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ORIGINAL ARTICLE

Morphological characters and SNP markers suggest hybridization and introgression in sympatric populations of the pleurocarpous mosses *Homalothecium lutescens* and *H. sericeum*

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Abstract

Hybridization in bryophytes involves a fusion of gametes produced by haploid parental gametophytes of different species. The primary hybrid is thus the short-lived diploid sporophyte, which soon undergoes meiosis prior to the formation of large amounts of haploid spores. We compared morphology of gametophytes (branch leaves) and sporophytes (capsule inclination) from sympatric populations and allopatric populations of *H. lutescens* and *H. sericeum*. In addition, we used transcriptome data to select 85 nuclear SNP markers that were fixed for alternative alleles in the two species. The SNPs were used to estimate the degree of hybridization in diploid sporophytes. Our study shows that gametophytes from sympatric populations display intermediate morphology in a number of leaf characters, with exception for leaf sizes, which are markedly smaller than those in allopatric populations. None of the 100 sporophytes appeared to be primary hybrids, but 33 displayed admixing—heterozygotic expression of SNP markers or mismatch of occasional markers in homozygous condition—suggesting that extensive introgression takes place in the sympatric populations. Most sporophytes with intermediate capsule inclination, initially classed as putative hybrids, did not display admixture of nuclear SNP markers. Sixty-seven percent of admixed sporophytes have predominantly nuclear SNPs typical for *H. lutescens*. Our results suggest that interspecific hybridization and bidirectional introgression are relatively common in the studied sympatric populations, giving rise to viable recombinants, but not complete mixing of the parental genomes. Our study is one of the first detailed accounts of hybridization among pleurocarpous mosses, opening for future studies of gene transfer and introgression between bryophyte lineages and its role in local adaptation and long-term evolutionary diversification.

Keywords Homoploid hybridization · Admixture · Bryophyte · Introgression · Morphology · SNP haplotype

Introduction

It has been debated for a long time how common hybridization is among bryophytes and whether it is important for evolutionary processes. There is solid evidence that hybridization via allopolyploidy is fairly common in mosses and liverworts (reviewed by Wyatt et al. 1989; Stoneburner et al. 1991; Bishler and Boisselier-Dubayle 1997; Ricca and Shaw 2010; Shaw et al. 2013). On the other hand, there are few well-documented cases of hybridization at the homoploid level and most concern peat mosses *Sphagnum* (Natcheva and Cronberg 2007a, b; Ricca and Shaw 2010). In bryophytes, the diploid sporophyte is the primary hybrid, and spores formed in the sporophyte after meiosis are recombinants of the parental genomes, which, if viable, could germinate to form a new haploid gametophyte generation. Different kinds

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of evidence have been used to indicate hybridity in bryophytes, including observations of morphologically intermediate sporophytes (Khanna 1960; Pettet 1964; Anderson and Lemmon 1972), mostly in weedy bisexual species. Such an example is the cross between *Pleuridium subulatum* (Hedw.) Lindb. and *Ditrichum pallidum* (Hedw.) Hampe.—one parent having a stegocarpous spore capsule (*P. subulatum*) and the other a cleistocarpous capsule (*D. pallidum*) producing hybrids (*Pleuriditrichum marylandicum*) having intermediate capsule (Andrews and Harmann 1959). Artificial hybridization was early attempted (von Wettstein 1928; Burgeff 1943), and more recent evidence comes from molecular techniques (reviewed by Shaw 2009). However, recombinants of hybrid origin have rarely been documented in natural populations, and it has been suggested that homoploid hybridization is rare because hybrid sporophytes in most documented cases have failed to produce viable spores (reviewed by Natcheva and Cronberg 2004). Nevertheless, many examples of hybridization may remain undetected since molecular data suggest that recombinants inherit a skewed representation of the parental genomes (Shaw 1994, 1998; Natcheva and Cronberg 2007a, b), so that they display only occasional "misplaced" morphological character rather than complete intermediacy.

Homalothecium sericeum and *H. lutescens* (Brachytheciaceae) are perennial dioicous pleurocarpous bryophytes commonly found in Europe, showing a more regional distribution in North America (Nyholm 1965; Hofmann 1998; Huttunen et al. 2008; Lieske 2010; Rosengren et al. 2016; GBIF 2018a, b). The species occur in distinct habitats with a comparatively high pH (median and range of pH is 6.5 and 5.9–7.6 for *H. sericeum* versus 7.1 and 6.2–7.9 for *H. lutescens*; Tyler and Olsson 2016); *H. sericeum* grows epiphytically on trunks of broad-leaved trees or on calcareous stone or mortar in forests or parks, whereas *H. lutescens* grows on calcareous soil, usually in open habitats such as pastures. The two species display a number of distinguishing characters in both the gametophyte and sporophyte generations (Table 1). They have similar triangular and longitudinally plicate leaves. In dry condition, *H. sericeum* is easily recognized by its short secondary branches that form curled shoots with a typical golden lustre. In comparison, the branches of *H. lutescens* are rarely curled and less densely packed. It grows in loose mats with ascending shoots, whereas *H. sericeum* grows tightly connected to its substrate. The sporophytes differ in several traits, notably by *H. sericeum* having erect spore capsules and *H. lutescens* having horizontally oriented spore capsules. A partially intermediate taxon, *H. lutescens* var. *fallax*, has been considered to have a hybrid origin by some authors (Nyholm 1965; Hofmann 1998). Both species are facultatively nannandric, meaning that fertilization by swimming male gametes can be mediated by either normal-sized males or tiny dwarf males that grow epiphytically on females. An experimental study revealed that spores of both species germinate equally well on females of *H. lutescens* and form fertile dwarf males, suggesting an obvious route for hybridization (Rosengren and Cronberg 2015).

The purpose of this study is to test the hypothesis that *H. lutescens* and *H. sericeum* hybridize in mixed (sympatric) populations. Such populations occur on the Baltic island of Öland where Lars Hedenäs and Frida Rosengren (personal communications) have observed aberrant gametophytic morphotypes, proposed to be of hybrid origin. We compare morphological expression of leaf characters (gametophyte) and capsule (sporophyte) inclination between allopatric and sympatric populations. We also use 85 specifically selected SNP markers, with alternative alleles diagnostic for the two parental species, to identify hybrid sporophytes in sympatric populations. Based on earlier studies of hybrid sporophytes in other groups of bryophytes, we expected the intermediate spore capsules in *Homalothecium* to indicate primary hybridization, which means that they would show heterozygotic expression of species-specific SNPs markers.

Materials and methods

Sources of material Allopatric and sympatric populations were collected from different localities in south Sweden, including Öland and the mainland region of Skåne (Table 2). Populations from Öland were collected from the Great Alvar, a steppe-like and open area with a mosaic of base-rich and weakly acidic soils overlying the limestone bedrock (Königsson 1968; Bengtsson et al. 1988; Runyeon-Lager and Prentice 2000) or from the adjacent area with deciduous forest called Mittlandsskogen. The majority of the localities of Skåne were old, abandoned gravel or chalk pits (for *H. lutescens*, but not for *H. sericeum*), or artificial habitats created by remnants from such industrial activities (Rosengren 2015).

Sampling Most of the material was sampled from Öland in November 2014, along line transects, each sample consisted of a gametophyte colony with mostly several sporophytes, separated by a minimum of two metres to avoid double sampling of the same clone. The sampling sites represented (1) sympatric populations (Hb1–7) sampled where the two species grow in close vicinity and intermediate capsules were observed along calcareous stone walls; (2) allopatric populations of *H. lutescens* (Hl 1–2) collected from the ground at a calcareous sand steppe area and at a border area between the Great Alvar and the Midland forest; and (3) allopatric populations of *H. sericeum* (Hs 1–3) collected on the top of calcareous stone walls belonging to three different churchyards. The samples from the sympatric populations were deliberately subjective in that we actively chose to include colonies that had somewhat abnormal appearance in shoot architecture and

Table 1 Comparison of morphological characters of *H. lutescens* and *H. sericeum* (Hofmann 1998) and *H. lutescens* var. *fallax* (Nyholm 1965)—a variety suspected to be a hybrid between *H. lutescens* and *H. sericeum* by Hofmann (1998)

