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Polyploidization within the Funariaceae—a key principle behind speciation, sporophyte reduction and the high variance of spore diameters?

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Abstract

Although being recognized as a major force behind speciation in flowering plants, the evolutionary relevance of genome duplication (polyploidization) remains largely unexplored in mosses. Phylogenetic and-genomic insights from the model organism *Physcomitrella patens* and closely related species revealed that polyploidization, likely via hybridization (allopolyploidization), gives rise to new species within the Funariaceae. Based on the phylogenetic analysis of the nuclear single copy gene *BRK1* combined with the measurement of DNA content by flow cytometry, we identified *Entosthodon hungaricus* as such an allopolyploid species. Together with *Physcomitrium pyriforme*, *Physcomitrium eurystomum* and *Physcomitrium collenchymatum*, which were identified previously as species that likely arose by hybridization, *E. hungaricus* represents an additional allopolyploid lineage of a species complex that is characterized by convergent sporophyte reduction and a considerable variance in spore sizes. Based on morphological and cytological data from 18 species, we highlight the potential impact of polyploidization on the size of the spores and on sporophyte architecture.

Keywords: convergent evolution, genome duplication, hybridization, mosses, post-duplication diversification, *Entosthodon hungaricus*, *Physcomitrella patens*

Introduction

Exploring the driving forces behind speciation and diversification is one of the major topics in evolutionary biology. The increasing availability of genome sequences provides evidence for a high relevance of genome duplication (polyploidization) during land plant evolution. Analyses of the first sequenced embryophyte genomes revealed that polyploidization is frequent and a key process behind genome evolution, morphological complexity and speciation (Crow & Wagner 2006, Soltis & Soltis 2009; Rensing 2014, Van de Peer *et al.* 2017). Flowering plants are paleopolyploids descending from ancestors that multiplied their genomes through either auto-or allopolyploidy (Jiao *et al.* 2011, Van de Peer *et al.* 2017). In autopolyploids, chromosomal sets originate from the same parent, while in allopolyploids chromosomal sets are genetically different, which means these species result from hybridization of species or genetically divergent lineages.

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Genomic signs for polyploidy are present also in mosses (Rensing *et al.* 2007, Rensing *et al.* 2013, Beike *et al.* 2014). By now, allopolyploidization is widely accepted as the main source of polyploid mosses, as reflected by reports of hybrid species from different families, such as Mniaceae (Wyatt *et al.* 1988, Wyatt *et al.* 1992, Wyatt and Odrzykoski 1998), Sphagnaceae (Ricca and Shaw 2010, Stenøien *et al.* 2011), and Pottiaceae (Košnar *et al.* 2012). The observation that individual polyploid, hybrid species originate recurrently (Soltis & Soltis 1999) emphasizes the evolutionary role of this process and its impact on sympatric speciation. As hybridization implies the transfer of genetic material between different taxa, an inter-specific hybrid may experience altered reproductive compatibility with its parental species, especially in the case of polyploidization.

The model organism *Physcomitrella patens* (Hedwig 1801: 20) Bruch & Schimper (1849: 13) (or *Physcomitrium patens* (Hedwig 1801: 20) Mitten (1851: 363) as proposed by Beike *et al.* 2014 and Medina *et al.* 2019; *P. patens* in the following) is a polyploid species that underwent two whole-genome duplication events, 27–35 and 40–48 million years ago (Rensing *et al.* 2007, Rensing *et al.* 2008, Lang *et al.* 2018). It evolved from allopolyploid ancestors of the *Physcomitrium-Physcomitrella* species complex (McDaniel *et al.* 2010, Beike *et al.* 2014).

Funariaceae is a moss family of around 200 species (Crum & Anderson 1955) with considerable diversity in sporophyte architecture. The sporophytes of the different genera range from complex sporophytes on long setae with a diplolepidous-opposite peristome as found in *Funaria* to cleistocarpous sporophytes as present in *P. patens*. Many genera of the Funariaceae, like *Funaria*, *Entosthodon* and *Physcomitrium*, are classified according to their typical sporophyte complexity, although phylogenetic analyses showed that these traits are homoplasious (Liu *et al.* 2012). All members of the family are monoecious and intra-gametophytic self-fertilization may be the predominant mode of sexual reproduction, at least in isolates of *P. patens* (Perroud *et al.* 2011). Yet, allo-fertilization between non-conspecific gametophytes has been induced in the lab as well as it was observed in natural habitats where different species often grow in close proximity (von Wettstein 1924, Rensing *et al.* 2013).

Furthermore, Funariaceae show an enormous variety regarding chromosome numbers (as summarized in Fritsch 1991) and DNA content (Beike *et al.* 2014). The chromosome number of the *P. patens* model strain is n=27 (Reski *et al.* 1994, Lang *et al.* 2018) which is higher than the typical number of chromosomes among mosses (Fritsch 1991), due to at least two rounds of ancestral polyploidization. Chromosome numbers of Funariaceae range from n=5 in *Funariella curviseta* (Schwägrichen 1823: 17) Sérgio (1988: 10) up to n=56 in *Entosthodon hungaricus* (Boros 1924: 73) Loeske (1929: 115) (Györffy 1964). High chromosome numbers can be found in the genus *Physcomitrium*, like n=54 chromosomes in *Physcomitrium immersum* Sullivant (1848: 648) (Bryan 1957) or up to n=54 chromosomes in *Physcomitrium pyriforme* (Hedwig 1801: 38) Bridel (1827: 815) and *Physcomitrium eurystomum* Sendtner (1841: 142) (Danilkiv 1981). Nuclear DNA content differs threefold when comparing *Funaria hygrometrica* Hedwig (1801: 172) with *Physcomitrium collenchymatum* Gier (1955: 330), *P. eurystomum* or *P. pyriforme* (Beike *et al.* 2014).

