eppley et al. 2006).

Theory suggests that both population divergence and hybrid speciation may occur rapidly in self-fertile lineages (McCarthy et al. 1995; Barraclough et al. 2003; Coyne and Orr 2004). The evidence for more rapid speciation in self-fertile mosses than outcrossing mosses in general, however, is inconclusive. Nucleotide variation at the *adk* locus within the outcrossing species *Ceratodon purpureus* (McDaniel and Shaw 2005) among partially reproductively isolated populations (McDaniel et al. 2007, 2008) was nearly equal to that among the six species we sampled in the *Physcomitrella–Physcomitrium* complex ($\theta = 0.030$ and 0.033, respectively). The difference in number of species between these

genera suggests that the diversification rate, relative to the mutation rate, is higher in the self-fertile lineage in this one comparison. Consistent with this inference, experimental crossing studies showed that the hermaphroditic nematode *C. elegans* exhibits outbreeding depression at smaller genetic distances than its outcrossing congener, *C. remanei* (Dolgin et al. 2007). However, the situation is less clear in *Sphagnum* spp., where the mating system was not obviously correlated with patterns of genetic variation or reproductive isolation (Shaw and Cox 2005; Natcheva and Cronberg 2007), or *Polytrichum* spp., where F1 postzygotic barriers isolate the closely related and obligate outbreeders *P. commune* and *P. uliginosum* (van der Velde and Bijlsma 2004).

The differences between *P. pyriforme* and *P. patens* in nucleotide diversity and evidence of past recombination (Tables 5 and 6) also suggest that factors other than the arrangement of the gametangia may be critical for structuring variation within and among populations in the *Physcomitrella–Physcomitrium* complex; in fact, our estimates for *P. pyriforme* are almost certainly too low, given our limited sampling of this species. Species in the complex have evolved different temperature preferences and reproductive phenologies (Nakosteen and Hughes 1978; Furness and Grime 1982). Adaptation to these and other ecological factors, such as humidity or substrate preference, may be critical for limiting gene flow among diverging lineages, because the causative alleles participate in genetic incompatibilities or promote differential habitat specificity. Indeed, the putative hybrid *P. eurystomum* is rare and appears to be endemic to a marginal habitat which is different from that of its inferred parental species (Hill et al. 2006), similar to hybrid species of sunflowers and butterflies (Rieseberg et al. 2003; Gompert et al. 2006) and consistent with theoretical predictions (Buerkle et al. 2000; Gross and Rieseberg 2005). We anticipate that uniting the molecular techniques now well established in *P. patens* (Quatrano et al. 2007) with classical genetics (von Wettstein 1924, 1928, 1932) will provide important insights into the key genetic, ecological, and demographic factors that generate new species.

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Supporting Information may be found in the online version of this article.

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