Stage	Characters	*H. sericeum*	*H. lutescens* var. *fallax*	*H. lutescens*
Gametophyte	Size in general	Very small to robust, usually medium-sized	Robust like *H. sericeum*	Medium-sized to robust
	Main shoots	Green or golden green, grow closely appressed to substrate		Bright yellow to yellowish-green
	Branch	**Straight but curved when dry**	**Straight**	**Mostly straight**
	Branching	**Regularly and pinnately branched**		**Irregularly branched**
	Stem leaves	Triangular, often from a broadly ovate base abruptly attenuate to a narrow apex, 1.3–2.3 mm long, 0.5–0.9 mm wide		Triangular, similar to branch leaves, 2.3–3.5 mm long, 0.6–0.9 mm wide
	Branch leaves*	Appressed when dry and erectopatent when moist, triangular to lanceolate-triangular, 0.8–2.5 mm long, 0.2–0.7 mm wide		Loosely appressed when dry, erectopatent when moist, triangular to lanceolate or ovate-triangular, 2.0–3.2 mm long, 0.4–0.9 mm wide
	a. Leaf apex*	Acuminate, rarely long-filiform		Acuminate, often twisted
	b. Leaf base*	Slightly cordate with 4–6 decurrent cells		Slightly cordate with 2–4 decurrent cells
	c. Leaf margin*	Conspicuously denticulate at base, often with recurved teeth, only slightly denticulate towards apex, irregularly recurved	Less denticulate and shorter teeth (at base) than *H. lutescens*	Denticulate throughout, particularly at base and apex, irregularly recurved from base to below apex
	e. Costa	Extending 60–85% way up the leaf, sometimes ending in a spine	Similar to *H. sericeum*, sometimes ending in a spine	Extending 60–90% way up the leaf, frequently ending in a spine
	f. Median leaf cells*	Thin-walled and eporose, 50–100 μm long, 3.4–7.1 μm wide		Thin-walled and eporose, 35–90 μm long, 4.0–6.1 μm wide
	g. Cells at leaf insertion	Shortly rectangular, slightly incrassate, and usually eporose		Rectangular to shortly linear, incrassate, porose
	h. Alar cells*	Irregular in shape, isodiametric to shortly rectangular, slightly incrassate, forming a more or less distinct group, outline of cells not clearly visible		Irregular in shape, rectangular to quadrate, incrassate, frequently porose, forming a more or less distinct group, outline of cells not clearly visible
Sporophyte	Seta	Reddish-brown, rough, 1.0–2.0 cm long, when dry dextrorse		Orange to reddish-brown, rough throughout, rarely smooth above and rough below, 1.0–3.2 cm long, when dry often sinistrorse in upper part, extrorse below or throughout
	Capsule*	Erect, cylindrical, **more or less straight, rarely somewhat curved** below mouth, 2.2–3.2 mm long, 0.7–1.0 mm wide	**Almost erect**	Slightly inclined, more or less cylindrical to asymmetrical, **curved, rarely almost straight**, 1,9–3.0 mm long, 0.7–1.0 mm wide
	a. Annulus	–		Consisting of 2–3 cell rows
	b. Exothecial cells	Rectangular to isodiametric, 25–70 μm long, 12–35 μm wide, below mouth 2–3 cell rows		Rectangular to isodiametric or quadrate, 13–50 μm long, 10–25 μm wide, below mouth 1–3 rows of smaller cells
	c. Calyptra	Naked		Naked
	d. Operculum	Shortly rostrate to high conical, 0.8–1.0 mm long	Shorter than the type of *H. lutescens*	High conical to rostrate, ca 1.0 mm long
	e. Exostome	Yellowish-brown to hyaline above, ca 0.6 mm long, cross-striolate below, papillose above, transition zone intermediate, hyaline border narrow, vanishing at about two thirds of the length of teeth		Orange-brown to yellowish-orange below, yellowish-brown to hyaline above, 0.5–0.7 mm long, cross-striolate below, papillose above, transition zone intermediate, hyaline border very narrow, incomplete, best developed in middle part of teeth

Table 1 (continued)

Stage	Characters	*H. sericeum*	*H. lutescens* var. *fallax*	*H. lutescens*
	f. Endostome	Reduced, half as long as exostome or shorter, slightly papillose, without perforations; cilia lacking; basal membrane **two-thirds to three quarter of endostome height**, papillose		Segments as long as exostome teeth, papillose, perforate; *cilia* 1–2, short; basal membrane **one-third to half the height** of endostome, finely papillose
	g. Spores	8–22 μm, finely papillose		12–18 μm, finely papillose to almost smooth

The distinguishing characters between the two species are written in bold and characters used in our study are marked with asterisk (*)

branching pattern; no such colonies occurred in the allopatric populations. Additional material was later collected from allopatric populations in Skåne, to represent a reference area in which hybridization is less likely since sympatric populations are rare. Samples for each of these sites consisted of a more limited number of colonies from separate spots (i.e. not sampled along transects). Samples of *H. sericeum* came from tree trunks (Hs 4) and various kinds of stone walls (Hs 5–7), whereas *H. lutescens* came from the ground in former limestone quarries or gravel pits (Hl 3–6). Material for SNP marker extraction and selection (see below) was also collected from allopatric populations in Skåne. Gametophyte shoots with sporophytes were brought to lab for morphological studies and DNA extraction, and the remaining material of all samples was air-dried and preserved as voucher specimens. All voucher specimens are deposited at the Botanical Museum, Lund University, and are obtainable on request.

Morphological study: Leaf measurements From each female shoot, about 10 branch leaves (c. 2.5 cm from shoot tip) were dissected and mounted on a glass slide, from which a single complete representative leaf was selected to be photographed under a dissecting microscope (i.e., the whole leaf) and under a compound microscope (i.e., leaf cells). Images of leaves (and capsules, see below) were captured by a Nikon DS-2Mv digital camera and images of leaf cells were taken by a Nikon DS-Fi1 digital camera. A suite of 14 measured characters and 4 derived characters were scored for branch leaves (Fig. 1 and Table 3). In total, 240 samples were measured: 59 samples of *H. sericeum* from allopatric populations, 60 samples of *H. lutescens* from allopatric populations, and 121 samples from sympatric populations. All leaf image measurements were made using the Measure tool in the imaging software package NIS-Elements AR 3.0. **Capsule measurements**. Images of sporophytes were used to score capsule orientation in relation to the seta (inclination) by using the Angle tool in ImageJ (Schneider et al. 2012) (Fig. 2). Unopened ripe capsules characterized by dark green- to brown-coloured capsules with opercula but no calyptra were scored from the same individuals used for gametophyte morphology, but some individuals lacked mature sporophytes, and in some cases two spore capsules were measured from the same colony. In total, 213 living capsule samples—74 samples representing allopatric populations of *H. sericeum*, 25 samples from allopatric populations of *H. lutescens* and 113 samples from the seven sympatric populations—were measured and characterized. **Statistical analyses**. (1) The 14 morphological leaf characters were tested for normality using the Shapiro-Wilk test and equality of variance using one-way ANOVA with Student's *t* test for pairwise comparisons (ggplot2 package; Wickham 2009) in RStudio version 3.3.1 (RStudio Team 2016). All samples from one region were treated as a regional population. (2) A Pearson correlation test was used to display the relationship between leaf length (mm) and width (mm) in all leaf specimens from the three origins—allopatric populations of *H. lutescens*, allopatric populations of *H. sericeum*, and sympatric populations. (3) Patterns of variation in morphological characters were analysed using principal component analysis (PCA) on the 14 leaf characters using the R packages "FactoMineR" (Lê et al. 2008) and "Factoextra" (Kassambara 2017). (4) The inclination of capsules from the three origins—allopatric populations of *H. lutescens*, allopatric populations of *H. sericeum*, and sympatric populations—were tested for equality of variance by a one-way ANOVA followed by the Tukey test for the significance of differences between the means of capsule inclination. All analyses were performed in RStudio.

Genetic study: SNP marker extraction and selection We assessed the genetic relationships and degrees of genomic admixture of the two putative parental species in sporophytes using single nucleotide polymorphism (SNP) markers derived from transcriptomic data. Gametophyte shoots with sporophytes were collected from morphologically distinct *H. lutescens* and *H. sericeum* from allopatric populations in Skåne (see Table 2). The gametophyte shoots and sporophytes were acclimatized in the constant cold room (ca. 12 °C) for 3 days before RNA extraction. Total RNA was extracted by using the RNeasy Plant Mini Kit (Qiagen) with liquid nitrogen following the protocol used by Rosengren (2015). To get a high number of expressed genes, approximately 20 gametophyte and 20 sporophyte samples were separately pooled for

Table 2 The study sites of parental and putative hybrid populations

Population	Locality	Geographic coordinates (WGS84)	Habitat	Sample size		Date of sampling
				Shoot	Capsule	
Sympatric populations						
Hb1	Station Linné (Öland)	N 56° 37′ 08″ E 16° 29′ 52″	Limestone wall	24[d,f]	24[e]	3 Nov 2014
Hb2	Arontorp (Öland)	N 56° 38′ 42″ E 16° 33′ 22″	Limestone wall	38[d,f]	36[e]	4 Nov 2014
Hb3	Arontorp (Öland)	N 56° 38′ 37″ E 16° 36′ 02″	Limestone wall	19[d,f]	15[e]	4 Nov 2014
Hb4	Bostorp (Öland)	N 56° 38′ 16″ E 16° 35′ 03″	Limestone wall	15[d,f]	15[e]	4 Nov 2014
Hb5	Kåtorp (Öland)	N 56° 36′ 48″ E 16° 33′ 60″	Limestone wall	11[d,f]	11[e]	5 Nov 2014
Hb6	Lenstad (Öland)	N 56° 36′ 40″ E 16° 34′ 59″	Limestone wall	12[d,f]	12[e]	5 Nov 2014
Hb7	Gunnarstorp (Öland)	N 56° 36′ 44″ E 16° 34′ 27″	Limestone wall	2[d,f]	0[a]	5 Nov 2014
			Total	**121**	**113**	
Allopatric populations *H. lutescens*						
Hl1	Gårdby Sandhed (Öland)	N 56° 37′ 02″ E 16° 38′ 46″	Calcareous grassland	10[c,f]	0[a]	4 Nov 2014
Hl2	Lenstad (Öland)	N 56° 36′ 42″ E 16° 34′ 26″	Calcareous grassland	10[c,f]	8[a]	5 Nov 2014
Hl3	Bjärsjölagård (Skåne)	N 55° 43′ 33″ E 13° 42′ 17″	Limestone quarry	10[b,f]	6[a],10[b]	10 Sep 2014
Hl4	The peninsula Klagshamn (Skåne)	N 55° 31′ 18″ E 12° 54′ 10″	Calcareous grassland	10[b,f]	6[a],10[b]	10 Sep 2014
Hl5	Käglinge (Skåne)	N 55° 32′ 06″ E 13° 04′ 19″	Calcareous grassland	10[b,f]	10[b]	10 Sep 2014
Hl6	Arrie (Skåne)	N 55° 31′ 21″ E 13° 06′ 07″	Calcareous grassland	10[b,f]	10[b]	10 Sep 2014
Hl7	Östra Sönnarslöv (Skåne)	N 55° 53′ 51″ E 14° 00′ 48″	Limestone quarry	–	25[d,e]	10 Oct 2016
			Total	**60**	**85**	
H. sericeum						
Hs1	Torslunda kyrka (Öland)	N 56° 37′ 59″ E 16° 30′ 53″	Limestone wall	14[c,f]	9[a]	4 Nov 2014
Hs2	N. Möckleby kyrka (Öland)	N 56° 38′ 51″ E 16° 40′ 48″	Limestone wall	14[c,f]	4[a]	4 Nov 2014
Hs3	Gårdby kyrka (Öland)	N 56° 36′ 00″ E 16° 38′ 12″	Limestone wall	7[c,f]	6[a]	4 Nov 2014
Hs4	Dalby (Skåne)	N 55° 40′ 29″ E 13° 19′ 53″	Tree trunk	13[b,f]	10[b]	21 Feb 2015
Hs5	Övedskloster (Skåne)	N 55° 68′ 74″ E 13° 63′ 00″	Limestone wall	11[b,f]	10[b]	21 Feb 2015
Hs6	Öveds kyrka (Skåne)	N 55° 41′ 18″ E 13° 38′ 37″	Limestone wall	–	22[d,e]	17 Oct 2016
Hs7	Mölleröds slottsruin (Skåne)	N 56° 09′ 46″ E 13° 42′ 34″	Limestone wall	–	53[d,e]	10 Oct 2016
			Total	**59**	**113**	
			Grand total	**240**	**311**	