These diverse chromosome numbers and DNA contents provide evidence for widespread polyploidy within the Funariaceae. In a previous study, *P. pyriforme*, *P. eurystomum* and *P. collenchymatum*, which are species with increased DNA content and chromosome numbers, were identified as allopolyploids from which morphologically reduced taxa like *P. patens* arose convergently by sporophyte reduction and adaptation to specific habitats (Beike *et al.* 2014). Adding to this, traits that are directly associated with the mode and effectiveness of dispersal, such as spore size and seta length, might be correlated with alterations in ploidy. Although adaptive pressure on these traits by a specific lifestyle is to be expected, polyploidization might add to this by additionally favouring local recruitment, e.g. after loss of reproductive compatibility to parental species.

In this study, we expanded an earlier data set (Perroud & Quatrano 2008, Beike *et al.* 2014) by eight additional species to include some of the most common species of *Physcomitrium* and related genera *Funaria* and *Entosthodon.* Our aim was (1) to test whether *E. hungaricus*, which is reported to have high chromosome numbers, is another allopolyploid. For determination of the DNA content material from the herbarium was re-grown to establish *in vitro* cultures for flow cytometry. Furthermore, three additional accessions of *F. hygrometrica* and *P. patens* were included to (2) test for variability of the nuclear single copy marker *BRK1*. Morphological and cytological data from all 18 analysed taxa were compiled from the literature to analyse (3) whether allopolyploids share specific morphological traits in contrast to species that show no signs of allopolyploidization, by means of distinct *BRK1* homeologs (polyploidization-derived gene copies) and increased DNA content and/or chromosome numbers.

Material & Methods

Taxon sampling and *in vitro* cultivation

The specimens originated from different herbaria as well as from *in vitro* cultures available in the International Moss Stock Center (IMSC, www.moss-stock-center.org). The taxon set comprises 36 vouchers from 18 species. Detailed information about the species, voucher and Genbank identifiers are given in Table S1. The mosses derived from the IMSC were grown under axenic conditions on solid Knop medium (250 mg/L KH_2PO_4, 250 mg/L KCl, 250 mg/L $MgSO_4$ x 7 H_2O, 1000 mg/L $Ca(NO_3)_2$ x 4 H_2O, 12.5 mg/L $FeSO_4$ x 7 H_2O, pH = 5.8 with KOH; Reski & Abel 1985) in Petri dishes. In the case of *E. hungaricus*, plant material was either collected from the field prior to *in vitro* cultivation (Sabovljevic *et al.* 2012) or mature spores from herbaria samples were transferred to solid Knop medium to establish cultures for flow cytometric measurements (FCM). A *BRK1* sequence from *Encalypta streptocarpa* Hedwig (1801: 62) was used as an outgroup for phylogenetic analyses.

Phenotypic and cytological characterization

For phenotypic characterization of the sporophyte (capsule position and form, seta lengths, presence of the peristome, presence of the operculum), plant material from herbaria, *in vitro* culture, or from the field was analysed using a stereomicroscope (Leica Z16 APO, Leica Microsystems GmbH, Wetzlar, Germany) with an integrated CCD camera (Leica DFC 490, Leica Microsystems). To illustrate the sporophytes from *F. hygrometrica*, *E. hungaricus*, *P. collenchymatum* and *P. patens*, several images were combined into one picture using the LAS 3.8 Multifocus software via focus stacking (Leica Microsystems). For these taxa, the size of the spores was also analysed. We compiled additional morphological information for all taxa analysed in this work from the literature (Limpricht 1895, Williams 1901, Roth 1904, Gier 1955, Bartram 1972, Flowers 1973, De Sloover 1975, Crum & Anderson 1981, Sérgio 1988, Beever *et al.* 1992, Boros *et al.* 1993, Sharp *et al.* 1994, Ahrens 2000, Allen 2002, Frahm & Frey 2004, McIntosh 2007, Toren 2008, Fedosov *et al.* 2010). In the case of multiple ranges of lengths or diameters, the minima and maxima of all references were combined into one range. Chromosome numbers as summarized by Fritsch (1991) were used for calculation of the median chromosome number per species. All collected information is available in Table 1.

TABLE 1. Funariaceae morphological and cytological characteristics. The taxon set is listed on the left. Morphological data of sporophyte and spore size is compiled from Allen (2002), Bartram (1972), Beever *et al.* (1992), Boros *et al.* (1993), Crum & Anderson (1981), De Sloover (1975), Fedosov *et al.* (2010), Flowers (1973), Frahm & Frey (2004), Gier (1955), Limpricht (1895), McIntosh (2007), Roth (1904), Sérgio (1988), Sharp *et al.* (1994) and Toren (2008). *The median chromosome number was calculated from the literature compiled by Fritsch (1991). The minimum (min) and maximum (max) chromosome numbers reported for a certain taxon are depicted behind the median chromosome number. **The genome size is indicated as 1C value according to Beike *et al.* (2014) and as measured in this work for *Entosthodon hungaricus*. n.a. = not available.

Species	capsule position	seta length (mm)	capsule form	operculum	revoluble annulus	peristome	spore size (µm)	median chromosome number // min–max*	genome size (1C)**
Funaria flavicans	emergent	8.0–20.0	inclined	present	present	double	18–30	10	n.a.
Funaria hygrometrica	emergent	16.0–70.0	inclined	present	present	double	12.5–18.8	28 // 4–56	0.44 ± 0.03
Funaria calvescens	emergent	10.0–70.0	inclined	present	present	double	9–15	14	n.a.
Entosthodon americanus	emergent	6.0–10.0	inclined	present	absent	double	24–31	n.a.	n.a.