[a] No capsule inclination measurements since mature sporophytes were lacking

[b] Living gametophytes and sporophytes used for transcriptome analysis to develop SNP markers

[c] Living female gametophytes used for DNA extraction of allopatric reference populations

[d] Living female gametophytes used for DNA extraction from allopatric populations

[e] Capsules used for capsule inclination measurement and DNA extraction (only seta samples from sympatric populations)

[f] Living female gametophytes used for branch leaf morphometric study

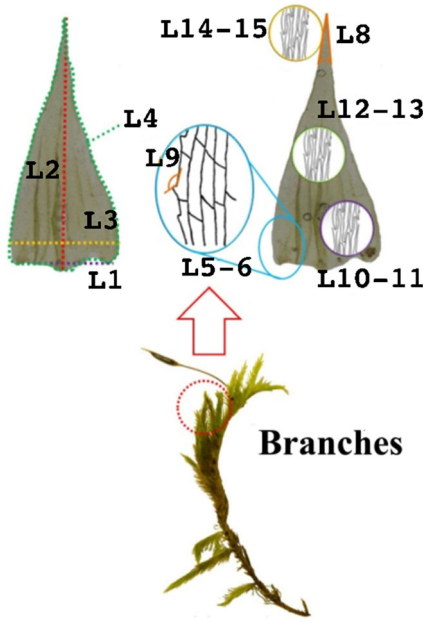


Fig. 1 Measurements of branch leaf characteristics of *Homalothecium* spp. Leaf characters (L1-L18) are explained in Table 3

construction of cDNA libraries of *H. lutescens* and *H. sericeum*. Six paired-end libraries were conducted by next-generation sequencing (NGS) on Illumina MiSeq Platform at the Sequencing Facility at the Department of Biology (Lund University). In total, we fed 34.5 megabase pairs (Mb) of raw sequences to run on a computer platform at the computational infrastructure UPPMAX, Uppsala University (http://www.uppmax.uu.se). The required programs consisted of several modules executed consecutively in an automated fashion. All scripts running in UPPMAX are provided in Supplementary materials Appendix I. We used the Trinity software (launched 2014-07-17) for de novo reconstruction of transcriptomes from RNA-seq data from gametophytes of *H. sericeum* to create a reference sequence (total no. of assembled bases: 51,692,526). After reference construction, we remapped short DNA sequences (reads) from both *H. lutescens* and *H. sericeum* to the reference using the aligner program Bowtie (version 1.1.2) and exported output alignments in the standard SAM format for SNP calling in SAMtools (version 0.1.19). Trinity, Bowtie and SAMtools were run on the command lines in UPPMAX. A final number of 79,314 contigs (total contigs created: 324,101) representing 42,481,248 reads (*H. lutescens*: 10,289,896; *H. sericeum*: 32,191,352) remained to be analysed from the remapping to the reference sequence. The SNP calling was performed by a custom made Perl-program (available upon request). In total putative 4631 SNPs with the read coverage of the SNP position at least 20, within 100 bp upstream or downstream of the targeted SNP positions, were extracted from the SAMtools. 133 SNPs which displayed species-specific variants (alleles) expressed in both the gametophyte and sporophyte generations were chosen for SNP genotyping. We blasted the contigs to predict their cellular compartmentalization (nuclear, mitochondrial or chloroplast) and to ensure that all three kinds were represented. The mitochondrial and chloroplast SNPs are expected to show maternal inheritance and therefore unlikely to show heterozygotic pattern in sporophytic tissue (setae). These plastid SNPs were primarily used to identify the maternal parent species in sporophytes (although they still would have a potential to signal hybridization if combined with alleles in other loci signalling origin from the other parent). **DNA extraction.** For allopatric populations of *H. lutescens* and *H. sericeum* (see Table 2), young gametophyte tissue (haploid) was sampled for DNA extraction using the Qiagen DNeasy Plant Minikit from the same individuals as used for the morphological study. These samples were used to check that the selected SNPs loci had alternative alleles specific for *H. lutescens* and *H. sericeum*. For samples from sympatric populations, we extracted DNA from setae from the same samples as used for capsule inclination and leaf morphology. The seta is the stalk supporting the capsule of a moss, and a part of the diploid sporophyte and therefore in principle containing a complete set of chromosomes from each parental gametophyte if hybridization occurs. The reason for not extracting also the capsule was that we planned to sow the spores to test spore germination in a separate study (Sawangproh et al. 2020; Sawangproh and Cronberg, in prep). Our initial plan was to also analyse the haplotypes of the maternal individuals, but this was not done due to a temporary problem with extraction failure (see Table 2). **SNP genotyping.** SNP genotyping was

Table 3 Branch leaf characters and scale units in morphometric analyses of two parental species, *H. sericeum* and *H. lutescens* from allopatric and sympatric populations

Leaf character number	Character
	Vegetative leaves of female shoot
L1	Width at base of leaf lamina (mm)
L2	Length of leaf lamina (mm)
L3	Width of leaf lamina (mm)
L4	Leaf surface area (mm^2)
L5	Tooth length at base of leaf lamina (μm)
L6	Tooth number at base of leaf lamina
L7	Ratio of leaf lamina length to width
L8	Lamina apical angle (degree)
L9	Tooth angle (degree)
	Leaf cells
L10	Length of basal leaf cell (μm)
L11	Width of basal leaf cell (μm)
L12	Length of median leaf cell (μm)
L13	Width of median leaf cell (μm)
L14	Length of apical leaf cell (μm)
L15	Width of apical leaf cell (μm)
L16	Ratio of basal leaf cell length to width
L17	Ratio of median leaf cell length to width
L18	Ratio of apical leaf cell length to width

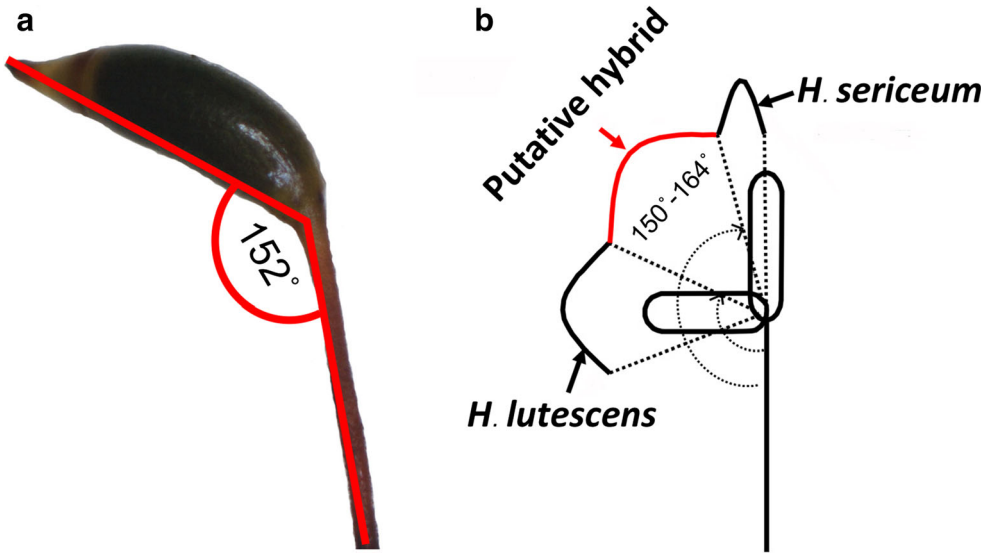


Fig. 2 (**a**) Measurement of capsule orientation in relation to the seta in *Homalothecium* as the angle (in degrees) between the seta and spore capsule at the basis of the spore capsule. (**b**) Capsule inclinations of individuals from the allopatric populations fell into the black ranges of variation; in the sympatric populations individuals occurred with capsule inclinations ranging outside typical capsule inclinations of the pure species (red zone: 150°-164°), indicating hybrid origin

performed on DNA samples of gametophytes and setae at SciLifeLab Uppsala with the multiplexed primer extension (SBE) chemistry of the iPLEX assay. The amount of DNA was measured by PicoGreen method by SciLifeLab prior to the analyses. The mean (±SD) DNA concentrations of extracts from gametophyte and seta samples were 1.79 ± 1.81 ng/μl and 0.26 ± 0.38 ng/μl, respectively. The preferred total DNA amount per sample in the analysis was 20 ng (5 ng of DNA added into the analysis of each of the four multiplex SNP panels). Since there was at least 20 μl of extract in microtubes, the amounts were usually enough for the analysis, although the DNA concentration varied a lot among samples. In some cases a lower amount was successfully used, although not all loci gave detectable signal.

Results

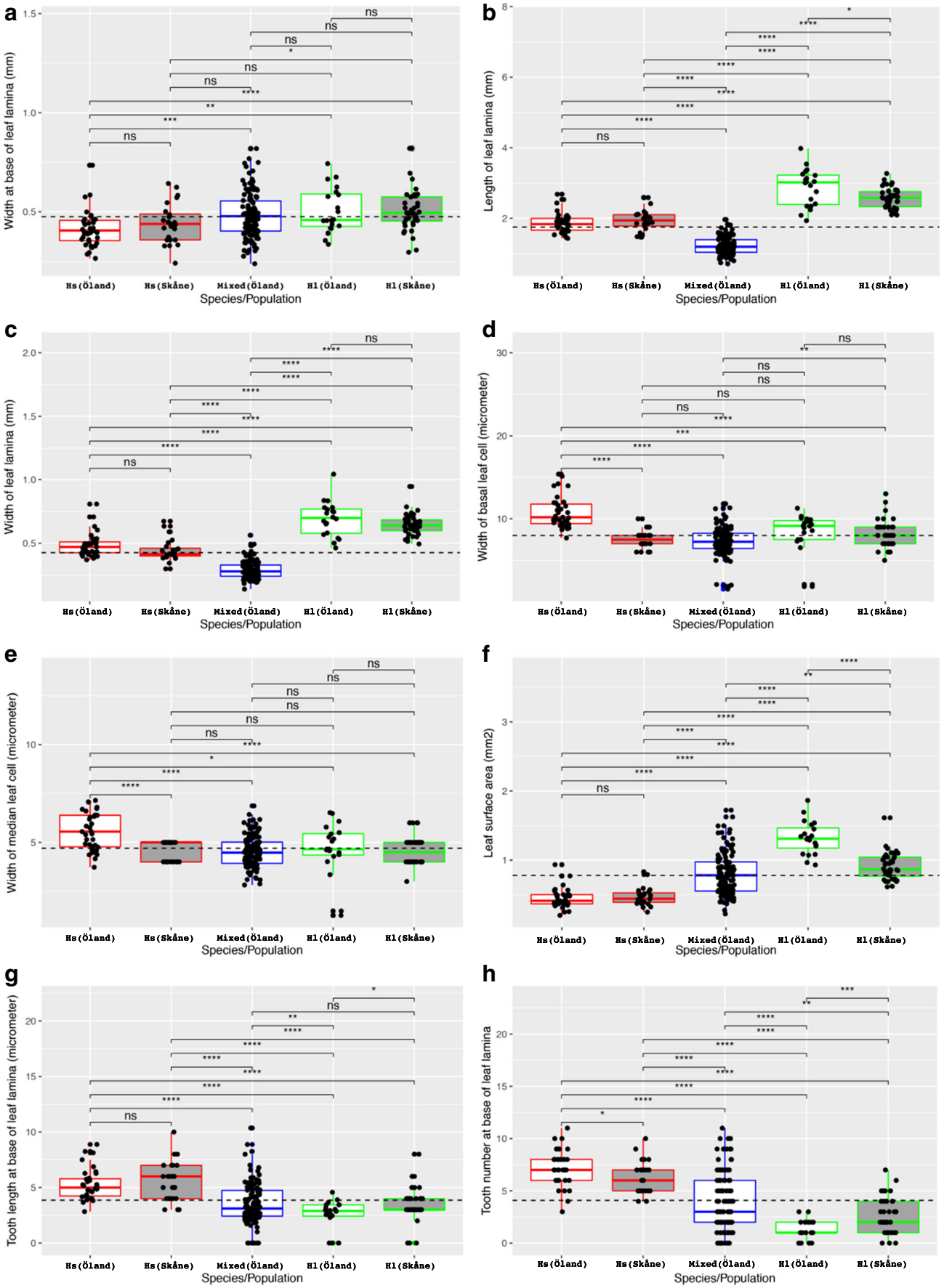
Leaf morphology

Results indicated that statistical techniques based on normality assumptions could be used for most morphological characters in our study of leaf morphology. Most of the leaf characters examined for the allopatric populations of *H. lutescens* and *H. sericeum* are similar within species; some leaf characters slightly differ between the two regional populations, i.e. Öland and Skåne, for example, width of leaf at base and width of median cell; and number of teeth at base is significantly larger for allopatric populations of *H. sericeum* from Öland than Skåne. Gametophytes from sympatric populations showed more or less clear intermediacy in several leaf characters such as width at base of leaf lamina, leaf surface area, tooth length at base of leaf lamina, and tooth number at base of leaf lamina (Fig. 3a, f, g, and h, respectively), whereas other characters fell out of range of both species, exemplified by the ratio between leaf length and width (Fig. 3b and c; Fig. 4). As for the general leaf dimensions, specimens from allopatric populations of *H. lutescens* had the longest and widest leaves, followed in size by specimens from allopatric populations of *H. sericeum*, whereas the sympatric population had the shortest and narrowest leaves (Fig. 4). All fourteen quantitative leaf characters for each allopatric and sympatric populations were showed in Table S1.

The PCA based on the leaf morphology data set reveals that 48.54% of the observed variation is explained by the first three factors (PC1 = 19.56%, mostly summarizing variation in general leaf size and traits related to leaf tooth; PC2 = 16.45% mostly reflecting width of leaf cells in different parts of the leaf; PC3 = 12.43% mostly determined by leaf size and length of apical leaf cells; Table 4). A plot of individual component scores along the first principal component (*x*-axis) and the second principal component (y-axis) (Fig. 5) showed almost complete separation of allopatric populations of *H. lutescens* and *H. sericeum* with individuals from the putative hybrid populations somewhat overlapping with the two clusters from allopatric populations, mostly filling the intermediate space between the allopatric populations.

Capsule inclination

In the allopatric populations, the capsules of *H. sericeum* were almost erect (152–180 degrees) whereas the capsules of *H. lutescens* were markedly inclined (125–154 degrees), in accordance with the generally accepted species characteristics. The capsule specimens from the sympatric populations, however, showed a wider range of variation in capsule inclination from strongly inclined to intermediate to erect (122–180°). The mean of capsule inclinations from the three different populations were significantly different (Table 5). The capsule inclinations falling between 150 and 160° were out of the


a
Width at base of leaf lamina (mm)
b
Length of leaf lamina (mm)
c
Width of leaf lamina (mm)
d
Width of basal leaf cell (micrometer)
e
Width of median leaf cell (micrometer)
f
Leaf surface area (mm2)
g
Tooth length at base of leaf lamina (micrometer)
h
Tooth number at base of leaf lamina
Hs(Öland)
Hs(Skåne)
Mixed(Öland)
Hl(Öland)
Hl(Skåne)
Species/Population

◀ **Fig. 3** Box plot of width at base of leaf lamina (mm) (**a**), box plot of length of leaf lamina (mm) (**b**), box plot of width of leaf lamina (mm) **(c),** box plot of width of basal leaf cell (μm) (**d**), box plot of width of median leaf cell (μm) (**e**), box plot of leaf surface area (mm^2) (**f**), box plot of tooth length at base of leaf lamina (μm) (**g**) and box plot of tooth number at base of leaf lamina (**h**) in allopatric regional populations of *Homalothecium sericeum* and *H. lutescens* and sympatric regional population. Red outline box plot = allopatric regional population of *H. sericeum*; blue outline box plot = sympatric regional populations; green outline box plot = allopatric regional population of *H. lutescens*; white-filled box plot = populations from Öland and grey-filled box plots = population from Skåne. Student's *t* test for pairwise comparisons (ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$)

capsule inclination range of the allopatric populations of both species and thus indicative of a hybrid origin (Table 5). Out of 101 capsule samples with leaf data from the sympatric populations, 44 capsules were *H. sericeum*-like (43%), 31 capsules were *H. lutescens*-like (31%), and 26 capsules were intermediate (26%). In the sympatric populations of *H. lutescens* and *H. sericeum* 12 out of 26 putative hybrid capsules fell into the morphospace of *H. lutescens* (46%), 4 into the morphospace of *H. sericeum* (15%), 9 into the overlapping morphospace of *H. lutescens* and *H. sericeum* (35%), and 1 outside the morphospace of both *H. lutescens* and *H. sericeum* (4%) (Fig. 6).

SNP variation

In total, 111 SNPs (out of 133 SNPs) distributed across 90 contigs were successfully genotyped. Out of 98 nuclear SNPs, 85 turned out to possess species-specific alleles for either *H. lutescens* or *H. sericeum*, and the remaining 16 were polymorphic in sympatric populations of one or the other species, or in one case, both. The 85 species-specific SNPs gametophytes were selected in our analysis to indicate hybrid influence ("admixture") of sporophytes in sympatric populations (Table 6). Admixture was indicated in sporophytes by (1) a presence of species-specific homozygous SNP expression representing both parental species in the same seta but in different loci (only seen in locus 31, 51, 70, and 80) and (2) heterozygous nuclear SNP expression in one or more loci (i.e. having species-specific alleles for both parental species; only occurring in locus 31, 51, 70 and 80). Analysis of 85 SNP markers in 100 seta samples (13 DNA samples failed to