...Continued on the next page

TABLE 1. (Continued)

Species	capsule position	seta length (mm)	capsule form	operculum	revoluble annulus	peristome	spore size (µm)	median chromosome number // min–max*	genome size (1C)**
Entosthodon attenuatus	emergent	6.0–14.0	erect	present	absent	single	22–35	28	n.a.
Entosthodon bonplandii	emergent	5.0–20.0	erect	present	absent	absent	20–34	n.a.	n.a.
Entosthodon convexus	emergent	10.0–20.0	inclined	present	absent	double	18–25	n.a.	n.a.
Entosthodon hungaricus	emergent	4.0–10.0	erect	present	absent	absent	30–40	54 // 22–56	1.96 ± 0.04
Entosthodon muhlenbergii	emergent	5.0–15.0	inclined	present	absent	double	19–30	28	n.a.
Entosthodon pulchellus	emergent	5.0–8.0	inclined	present	absent	double	18–25	n.a.	n.a.
Physcomitrium collenchymatum	emergent	2.0–3.0	erect	present	absent	absent	24–37	n.a.	1.51 ± 0.44
Physcomitrium eurystomum	emergent	4.0–7.0	erect	present	absent	absent	32–46	40 // 9–54	1.27 ± 0.32
Physcomitrium pyriforme	emergent	3.0–14.0	erect	present	absent	absent	25–50	36 // 9–54	1.33 ± 0.42
Physcomitrella magdalenae	immersed	0.25	erect	absent	absent	absent	22–29	n.a.	0.92
Physcomitrella patens	immersed	0.2	erect	absent	absent	absent	24–34	27 // 14–28	0.96 ± 0.15
Physcomitrella readeri	immersed	0.2	erect	absent	absent	absent	27–42	n.a.	0.96 ± 0.05
Aphanorrhegma serratum	immersed	0.2	erect	present	absent	absent	26–40	27	0.9
Funariella curviseta	immersed	1.2–3.0	inclined	present	absent	absent	15–24	5	n.a.

DNA extraction

In the case of herbarium samples, single gametophores were washed and dried overnight in a paper envelope in a screw top jar filled with silica gel (Carl Roth GmbH, Karlsruhe, Germany). The dried moss tissue was disrupted in a Tissue Lyser (Qiagen, Hilden, Germany) for 2 min at 20 Hz. Genomic DNA was extracted from approximately 5–10 mg dry weight using the Genomic DNA from Plant Kit II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's protocol. In the case of material grown from *in vitro* culture, fresh material was used for DNA extraction. DNA from up to 50 mg fresh weight was isolated using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1990).

Amplification, cloning and sequencing of *BRK1*

The nuclear single copy gene *BRK1* (*P. patens* gene ID: Pp3c8_2740V3.1, Lang *et al.* 2018) was used as a phylogenetic marker. For the amplification of a part of *BRK1* the primers BRICK1_for (GTCGGCATTGCTGTACAA) and BRICK1_rev (CTCCAGCTGACGCTCCAG) were used (Beike *et al.* 2014). In a final reaction volume of 25 µL, the PCR was set up using 2.5 µL 10×DreamTaq Green Buffer (Thermo Scientific, St. Leon-Rot, Germany), 2.5 µL deoxyribonucleotide triphosphates (dNTPs, 2 mM, Thermo Scientific), 0.2 µL DreamTaq polymerase (5 U/µl, Thermo

Scientific), 0.5 µL of each primer (10 pmol/µL) and 1 µL genomic DNA. After an initial denaturation at 94 °C for 5 min, the cycling conditions consisted of a denaturation step of 45 sec at 94 °C, an annealing step at 53 °C for 1 min and an elongation step for 1 min at 72 °C for 30–33 cycles. For final elongation, the reaction was incubated at 72° C for 5 min. The PCR was controlled via agarose gel electrophoresis. The PCR products were purified using the Nucleo Spin Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's protocol. The purified PCR product and both primers were sent to LGC genomics (Berlin, Germany) or to GATC Biotech (Konstanz, Germany) for Sanger sequencing. *BRK1* sequences from *E. hungaricus* showed nucleotide polymorphisms in the electropherogram of the sequenced PCR product, providing evidence for paralogous (probably homeologous) sequences. To analyse the *BRK1* homeologs from *E. hungaricus*, the PCR products were cloned into the pJET1.2/blunt vector (CloneJET PCR Cloning Kit, Thermo Scientific, St. Leon-Roth, Germany) following the manufacturer's instructions. The vector was transfected into chemically competent *E. coli* cells that were grown on ampicillin-containing (Carl Roth GmbH, Karlsruhe, Germany) solid LB-medium for selection. Bacterial colonies were screened via PCR for the insertion of plasmid DNA using the gene-specific primers. Plasmid DNA extraction was done after an overnight incubation in liquid ampicillin-containing LB-medium at 37 °C using the GeneJET Plasmid Miniprep-Kit (Thermo Scientific). For each accession of *E. hungaricus* at least three plasmid DNAs from independent bacterial clones were sequenced.

Sequence analysis and phylogenetic reconstructions

The sequence chromatograms were analysed using Geneious 8.0.5 (Kearse *et al.* 2012). Each chromatogram was checked for sequence polymorphisms, i.e. overlapping sequence peaks. In the case of polymorphisms, the PCR product was cloned as described above. Consensus sequences were generated from both sequences obtained from Sanger sequencing using the primers BRICK1_for and BRICK1_rev. Multiple sequence alignments were generated with MAFFT (Katoh & Standley 2013). Phylogenetic trees were calculated with Bayesian Inference (BI) and Maximum Likelihood analyses (ML) as described in Beike *et al.* (2014). BI was done with MrBayes 3.2.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) using the GTR substitution model with eight gamma distributed rates, two heated chains and a heated chain parameter of 0.2. Two million cycles for the MCMC algorithm with a sampling frequency of 1,000 were applied, until the average standard deviation of split frequencies dropped below 0.01. The ML analysis was performed with RAxML 7.2.8 using the GTR gamma nucleotide model and a rapid bootstrapping analysis of 1,000 replicates. The phylogenetic tree was visualized with Geneious 8.0.5. The figure was generated using Inkscape (www.inkscape.org).

Character trait mapping and statistical analysis

The morphological information from the literature was mapped onto the phylogenetic tree for illustration purposes. Character state mapping of the continuous characters spore size and chromosome number (Table 1) was done with Mesquite 3.03 (Maddison & Maddison 2006) using unordered parsimony tracing. Correlations between continuous traits (spore size, seta length, chromosome number) were calculated with phylogenetically independent contrasts (PIC) (Felsenstein 1985), which is applicable to phylogenetic trees with polytomies (Garland & Diaz-Uriarte 1999), using the phenotypic diversity analysis module PDAP:PDTREE for Mesquite 3.03 (Midford *et al.* 2005, Maddison & Maddison 2006).