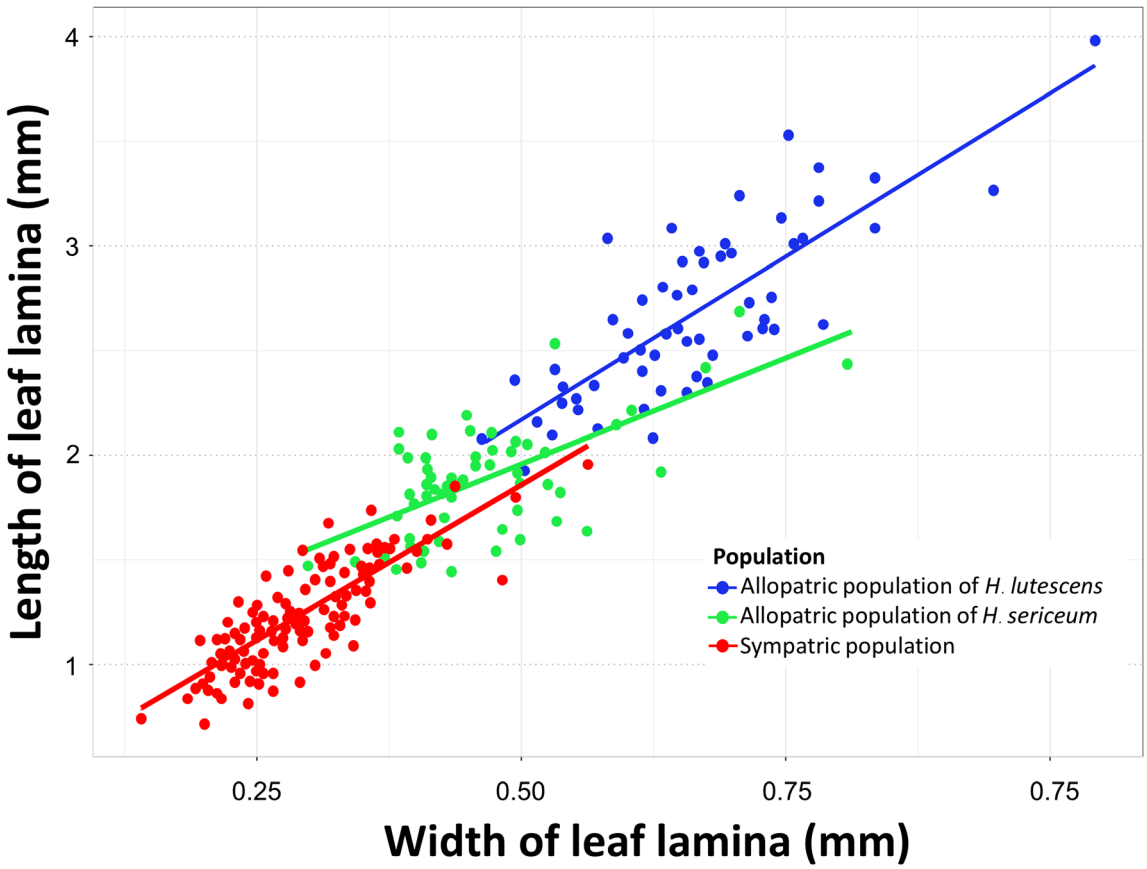


Fig. 4 Relationship between width and length of leaf lamina of *Homalothecium* leaf specimens ($N = 240$) collected from the allopatric populations of *H. lutescens* ($N = 60$) and *H. sericeum* ($N = 59$) and the sympatric population ($N = 121$). The regression line of data points from each population is also displayed

Table 4 Factor loadings for the first three principal axes of a PCA based on leaf morphology of *Homalothecium lutescens* and *H. sericeum* from allopatric and sympatric populations

Character	Factor loadings		
	PC1	PC2	PC3
Width at base of leaf lamina (mm)	**0.521**	−0.138	0.373
Length of leaf lamina (mm)	0.450	0.104	**0.766**
Width of leaf lamina (mm)	**0.546**	0.207	**0.719**
Ratio of leaf lamina length to width	−0.459	−0.386	−0.037
Length of basal leaf cell (μm)	0.399	0.259	−0.198
Width of basal leaf cell (μm)	−0.128	**0.731**	0.239
Ratio of basal leaf cell length to width	0.480	−0.437	−0.378
Length of median leaf cell (μm)	−0.351	−0.147	0.189
Width of median leaf cell (μm)	−0.089	**0.753**	0.000
Ratio of median leaf cell length to width	−0.182	**−0.733**	0.167
Length of apical leaf cell (μm)	**−0.538**	0.028	**0.402**
Width of apical leaf cell (μm)	0.093	**0.538**	−0.114
Ratio of apical leaf cell length to width	**−0.535**	−0.321	**0.415**
Lamina apical angle (degree)	0.462	0.390	−0.326
Leaf surface area (mm^2)	**0.667**	−0.323	0.382
Tooth length at base of leaf lamina (μm)	**−0.549**	0.277	0.237
Tooth angle (degree)	−0.098	0.057	−0.162
Tooth number at base of leaf lamina	**−0.642**	0.304	0.158
Eigenvalue	3.52	2.96	2.25
Cumulative variance (%)	19.56	36.01	48.54

The characters that have high scores are written in bold

produce SNP genotypes) indicated 53 sporophytes as pure *H. lutescens*, 14 sporophytes as pure *H. sericeum*, and 33 as admixed sporophytes (only putative hybrid sporophytes with intermediate capsule inclination and/or admixed sporophytes inferred by SNP markers are shown in Table 7**)**. Thus, no sporophytes appeared to be primary hybrids because heterozygotic expression was rare. The results, rather, suggest secondary back-crossing or other kinds of introgression (admixing). Overall, admixed sporophytes showed biased SNP markers towards either *H. lutescens* (22 sporophytes = 67%) or *H. sericeum* (11 sporophytes = 33%). The positions of SNP-inferred admixed sporophytes superimposed on leaf sample of the associated female gametophyte ordination are shown in Fig. 6.

Discussion

The results from our analyses show a clear signature of hybridization and introgression in sympatric populations of the two *Homalothecium* species in traits of gametophytes (branch leaves) and sporophytes (capsule inclination) as well as genetic markers (SNP genotypes). These three indicators of hybridization show a non-overlapping pattern, with individuals displaying hybrid character states in one or two indicators, rather than all three. We believe that the non-overlapping pattern of the three indicators of hybridization is explained by extensive introgression and in the following we will discuss this conclusion, what assumptions and limitations are found in the data sets and how the results fit into a wider context.

Morphological evidence of hybridization

Homalothecium lutescens and *H. sericeum* are sometimes considered difficult to separate (Lieske 2010) since they are closely related and share some common morphological characters (Ignatov and Huttunen 2002). Our results show that allopatric populations of *H. lutescens* and *H. sericeum* are morphologically well differentiated, as demonstrated by the PCA (Fig. 5), which confirms the characters that have traditionally been used for species identification. For example, *H. sericeum* is conspicuously toothed at leaf basal margin and produces more or less straight capsules, whereas *H. lutescens* is denticulate at leaf basal margin and produces curved capsules (Nyholm 1965; Hofmann 1998). In contrast, gametophytes from putatively hybridizing sympatric populations showed more or less intermediate character states for several leaf characters such as width at base of leaf lamina, width of basal leaf cell, width of median leaf cell, leaf surface area, tooth length at base of leaf lamina, and tooth number at base of leaf lamina (Fig. 3). Notably, the general leaf shape was relatively shorter and narrower in the sympatric populations as compared with any of the allopatric populations (Fig. 4). It could be argued that the morphological expression in our leaf samples represents the wild phenotypes (without prior common garden culturing) and consequently that the phenotypes from the sympatric populations result from some specific environmental conditions, rather than hybridization. However, we want to point out that the morphology of each species, collected in allopatric populations of environmentally different regions such as in Öland and Skåne, does not differ substantially, suggesting that the studied branch-leaf traits are largely species-specific rather than environmentally controlled. Thus, we have reason to believe that the morphological analysis of branch leaves provides evidence for hybridization between *H. lutescens* and *H. sericeum* in sympatric populations.

Intermediate spore capsules have been used to infer hybridization in bryophytes and primary hybrids are expected to display characters of both parental species. In our study, we focussed on the inclination of capsules, as this is a major distinguishing character between the species, *H. lutescens* having inclined capsules and *H. sericeum* having erect capsules (Hofmann 1998). *H. sericeum* grows mainly on walls, rocks and tree trunks,

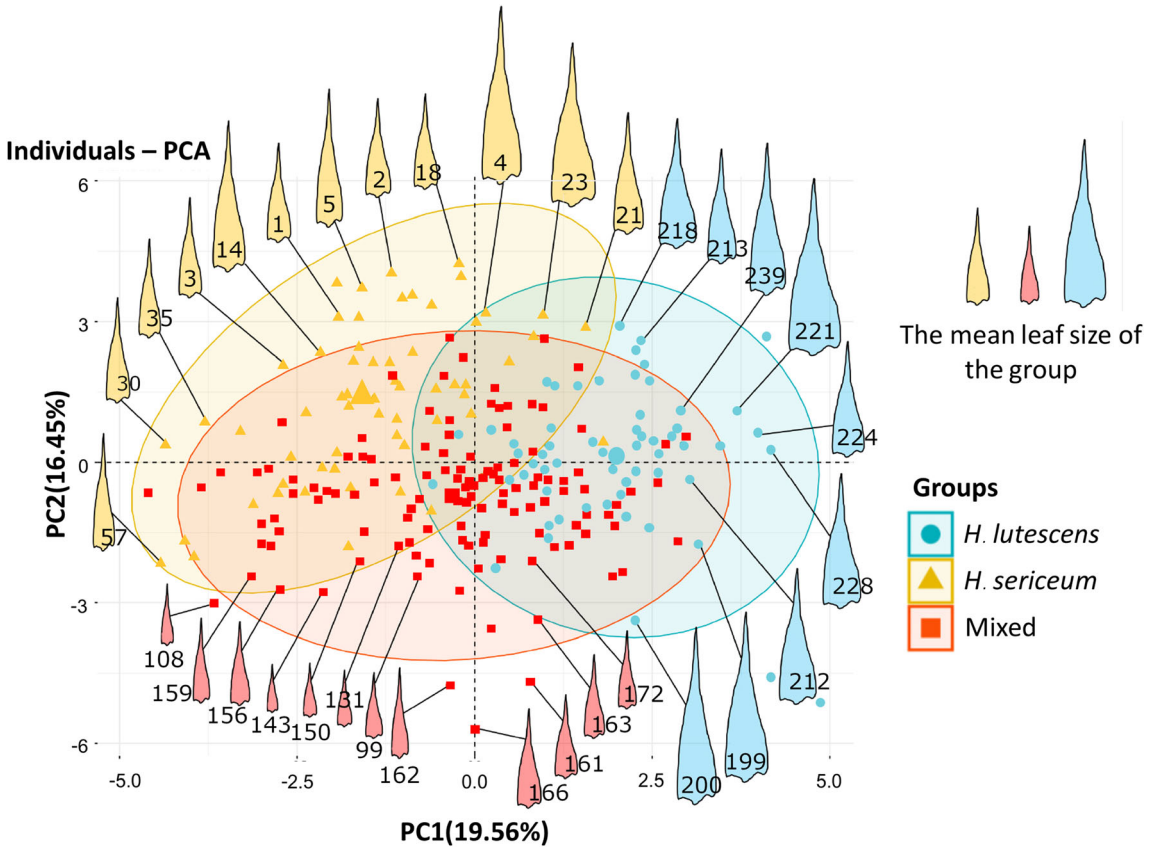


Fig. 5 Principal component analysis of 14 leaf characters from 240 specimens representing allopatric and sympatric populations of *Homalothecium lutescens* and *H. sericeum*. The first two axes (PC1 and PC2) representing together 36% of variation are shown. The colours and shapes of data points correspond to the population of the specimens. Leaf shapes are exemplified for selected leaves (with sample ID no). The encircled surfaces represent 95% confidence level of the sample means with the centre of each cluster marked by a larger symbol. Factor loadings are presented in Table 4

whereas *H. lutescens* grows mainly on soil and sand but rarely epiphytically (Hofmann 1998). The contrasting capsule orientation in the two species is therefore likely explained as a consequence of selection pressures imposed by their particular habitat preferences. Hedenäs (2001) showed that erect and straight capsules types are overrepresented among epiphytes, whereas curved and inclined capsule types are more common among species

Table 5 Capsule inclination of specimens from allopatric and sympatric populations of *H. lutescens* and *H. sericeum* collected in Sweden

Population	Capsule inclination (degrees)		
	H. lutescens (allopatric)	Mixed (sympatric)	*H. sericeum* (allopatric)
N	25	113	75
Min-Max	125–154	122–180	152–180
Mean ± SD	143 ± 7	158 ± 14	171 ± 7
Lower limit	143–7 = 136	**150–164** (putative capsule)	**171–7 = 164**
Upper limit	**143 + 7 = 150**		171 + 7 = 178
One-way ANOVA	$F_{2,210} = 64$, $P < 0.001$		
Tukey multiple comparisons	A	B	C

Data met normality test (Shapiro-Wilk normality test: W = 0.981, *P* value = 0.006). One-way ANOVAs with Tukey multiple comparisons; Tukey multiple comparisons of means shown by the different letters are significantly different at $P < 0.05$

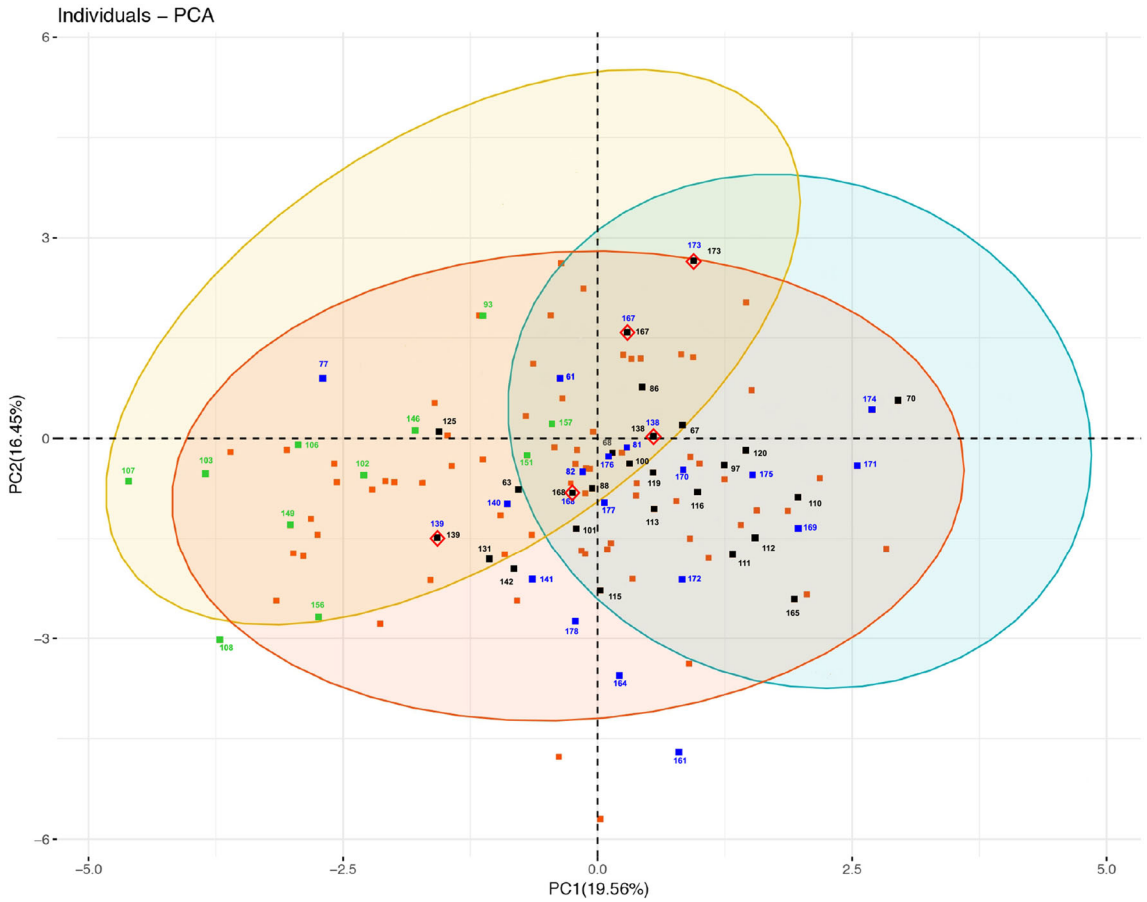


Fig. 6 The positions of putatively hybrid sporophytes from the sympatric populations of *H. lutescens* and *H. sericeum* superimposed in the PCA from Fig. 5, based on leaf morphology of the maternal gametophytes. The morphospace of leaves from allopatric populations of *H. lutescens* and *H. sericeum* are shown as encircled surfaces (blue circle = *H. lutescens* and yellow circle = *H. sericeum*). The red circle represents the morphospace of individuals from the sympatric populations. Each square represents a hybrid sporophyte specimen, collected on its corresponding gametophyte (sample ID number). The colours of the square corresponds to different hybrid indications, i.e. hybrid capsules inferred by intermediate capsule inclination (■), hybrid capsules inferred by SNP markers but SNPs mainly typical for either *H. lutescens* (■) or *H. sericeum* (■), hybrid capsules inferred by both intermediate capsule inclination and SNP markers but SNPs mainly typical for *H. lutescens* (◆)

growing on the ground. A probable explanation is that spore dispersal is most efficient at a horizontal capsule orientation—for epiphytic species with a straight capsule growing on a vertical substrate, the capsule orientation

Table 6 Frequency of polymorphism of different forms within and between SNPs of gametophyte individuals of *H. lutescens* and *H. sericeum*

Polymorphism type	SNPs	Locus
A. Nuclear SNP markers		
1) Not alternatively fixed SNPs	26	
SNP polymorphic in *H. lutescens* but one allele fixed in *H. sericeum*	5	18,30,48,55,78
SNP polymorphic in *H. sericeum* but one allele fixed in *H. lutescens*	20	20,21,22,23,35,40,45,52,61,64, 68,73,74,77,81,82,90,93,94,96
H. lutescens and *H. sericeum* share polymorphism	1	79
2) Alternatively fixed SNPs[§]	85	1–17, 19, 24–29, 31–34, 36–39, 41–44, 46–47, 49–51, 53–54, 56–60, 62–63, 65–67, 69–72, 75–76, 80, 83–89, 91–92, 95, 97–111
Total	111	

[§] 85 fixed nuclear SNPs are only used for identified hybrid sporophytes

Table 7 Distribution of species-specific alleles in 85 nuclear SNP loci for 33 out of 100 sporophyte (seta) samples collected from 18 sampling points (Patch No. I-XVIII) from sympatric populations (Hb1-Hb6) that showed signs of hybrid origin by SNP markers or spore intermediate capsule inclination in sympatric *Homalothecium* populations