Flow cytometric measurements

Flow cytometric measurements were carried out according to Schween *et al.* (2003) with modifications to analyse the DNA content of three *E. hungaricus* accessions in comparison to the DNA content of *P. patens*. Gametophores were chopped with a razor blade in 4.6 Diamidino-2-Phenylindol (DAPI) buffer (0.107 g $MgCl_2$*6 H_2O, 0.5 g NaCl, 1.211 g Tris, 0.1 mL Triton X-100 in 100 mL water, 1mg/L DAPI, pH 7.0 with HCl). The remaining plant debris was filtered and the measurements were made on an Aria III cell sorter system (Becton, Dickinson and Company, BD Biosciences, New Jersey, USA). As configuration, a 85 µm nozzle was used with a pressure of 45 psi. DAPI was excited with a 405 nm 50 mW diode laser and the emitted signals were collected with a 450/40 band pass filter. To monitor the records, identified positive signals were gated from SSC-/FSC-plot and displayed as counts in a histogram with linear scale. For the record and analysis, the DIVA version 8.0.1 was used.

Results

In vitro cultivation and flow cytometry of *Entosthodon hungaricus* from herbaria material

In vitro cultivation of *E. hungaricus* from mature spores was possible after six years of herbarium storage. It took between ten days to three weeks until protonema filaments emerged. First gametophores were observed after 45 days. As determined with FCM from these *in vitro* cultures, the DNA content of *E. hungaricus* is higher than in *F. hygrometrica* and *P. patens*. The 1C value is 1.96 ± 0.05 (Table S2), while it is 0.96 ± 0.15 in *P. patens* and 0.44 ± 0.03 in *F. hygrometrica* (Beike *et al.* 2014). *E. hungaricus* has the highest DNA content so far recorded within the Funariaceae, even higher than that of *P. pyriforme*, *P. collenchymatum* and *P. eurystomum* (Table 1).

BRK1 sequence analysis shows no variability between different accessions of the same taxon, even if homeologs are present, and identifies *E. hungaricus* as allopolyploid

We complemented a published set of 20 accessions representing 10 species (Beike *et al.* 2014) with 16 additional accessions resulting in a total set of 36 accessions covering 18 distinct species of the Funariaceae (Table S1). For all species, the amplified sequence of the nuclear single copy marker gene *BRK1* has a length of approximately 600 bp (Figure 1a). Additional accessions of *F. hygrometrica* from Iceland and Germany, and *P. patens* from Germany were included to test for marker variability within these taxa. Sequence variability was not detected; the *BRK1* sequences are 100 % identical in all three accessions of *P. patens* and *F. hygrometrica*, respectively. Except for *Entosthodon attenuatus* (Dickson 1801: 8) Bryhn (1908: 25), where one single nucleotide polymorphism (SNP) within the intron occurs when comparing both accessions, none of the other species showed sequence variability when comparing multiple accessions of the same species.