Pop	Sporophyte ID	Patch No[#]	SNPs (%)				Capsule inclination[§]
			Homozygous (*H. lutescens*)	Homozygous (*H. sericeum*)	Heterozygous	Missing SNPs	
Hb1	61	I	15 (18%)	1 (1%) (80)	0 (0%)	69 (81%)	Inclined
	63		16 (19%)	0 (0%)	0 (0%)	69 (81%)	Intermediate
Hb1	67	II	82 (96%)	0 (0%)	0 (0%)	3 (4%)	Intermediate
	68		81 (95%)	0 (0%)	0 (0%)	4 (5%)	Intermediate
	70		82 (96%)	0 (0%)	0 (0%)	3 (4%)	Intermediate
Hb1	77	III	61 (71%)	0 (0%)	1 (1%) (31)	23 (28%)	Erect
Hb1	81	IV	62 (72%)	0 (0%)	1 (1%) (31)	22 (27%)	Inclined
	82		62 (73%)	1 (1%) (31)	0 (0%)	22 (26%)	Inclined
Hb2	86	V	81 (95%)	0 (0%)	0 (0%)	4 (5%)	Intermediate
	88		16 (19%)	0 (0%)	0 (0%)	69 (81%)	Intermediate
Hb2	93	VI	0 (0%)	32 (38%)	1 (1%) (51)	52 (61%)	Erect
Hb2	97	VII	NA	NA	NA	NA	Intermediate
	100		61 (72%)	0 (0%)	0 (0%)	24 (28%)	Intermediate
	101		78 (93%)	0 (0%)	0 (0%)	7 (8%)	Intermediate
Hb2	102	VIII	0 (0%)	82 (97%)	1 (1%) (70)	2 (2%)	Erect
	103		0 (0%)	84 (99%)	1 (1%) (70)	0 (0%)	Erect
	106		0 (0%)	73 (86%)	1 (1%) (51)	11 (13%)	Erect
	107		0 (0%)	82 (97%)	2 (2%) (51,70)	1 (1%)	Erect
	108		0 (0%)	81 (95%)	1 (1%) (70)	3 (4%)	Erect
Hb2	110	IX	65 (76%)	0 (0%)	0 (0%)	20 (24%)	Intermediate
	111		81 (95%)	0 (0%)	0 (0%)	4 (5%)	Intermediate
	112		54 (64%)	0 (0%)	0 (0%)	31 (36%)	Intermediate
	113		84 (99%)	0 (0%)	0 (0%)	1 (1%)	Intermediate
	115		16 (19%)	0 (0%)	0 (0%)	69 (81%)	Intermediate
Hb2	116	X	83 (98%)	0 (0%)	0 (0%)	2 (2%)	Intermediate
	119		80 (94%)	0 (0%)	0 (0%)	5 (6%)	Intermediate
	120		17 (20%)	0 (0%)	0 (0%)	68 (80%)	Intermediate
Hb3	125	XII	58 (68%)	0 (0%)	0 (0%)	27 (32%)	Intermediate
	131		NA	NA	NA	NA	Intermediate
Hb3	138	XIV	83 (98%)	1 (1%) (51)	1 (1%) (31)	0 (0%)	Intermediate
	139		82 (97%)	1 (1%) (51)	1 (1%) (31)	1 (1%)	Intermediate
	140		82 (96%)	0 (0%)	1 (1%) (31)	2 (3%)	Inclined
Hb4	141	XV	84 (99%)	0 (0%)	1 (1%) (51)	0 (0%)	Inclined
	142		85 (100%)	0 (0%)	0 (0%)	0 (0%)	Intermediate
Hb4	146	XVI	0 (0%)	34 (40%)	1 (1%) (51)	50 (59%)	Erect
	149		0 (0%)	80 (94%)	1 (1%) (51)	4 (5%)	Erect
	151		0 (0%)	82 (97%)	1 (1%) (51)	2 (2%)	Erect
Hb5	156	XVII	0 (0%)	84 (99%)	1 (1%) (70)	0 (0%)	Erect
	157		0 (0%)	83 (98%)	1 (1%) (70)	1 (1%)	Erect
Hb5	161	XVIII	82 (97%)	0 (0%)	1 (1%) (31)	2 (2%)	Erect
	164		84 (99%)	0 (0%)	1 (1%) (31)	0 (0%)	NA
	165		85 (100%)	0 (0%)	0 (0%)	0 (0%)	Intermediate
Hb6	167	XIX	78 (92%)	1 (1%) (31)	0 (0%)	6 (7%)	Intermediate
	168		78 (92%)	1 (1%) (31)	0 (0%)	6 (7%)	Intermediate
	169		78 (92%)	1 (1%) (31)	0 (0%)	6 (7%)	Erect

Table 7 (continued)

Pop	Sporophyte ID	Patch No[#]	SNPs (%)				Capsule inclination[§]
			Homozygous (*H. lutescens*)	Homozygous (*H. sericeum*)	Heterozygous	Missing SNPs	
	170		14 (17%)	1 (1%) (31)	1 (1%) (80)	69 (81%)	Erect
	171		84 (99%)	1 (1%) (31)	0 (0%)	0 (0%)	Inclined
	172		83 (98%)	1 (1%) (31)	0 (0%)	1 (1%)	Erect
	173		83 (98%)	1 (1%) (31)	0 (0%)	1 (1%)	Intermediate
	174		84 (99%)	1 (1%) (31)	0 (0%)	0 (0%)	Erect
	175		84 (99%)	1 (1%) (31)	0 (0%)	0 (0%)	Erect
Hb6	176	XX	78 (92%)	2 (2%) (31,51)	0 (0%)	5 (6%)	Inclined
	177		83 (98%)	2 (2%) (31,51)	0 (0%)	0 (0%)	Erect
	178		82 (97%)	2 (2%) (31,51)	0 (0%)	1 (1%)	Inclined

Hybridization was signalled by SNPs either through heterozygotic expression in certain SNP loci or homozygotic expression of alleles representing both species, but in different loci. Numbers of SNP loci specific to *H. lutescens* or *H. sericeum* with homozygotic or heterozygotic expression are provided with the percentages in parentheses, followed by the respective SNP locus number corresponding to the locus identification number shown in Supplementary materials Appendix II

[#] Patch number = the position along transects where samples (colonies) were collected

[§] Inclined capsule = typical capsule of *H. lutescens*, Erect capsule = typical capsule of *H. sericeum*

NA = missing data

will effectively be horizontal. We confirmed the specific characteristics in allopatric populations, whereas sporophytes displayed a high variability of capsule inclination when the two species grew intermixed in sympatric populations. We see no reason other than hybridization that capsules with intermediate inclination should occur spontaneously in six separate sympatric populations.

Genetic evidence for hybridization

Before discussing the interpretation of the genetic data, it is worth noting that there is a fairly high and variable failure rate for SNP markers in sporophyte samples (see Supplementary materials Appendix II). We must ask if we can trust the markers and why there is such a high failure rate. Primarily, the failure rate for SNP markers can be explained by the low DNA concentration in an individual seta. As far as we know, nobody has tried to extract SNP haplotypes from only setae before, which contains much less tissue than complete sporophytes. We knew beforehand that we were close to the detection limit, although we used a method for SNP genotyping that required low amounts of DNA. The reason for extracting setae was that we wanted to sow out the spores in the spore capsules for germination tests (Sawangproh et al. 2020; Sawangproh and Cronberg, in prep).

It is possible that heterozygotic loci are overrepresented among the failing loci, since each SNP allele only represents half the amount of DNA as compared with the single allele in a homozygotic locus. A low signal level for individual alleles could therefore be a partial explanation for the low incidence of loci with heterozygotic expression. On the other hand, the genotype distribution among diploid sporophytes agrees well with their maternal origin, in cases where several sporophytes have been analysed from the same mother. Typically, rare allelic combinations (and even instances of detection failure) are similar or identical in such sibling sporophytes, supporting the credibility to of detected SNP variants.

Contrary to our initial expectation, the analysis of SNP genotypes in sporophytes with intermediate capsule inclination using a combination of 85 species-specific nuclear markers did not reveal primary hybridization in sporophytes from sympatric populations. Instead, we found only four nuclear SNPs (i.e. locus 31, 51, 70 and 80 from four separate nuclear sequences) out of 85 SNPs that appear to signal secondary hybridization (introgression). Taken alone, a low frequency of heterozygotes is a somewhat weak evidence for hybridization, since it could be argued that these four SNP loci may also be heterozygotic in one or the other of the parental species, although not in our allopatric reference populations. We also observed that individuals with mixed SNP markers generally fell into the leaf morphospace of the parent that had the strongest genomic representation in the SNP genotype, whereas those with both intermediate capsule inclination and mixed SNP markers more clearly fell into the intermediate morphospace (Fig. 6). Finally, sporophytes with intermediate capsule inclination were more commonly borne on gametophytes with a maternal origin from *H. lutescens*, as indicated by the SNP markers. In a subsequent larger study,

comparing hybridization across different stages in the life cycle (Sawangproh et al. 2020), we did find a small fraction of individuals (3.8%) with strongly admixed genomes suggesting primary hybridization.

Evidence from spore capsule inclination

Our study is the first, to our knowledge, to report intermediate sporophytes (capsule inclination) in bryophytes supported by genetic data analysis (SNP markers). Closely related (congeneric) species of most pleurocarps, many acrocarps, peat mosses, and almost all liverworts have similar sporophytes, and there is little chance of recognizing F_1 hybrids (hybrid sporophytes) by morphological examination of sporophytes (Shaw 1994; Hofmann 1997; Natcheva and Cronberg 2004). One exception is the closely related species pair *Orthotrichum gymnostomum* and *O. obtusifolium* in which Hedderson (1986) inferred hybridization based on the basis of intermediate characters of sporophytes, such as capsule shape, ribbing, seta length, peristome, exothecial cells and stomata. Other studies show that hybrid sporophytes do not necessarily have intermediate shapes but rather tend to be closer or identical to one or the other parent. For example, when crossing *Physcomitrium pyriforme* (Hedw.) Hampe and *Funaria hygrometrica* Hedw., the sporophyte morphology, including peristomal structure, appeared to be more similar to the maternal parent (von Wettstein 1923). Naturally occurring hybrid sporophytes among several species of *Weissia* also tended to show morphologies that were more similar to the maternal parent (Nicholson 1905, 1906), suggesting complex inheritance involving maternal effects, non-nuclear genetic factors and/or nuclear-cytoplasmic interaction (Shaw 1998). In most cases, the spores in such sporophytes have proved to be more or less completely inviable, probably because the parental species were too distantly related (Natcheva and Cronberg 2004) and therefore genomically incompatible.

In our study, intermediate sporophytes tended to occur in individuals with genomes dominated by one of the parents as indicated by SNP markers, and they are clearly not indicative of primary hybridization. In primary hybridization, we expect genes that are responsible for parental differences in sporophyte morphology to be represented by species-specific alleles in the F_1 hybrid sporophytes. In such cases, intermediacy may be visible if the traits (e.g., capsule inclination) are controlled by few genes and show co-dominance. In secondary backcrossing (introgression), the alleles that are responsible for the sporophyte morphology may be recombined in a different way. If inheritance of genes controlling sporophyte morphology is simple (involving few genes) and these genes not strongly linked to other traits, their distribution could be more or less random in a population of introgressed individuals and, as we experience in our study, decoupled from other morphological indicators of hybridization. We therefore urge against uncritical use of intermediate sporophytes as evidence for primary hybridization.