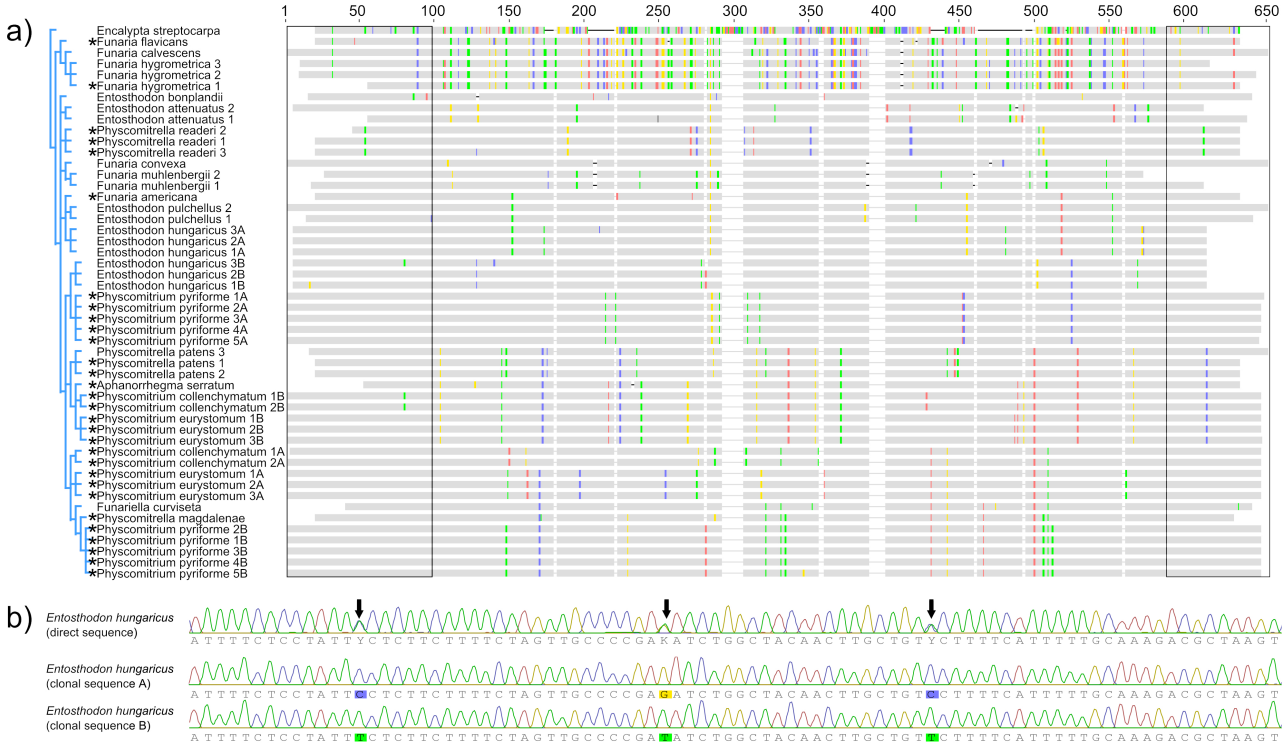

FIGURE 1. Multiple sequence alignment and sequence polymorphisms of ***BRK1.*** The nuclear single copy gene *BRK1* (*P. patens* gene ID: Pp3c8_2740V3.1) was amplified and sequenced from 18 Funariaceae. *Encalypta streptocarpa* (Encalyptaceae) was used as outgroup. (a) Multiple sequence alignment of *BRK1*. The exon regions flanking the intron are marked in black boxes. Nucleotide positions identical in all species are shown in grey, while nucleotide disagreements are marked with green (T), red (A), blue (C) and yellow (G). The taxa and the Bayesian Inference tree topology are shown on the left. The number of nucleotides is depicted at the top. The sequences previously published by Beike *et al*. (2014) are highlighted with asterisks. (b) In the *BRK1* sequences from all three accessions of *Entosthodon hungaricus*, identical sequence polymorphisms were detected and highlighted with arrows. The sequences obtained from the cloned PCR products separated two homeologs of *BRK1* (clonal sequence A and clonal sequence B).

As previously shown for *P. collenchymatum*, *P. eurystomum* and *P. pyriforme* (Beike *et al.* 2014), the *BRK1* sequences from *E. hungaricus* contain SNPs within all three accessions from Germany and Hungary, respectively (Figure 1b). Cloning and sequencing of the corresponding PCR products revealed two distinct *BRK1* homeologs (i.e., polyploidization-derived gene copies) in all analysed *E. hungaricus* accessions (Figure 1b). In all species with multiple *BRK1* homeologs more SNPs were observed in the intron than in the exon (Figure 1a). In *E. hungaricus* no SNPs were observed within the exon, while 14 SNPs were found in the 487 bp intron.

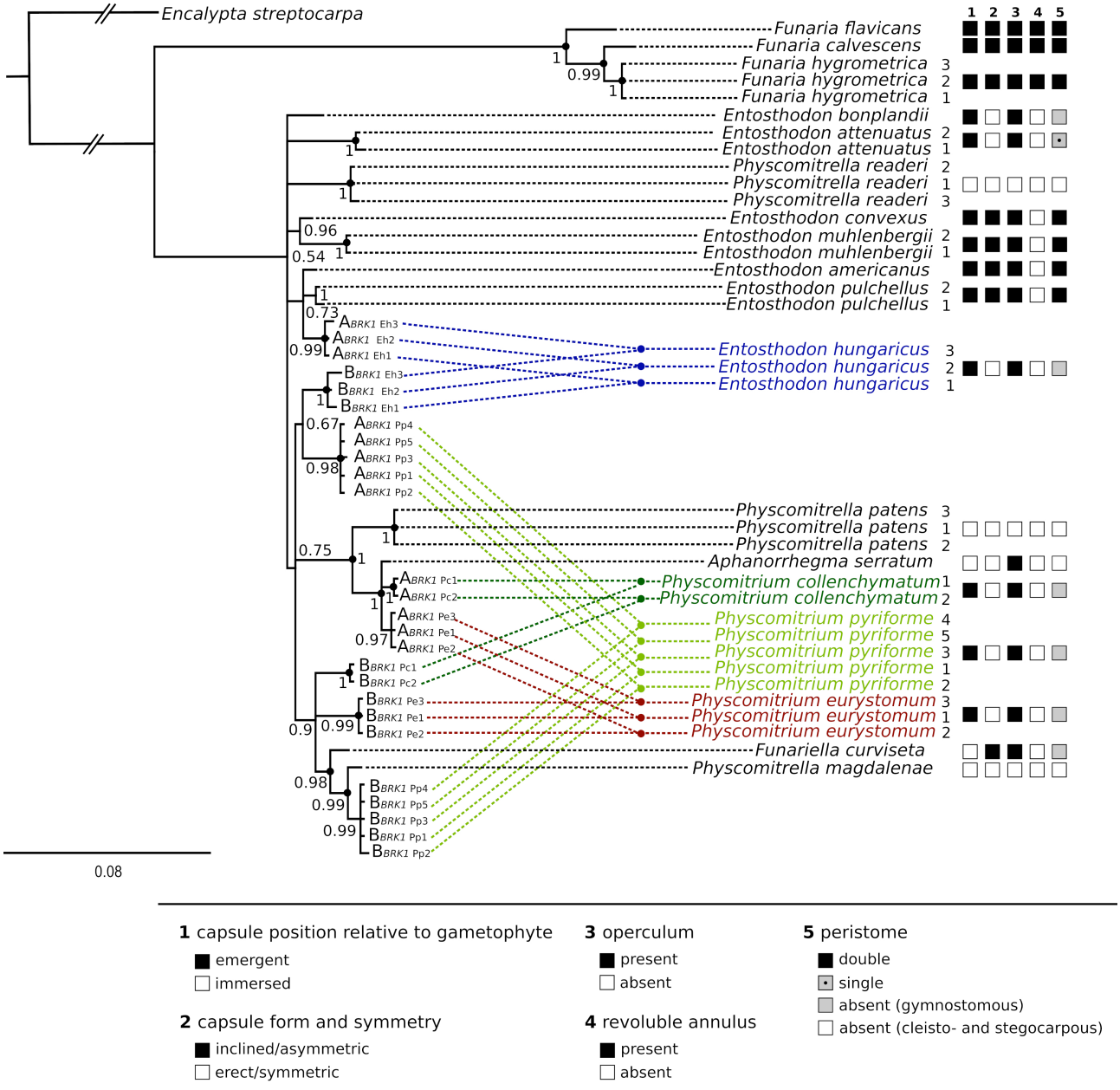

FIGURE 2. Trait mapping of sporophytic traits onto the ***BRK1***-based phylogeny. The phylogenetic tree based on *BRK1* sequences was calculated with Bayesian Inference (BI) and Maximum Likelihood (ML). The BI tree is shown in this figure, the support values show posterior probabilities, while nodes with additional ML bootstrap support > 90 are marked with black dots. The scale bar depicts substitutions per site. *BRK1* amplified from *Encalypta streptocarpa* was used as outgroup. Due to unresolved branching order, the backbone of the major clade is partly shown as multifurcating. Species with two distinct homeologs of *BRK1* are shown in blue (*Entosthodon hungaricus*), dark green (*Physcomitrium collenchymatum*), light green (*Physcomitrium pyriforme*) and red (*Physcomitrium eurystomum*). The two distinct *BRK1* homeologs are labelled "A" and "B", followed by the gene name, the initials of the species ("Eh", "Pc", "Pp", "Pe") and the accession numbers (1–5). The branches of the tree are lengthened by dotted lines for graphical representation. Black lines depict species without polymorphisms in the *BRK1* sequence; coloured lines depict species with two distinct *BRK1* loci. Sporophytic traits are shown on the right; black denotes the characteristics of the type species *Funaria hygrometrica*.

FIGURE 3. Typical Funariaceae sporophytes and spores. Four Funariaceae species are shown, from the left to the right: *Funaria hygrometrica*, *Entosthodon hungaricus*, *Physcomitrium eurystomum* and *Physcomitrella patens*. The mosses are shown in equal magnification, scale bar = 2.5 mm. The corresponding mature spores are shown below in equal magnification, scale bar = 20 µm.

The *BRK1*-phylogeny separates *Funaria* from an unresolved species complex with diverse sporophyte architecture

Based on phylogenetic analysis of *BRK1*, two major clades are separated. One clade includes the sequences of *Funaria flavicans* Michaux (1803: 303), *Funaria calvescens* Schwägrichen (1816: 77) and *F. hygrometrica*. The sister

clade contains sequences of the genera *Entosthodon* (many species herein are also frequently classified as *Funaria*), *Physcomitrium* (including *P. patens*, *Aphanorrhegma serratum* (Wilson & Hooker in Drummond 1841) Sullivant (1848: 647)) and *Funariella* (Figure 2). In this clade we observe several polytomies, which are also found in multiple gene consensus trees (e.g., McDaniel *et al.* 2010, Liu *et al.* 2012) but were resolved in more recent studies (Medina *et al.* 2018, Medina *et al.* 2019).

Mapping sporophyte traits onto the *BRK1*-based phylogeny shows that species from the *Funaria*-clade share an inclined, emergent sporophyte with operculum, revoluble annulus and completely developed peristome. None of these species shows signs of allopolyploidy by means of multiple *BRK1* homeologs (Figure 2).

In the multifurcating clade, sporophyte morphology is highly variable. *Entosthodon* species have emergent sporophytes with operculum, but are lacking a revoluble annulus. Within the present taxon set all *Entosthodon* spore capsules are emergent on setae between 4–20 mm, however they are either inclined and asymmetric, or emergent and symmetric (Table 1). *Physcomitrium* has a gymnostomous, emergent and symmetric spore capsule. The allopolyploids *E. hungaricus*, *P. pyriforme*, *P. eurystomum* and *P. collenchymatum* share a similar sporophyte complexity, namely an emergent sporophyte with symmetric capsule, operculum, but without a peristome. A noteworthy exception is *Entosthodon bonplandii* (Hooker 1816) Mitten (1869: 245), which shares a sporophyte complexity but shows no signs for allopolyploidy by means of multiple *BRK1* gene copies (Figure 2).

Aphanorrhegma serratum, *P. patens* and *F. curviseta* have immersed capsules on setae between 0.2–3 mm (Table 1). While the capsules are emergent and symmetric in *A. serratum* and *P. patens*, they are inclined and asymmetric in *F. curviseta*. An operculum is present in *A. serratum* and *F. curviseta*, while peristomes and revoluble annuli are lacking in these genera. In none of these taxa multiple *BRK1* gene copies were detected.