Introgression—the ghost of hybridization

It is sometimes assumed that individuals of hybrid origin should also display intermediacy in vegetative morphological characters, but this assumption may be misleading and result in failure to recognize hybridization and introgression, especially if supporting molecular data is missing (Rieseberg et al. 1993). Rieseberg (1995) suggested that non-intermediate morphology is common where past hybridization and intensive introgression occur in sympatric populations of vascular plants. Bryological studies have shown that morphology of hybrid gametophytes is more similar to either the female (more common) or the male parent (Nicholson 1910; Allen 1935, 1945; Pettet 1964; Holmen and Scotter 1971; Delgadillo 1989; Shaw et al. 2012, 2013; Werner et al. 2014) or displays to some degree a mixed combination of parental characters (Burgeff 1943; Boisselier-Dubayle et al. 1998; Såstad et al. 1999, 2001; Flatberg 2005; Orzechowska et al. 2006) so that only occasional traits seem to be misplaced (Cronberg and Natcheva 2002; Natcheva and Cronberg 2007a, b).

Our interpretation is that introgression is extensive in the sympatric populations of *Homalothecium*, most of the admixed individuals display a majority of SNPs from one parent and occasional SNPs that appear to be misplaced. Pereira et al. (2019) recently found evidence for extensive introgression in the sibling moss species *Syrrhopodon annotinus* and *S. simmondsii* that occur in sympatry but in different habitats in lowland Amazonian rainforests. They observed that the two species show a clear morphological differentiation and a low but significant genetic differentiation using 183 SNPs, but interspecific pairs of individuals were more closely related when they were geographically closer, suggesting that isolation-by-distance is stronger than the interspecific reproductive barrier and pointing to extensive introgression between the two species. Our observation of occasional misplaced SNPs resembles earlier studies of peat mosses, which display limited mixing of genetic markers in hybridizing populations. Without individuals that show a strong mixing of the parental genomes, it is difficult to demonstrate hybridization in the absence of additional evidence. In studies of the species pair *Sphagnum capillifolium* and *S. quinquefarium*, Natcheva and Cronberg (2007b) were able to show that hybrid capsules could produce viable spores, which turned out to inherit the chloroplast genes from the maternal parent, whereas most of the nuclear markers came from the father. Since a very small fraction of spores

from the hybrid capsules germinated, it was suggested that only those spores that for stochastic reasons happened to recombine a majority of the genome from one parent were viable, i.e. most of the spores were aborted due to incompatibilities between the genomes. In other words, individuals with a more balanced genomic composition of the parental species may be absent when homoploid hybridization takes place. A similar situation may occur in the *Homalothecium* species, but we could imagine other, mutually non-exclusive, explanations for limited genomic mixing in this study. Firstly, both species are nannandric, which means that dwarf males can be generated by spores that germinate on normal-sized females. Spore dispersal by wind in bryophytes is strongly leptokurtic (Miles and Longton 1992), which means that many spores may actually land directly on the sporulating maternal gametophyte. Rosengren et al. (2016) inferred that repeated back-crossing by son-mother fertilization occurs from time to time in *H. lutescens*. For statistical reasons, each back-crossing event will mean that half of the paternal genetic variation is lost. Since dwarf males of *Homalothecium* are annual or possibly biannual and therefore have a high turnover rate, this kind of genome homogenisation is bound to be faster than in species with normal-sized males or females. On the other hand, dwarf males are most likely to be exposed to different selection pressures than normal-sized individuals, which is important since both represent the haploid generation—no variation is hidden as recessive alleles. Secondly, our method of selecting species-specific SNP markers may also play a role in our potential to detect hybrids. If hybridization has occurred episodically in the past, we may have excluded markers that occur in parts of the genome that are interchangeable between the species and therefore potentially polymorphic in one or both species. Recent studies of various organisms at the genomic level have stressed the semipermeable nature of species boundaries (Harrison and Larson 2014), meaning that some parts of the genomes are mixed between sister lineages in hybrid zones and others not. Finally, our approach to include only individuals with mature sporophytes from the sympatric populations may mean that we have excluded individuals that are sterile (no sex organs) or failing to produce sporophytes after cross-fertilization (aborted sporophytes). Such individuals may possess more strongly mixed genomes. In additional data sets from the same sympatric populations we include non-sporulating gametophytes, a few of which appear to be more close to primary hybrids, and we also perform germination tests to estimate spore germination frequencies (Sawangproh et al. 2020; Sawangproh and Cronberg, in prep).

Despite limited genomic mixing and strong genomic representation of one or the other parent in admixed SNP genotypes, individuals from the sympatric populations had characteristics that were out of range of any of the parental species from the allopatric populations, in leaf shape, having smaller leaves. These individuals also tended to look habitually intermediate (in terms of branching and general growth pattern) although this was not recorded. Similarly, hybrids of *Sphagnum capillifolium* and *S. quinquefarium* differed systematically from the parental species in a controlled growth experiment with different watering and shading regimes. The hybrids performed worse under drought stress but tended to regenerate faster than either of the parental species (Natcheva and Cronberg in Natcheva 2006) and as a possible explanation, it was proposed that adaptive gene combinations may have been disrupted during recombination.

Because species of the genus *Homalothecium* sometimes grow sympatrically, it has been speculated that hybridization plays an evolutionary role within the genus (Hofmann 1998; Hedenäs et al. 2009). Variable gametophyte characters could suggest multiple origins and interspecific crossing; for example, *Homolothecium aeneum* shares characters with *H. nevadense* and *H. aureum* as well as *H. pinnatifidum*, without having any diagnostic characters of its own. To investigate this, Hofmann (1997) made a biometrical analysis, which revealed that branch leaves of *H. aeneum* are intermediate between *H. aureum* and *H. nevadense*, in accordance with the theory of a hybrid origin and also similar to our results from the sympatric populations of *H. lutescens* and *H. sericeum*. Although *H. lutescens* and *H. sericeum* have been suspected to hybridize, and specimens with intermediate gametophyte morphology have been recognized as *H. lutescens* var. *fallax* (Hofmann 1998, see Table 1), hybrid sporophytes have not been documented. We cannot verify that var. *fallax* is a hybrid taxon because the critical characters do not completely agree with the morphospace of individuals in our sympatric populations. The original description (by Philibert in Schimper 1876, as *H. fallax*) states that the vegetative characters resemble *H. philippeanum* but gives no specific information about the traits of normal leaves. Having inspected the type specimen, Hofmann (1998) notes that the only difference between *H. fallax* and *H. lutescens* is the erect, straight capsule, which led her to suggest that *H. fallax* is a hybrid between *H. lutescens* and *H. sericeum*, or possibly that the straight capsule is caused by a rare mutation. She does not mention anything regarding intermediate leaf characters, although she had observed occasional specimen of *H. lutescens* with variable capsule shapes from a number of countries in Europe. Nyholm (Nyholm 1965; Table 1) having not seen the type of *H. fallax* states that Scandinavian plants referred to this taxon has been found from Öland and some other regions, not including Skåne. She mentions that they have more or less creeping stems with few rhizoids and crowded erect branches, like a robust *H. sericeum*. In agreement with our observations from sympatric populations, she points out that the angular cells have less denticulate and shorter teeth than

H. sericeum (i.e. intermediate between the species). She also mentions that the capsule is almost erect and with shorter operculum than *H. lutescens*. To summarize, it is difficult to directly link var. *fallax* to hybrid origin(s), but it is probable that the taxon sometimes has been applied for putative hybrids.

The predominance of SNP markers typical of one or the other of the parental species in admixed sporophytes suggests that introgression is bidirectional—meaning that hybrid gametophytes can backcross repeatedly with either *H. lutescens* or *H. sericeum*. Bidirectional hybridization is a possible explanation why some leaf samples of gametophytes from sympatric populations showed similar morphology typical to one of the parents although the molecular data revealed signs of hybridization. In sympatric populations on Öland, *H. sericeum* generally grows above *H. lutescens*. Therefore, *H. sericeum* is more likely to act as paternal plants, since the sperm cells are more easily transported downwards with flowing or splashing water. Also spores of *H. sericeum* could be expected to more easily disperse downwards at the local scale, and therefore deposit on female shoots of *H. lutescens* and dominate the population of dwarf males. A tendency for spores to be deposited close to the sporulating female shoot could be even more pronounced if spore dispersal is negatively affected by intermediate capsule inclination or peristomal dysfunction as a consequence of hybridization.

Conclusions

Our study shows that *H. lutescens* and *H. sericeum* are incompletely reproductively isolated and that interspecific hybridization and introgression occur in sympatric populations. Branch leaves of *Homalothecium* plants in sympatric populations show intermediate morphology in a number of characters but are generally smaller in size compared with those of either species in allopatric populations. Contrary to our expectations, intermediate capsule inclination is not a morphological marker for primary hybridization, but rather an indication of diffuse introgression. Most of the sporophyte genotypes that contained SNP markers from both species displayed asymmetrical genomic contribution from the two parents, indicating that hybrid sporophytes with low recombination of parental genomes can develop normally on maternal gametophytes and perhaps also produce viable hybrid spores. Overall, sometimes stronger morphological affinity of branch leaves to one or the other of the parents of admixed individuals and asymmetrical SNP markers in sporophytes suggest bidirectional hybridization and introgression. It appears that ecological isolation is a stronger barrier against hybridization than post-zygotic genomic incompatibilities—in the vast majority of populations of *H. lutescens* and *H. sericeum* in south Sweden, the species do not occur together at all or at least not in immediate vicinity.

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