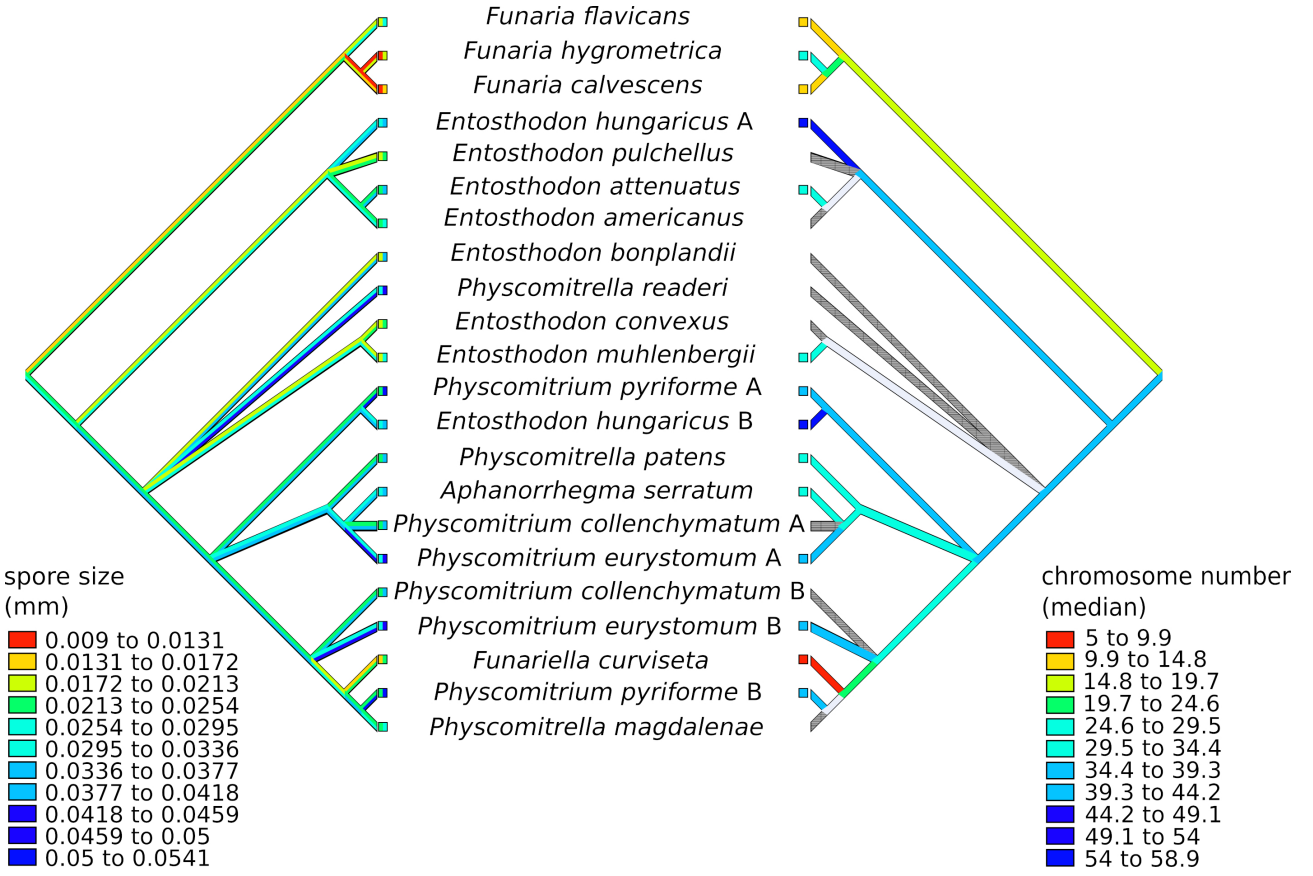

FIGURE 4. Spore size and chromosome numbers mapped onto the ***BRK1***-based phylogeny. The mirrored *BRK1*-based phylogenetic trees were inferred by Bayesian Inference including only one randomly chosen accession per species. Multiple *BRK1* homeologs of the allopolyploid species are labelled "A" and "B". Phylogenetically independent contrasts revealed a positive correlation (p=0.0019, FDR-corrected p=0.0058) between the size of the spores as shown on the left and the median of chromosome counts according to Fritsch (1991) depicted on the right. For species with unknown chromosome numbers, the branches are depicted in grey.

The size of the spores, but not the length of the seta correlates with the number of chromosomes

Besides sporophyte architecture, size and ornamentation of spores are quite variable within the Funariaceae (Figure 3). According to the literature, spores from *F. hygrometrica* are 12.5–18.8 µm, while chromosome numbers range from n=14 to n=28, and the 1C value is 0.44 (Table 1). In *E. hungaricus*, spores are more variable with diameters of 30–40 µm (Figure 3), the median chromosome number and DNA content is higher than for example in *F. hygrometrica*. For *P. eurystomum*, *P. pyriforme* and *P. collenchymatum* we find a comparable situation of large spores along with increased DNA content or chromosome numbers (Table 1). In *P. patens*, the spores are slightly smaller with 24–34 µm (Figure 3), while the chromosome number is n=27 (Reski *et al.*1994) and the genome size is 1C=0.96 (Table 1). A possible correlation between spore size and chromosome number becomes even more evident when comparing data from the literature for all Funariaceae included in this work (Table 1). Phylogenetic independent contrast (PIC) analysis based on the *BRK1* phylogeny revealed a positive correlation (p=0.0019, FDR-corrected p=0.0058) between median chromosome number and spore size (Figure 4). In contrast, we did not detect a correlation between chromosome number and the length of the seta.

Discussion

Based on molecular phylogenetics, it was postulated that *Physcomitrium* and *Physcomitrella* under traditional nomenclature are polyphyletic, and that *P. patens*-like, cleistocarpous sporophytes evolved several times convergently from allopolyploid ancestors (McDaniel *et al.* 2010, Beike *et al.* 2014). Medina *et al.* (2018) combined morphological and molecular data for a large Funariaceae taxon set to show that *Entosthodon* is also polyphyletic and named the evolutionary young, rapidly evolving *Entosthodon-Physcomitrium* (E-P) species complex. However, in phylogenetic studies based on typically uniparental inherited plastid and mitochondrial markers, the relevance of polyploidy, e.g. based on hybridization, can easily be underestimated. Medina *et al.* (2019) analysed 648 nuclear loci to infer the evolutionary relationships of five taxa of the Funarioideae. The authors proposed a new monophyletic delineation of *Physcomitrium* and found *P. patens*-like (or *A. serratum*-like) sporophytes to have emerged several times within the *Physcomitrium* complex, yet they only included haploid accessions in their analyses. We chose to analyse a nuclear single copy gene instead to disclose extant species of hybrid origin based on the presence of both homeologs.

We chose the nuclear single copy gene *BRK1* as a marker since it was established in an earlier study within the Funariaceae (Beike *et al.* 2014) and proved its broad applicability in a molecular fingerprinting of 19 *Sphagnum* accessions (Heck *et al.* 2021). In congruence with Medina *et al.* (2019), our analysis of *BRK1* confirms the polyphyly of *Entosthodon*, *Physcomitrium* and *Physcomitrella* under traditional nomenclature, while *Funaria* forms a monophyletic entity in a sister group position. Additionally, we provide evidence that *E. hungaricus* is a species of hybrid origin, as it has two different homeologs of *BRK1* and an increased genome size as compared with other Funariaceae. *E. hungaricus* is a very rare species. Its growth in axenic *in vitro* culture from herbaria material highlights the potential of such collections for scientific analyses. The regeneration ability of moss spores after long time of dormancy combined with cell culture techniques enables the study of rare, endangered or even extinct species. Moreover, long-time storage over liquid nitrogen has been established (Schulte & Reski 2004), so that cryopreservation of *in-vitro* cultures further facilitates preservation of endangered moss species.

E. hungaricus was first recorded as *Funaria hungarica* Boros (1924: 73) in saline areas in Hungary (Boros 1924). For a long time, it was considered endemic for Europe (Sabovljevic *et al.* 2012), but more recently it has been recorded at many locations worldwide. Until now, it has been found two times in Germany and at the Neusiedler See in Austria (Zechmeister 2005). Other locations are steppe regions in Europe and Russia as summarized in Pisarenko *et al.* (2001), Israel (Herrnstadt *et al.*1991), Central Asia (Pisarenko *et al.* 2001), the Mediterranean region including Sicily (Cano *et al.* 1999), Northern Africa (Cano *et al.* 1999, Ros & Cano 2008) and the Canary Islands Fuerteventura and Lanzarote (Dirkse *et al.* 2011). Some of these records are a consequence of taxonomic revisions, in which some taxa were synonymized with *E. hungaricus*, e.g. *Entosthodon maroccanus* (Meylan 1937: 426) Hébrard & Lo Giudice (1997: 145) from Northern Africa (Cano *et al.* 1999), or *Physcomitrium longicolle* Trabut (1922) from Northern Africa (Ros & Cano 2008) and Macaronesia (Dirkse *et al.* 2011). Based on our work, we postulate allopolyploidy for populations from Germany and Hungary. Whether or not *E. hungaricus* represents an allopolyploid entity of single origin and worldwide dispersal or whether it is of recurrent origin remains unclear at present.

All allopolyploids detected so far, namely *E. hungaricus*, *P. pyriforme*, *P. eurystomum* and *P. collenchymatum*,

share an emergent, symmetric and erect sporophyte with a reduced peristome. A sporophyte like this is the ancestral state in the genus *Physcomitrium*. A close relationship between *E. hungaricus* and *Physcomitrium* was postulated based on the shape of the operculum and the calyptra (Ahrens 1996). Due to this shared sporophytic morphology, polyploidization seems to coincide with this degree of sporophyte complexity. However, the genus *Physcomitrium* includes both, hybrid taxa with increased chromosome numbers, as for example *P. immersum* with n=54 chromosomes (Bryan 1957), and taxa with smaller chromosome numbers, such as *Physcomitrium sphaericum* (C. Ludwig 1810: 26) Bridel (1827: 815) or *Physcomitrium japonicum* (Hedwig 1801: 34) Mitten (1891: 164) with 26 or even less chromosomes (Fritsch 1991), which is rather comparable to *P. patens* with 27 chromosomes (Reski *et al.* 1994). According to our work, the length of the seta is not correlated with the median chromosome number. Furthermore, *E. bonplandii*, which also has a sporophyte complexity comparable to *Physcomitrium*, has only one *BRK1* copy, which is proposedly a consequence of haploidization, i.e. gene loss after polyploidization. Only the relatively recent polyploids contain two *BRK1* homeologs. In consequence, genome size and chromosome number do not always seem to be in alignment with sporophyte complexity.

In contrast to this, the size of the spores correlates with the chromosome number. According to Crawford *et al.* (2009), spore sizes and chromosome numbers in mosses are correlated with the sexual system, i.e. mosses with separate sexes have smaller spores, while monoicous mosses, like the Funariaceae, tend to have higher chromosome numbers. Although in many plants polyploids have larger diaspores, a correlation like this was not reported for mosses in general (Crawford *et al.* 2009). Here, we provide evidence that spore size is correlated with chromosome numbers in the 18 Funariaceae analysed in this study. Interestingly, the bigger spore sizes probably hinder dispersal by wind, and might lead to adaptation to peculiar niches and modes of dispersal. Alternatively, adaptation to open, often disturbed habitats and a short annual lifestyle might select bigger spore sizes to ensure regular, local re-emergence after disturbance (During 1979). For *P. patens*, it has been hypothesized that spore capsules remain in the muddy ground and are distributed by migratory birds (Beike *et al.* 2014), a mode of dispersal that has been proven for bryophyte diaspores (Lewis *et al.* 2014).

All taxa with stego-or cleistocarpous sporophytes, like *F. curviseta*, *Physcomitrella* (*sensu lato*) or *A. serratum*, contain only one *BRK1* copy. *P. patens* is in fact a polyploid species that underwent two whole-genome duplication events (Rensing *et al.* 2007, 2008, Lang *et al.* 2018). Because duplicated chromosomal regions are either retained or lost after duplication (Rensing 2014, Van de Peer *et al.* 2017), the lack of multiple *BRK1* homeologs can obviously not rule out an allopolyploid origin of a species. Alternatively, identical homeologs of a single marker might also conceal signs of polyploidization. The E-P species complex evolved during the last 28 million years (Medina *et al.* 2018). The two whole-genome duplications found in the *P. patens* genome were dated to 27–35 and 40–48 Mya (Lang *et al.* 2018). Transcriptome-based analyses of whole genome duplication events suggest that *Physcomitrium* and *Encalypta* share both events with *P. patens* (Lang *et al.* 2018). Whether and which of the whole genome duplication events visible in the *P. patens* genome are shared by all members of the E-P species complex requires genome-wide analyses from a broad taxon set. Genome duplication followed by the reduction of chromosomal regions is a key evolutionary principle, probably also applicable to *Entosthodon*, *Physcomitrium* and *Physcomitrella* (and other cleisto-or stegocarpous taxa). Hence, progressive reduction of Funariaceae sporophyte complexity likely results from genomic and morphological reduction after polyploidization.

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Data Availability Statement

Nucleic acid sequences are available in Genbank (KY660687, KY660688, KY660689, KY660690, KY660691, KY660692, KY660693, KY660694, KY660695, KY660696, KY660697, KY660698, KY660699, KY660700, KY660701, KY660702, KY660703, KY660704, KY660705).

The remaining data generated or analysed during this study are included in this published article and its supplementary information files (Supplemental Tables S1, S2, S3).

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SUPPLEMENTAL TABLE S1. Taxon Set

SUPPLEMENTAL TABLE S2. Flow cytometry of *Entosthodon hungaricus* in comparison to *Physcomitrella patens*

SUPPLEMENTAL TABLE S3. Phylogenetic independent contrasts calculated from the continuous characters seta length, spore size and chromosome number