Gene transfer by interspecific hybridization in bryophytes

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract
The role of hybridization in evolution has been debated for more than a century regarding bryophytes (mosses, liverworts, and hornworts) as well as most other organisms. Bryophytes have haplodiplontic life cycles with a dominant haploid generation. Hybridization in bryophytes involves fusion of gametes produced by haploid parental gametophytes of different species. The hybrid is thus the short-lived diploid sporophytes, which soon undergoes meiosis prior to forming a large amount of haploid recombinant spores. In this study, two moss species (*Homalothecium lutescens* and *H. sericeum*) and three subspecies of liverwort *Marchantia polymorpha* were investigated for evidence of gene transfer by hybridization. Firstly, we compared the morphology of gametophytes and sporophytes from allopatric and sympatric populations of *H. lutescens* and *H. sericeum.* Secondly, we used species-specific SNP markers to estimate the degree of genetic mixing in three generations (i.e., haploid maternal gametophytes, diploid sporophytes, and haploid sporelings) in samples from sympatric populations of *H. lutescens* and *H. sericeum.* Thirdly, we assessed fitness traits in relation to the degree of genetic admixture in sporophytes of *H. lutescens* and *H. sericeum*, including non-admixed, mildly and strongly admixed genotypes. Finally, we investigated the genome-wide scale phylogenetic relationship between the three subspecies of *M. polymorpha* to test the hypothesis that subsp. *ruderalis* has originated as a homoploid hybrid species between subsp. *polymorpha* and subsp. *montivagans*. Our study of *Homalothecium* shows that gametophytes from sympatric populations display intermediate morphology in a number of leaf characters, with the exception for leaf dimensions, which are strikingly smaller than those in allopatric populations. Most sporophytes with intermediate capsule inclination, initially classified as putative hybrids, did not display admixture of SNP markers. Many sporophytes appeared to be secondary hybrids by displaying asymmetrical admixture of SNP markers except five sporophytes, which were found to be primary hybrids. Admixture analyses using SNP markers identified 76 samples (17%) as mildly admixed and 17 samples (3.8%) as strongly admixed. Admixed samples represented all three generations and were found in all sympatric populations. Hybridization and introgression were bidirectional. Admixed sporophytes gave rise to viable recombinant spores and sporelings. Sporophytes with mildly admixed *H. lutescens* tended to show lower fitness, whereas sporophytes with mildly admixed *H. sericeum* showed signs of heterosis. Some strongly admixed sporophytes showed high spore counts, intermediate spore diameters and high spore germination rates. Genomic analysis showed three distinct taxa within the *M. polymorpha* complex, coinciding with the three generally accepted subspecies. All three possible topologies were frequent across the genome but species tree analyses using *M. paleacea* as outgroup recovered an overall branching order where subsp. *montivagans* diverged first and subsp. *ruderalis* and subsp. *polymorpha* were placed as sister species. The high degree of inconsistent gene trees suggests frequent incomplete lineage sorting (ILS) and/or recent or intermittent introgression. Evidence for recent introgression was found in two samples of *M. polymorpha.* Remarkably, pseudo-chromosome 2 in subsp. *montivagans* differed by being more diverged than other parts of the genomes. This could either be explained by specific capture of chromosome 2 from an unknown related species through hybridization or by conservation of chromosome 2 despite intermittent or ongoing introgression affecting more permeable parts of the genomes. A higher degree of chromosomal rearrangement in pseudochromosome 2 of subsp. *montivagans* provide some evidence for the latter explanation. Our results show that gene transfer between lineages occurs in sympatric populations of both the *Marchantia polymorpha* complex and among the *Homalothecium* species. This supports the hypothesis that homoploid hybridization is more widespread among bryophytes than earlier assumed. Moreover, the population-level studies of sympatric populations of *H. lutescens* and *H. sericeum* demonstrate that they behave as true hybrid zones, where genetic material is transferred across species boundaries and secondarily backcrossed. Presence of hybrid zones has strong evolutionary implications because genetic material transferred across species boundaries can be directly subject of natural selection in the dominant haploid generation of the bryophyte life cycle, and contribute to local adaptation, survival and speciation.

Key words
homozygote, heterozygote, admixture, single nucleotide polymorphism, STRUCTURE, PCoA, hybrid index, inbreeding, outbreeding, hybrid depression, heterosis, phylogeny, purging selection, genetic purging, genotype, phenotype, sympatry, allopatry

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To my family:
Boonsong, Noo-chan, Tom, Kung, Dirk, little Patrick

The role of hybridization in evolution has been debated for more than a century. Two highly polarized viewpoints have emerged. At one extreme, hybridization is considered to be a potent evolutionary force that creates opportunities for adaptive evolution and speciation. In this view, the increased genetic variation and new gene combinations resulting from hybridization promote the development and acquisition of novel adaptations. The contrasting position accords little evolutionary importance to hybridization (aside from allopolyploidy), viewing it as a primarily local phenomenon with only transient effects – a kind of "evolutionary noise". Unfortunately, definitive support for either viewpoint is lacking.

–A quote from a paper by Loren Rieseberg and colleagues.

(Rieseberg LH, Raymond O, Rosenthal DM, Lai Z, Livingstone K, Nakazato T, Durphy JL, Schwarzbach AE, Donovan LA, Texer C. 2003. Major ecological transitions in wild sunflowers facilitated by hybridization. *Science* 301: 1211-1216)

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List of papers

This thesis is based on the following papers, referred to by their roman numerals:

Paper I **Sawangproh, W.**, Lang, A.S., Hedenäs, L. & Cronberg, N. Morphological characters and SNP markers suggest hybridization and introgression in sympatric populations of the pleurocarpous mosses *Homalothecium lutescens* and *H. sericeum*. Submitted.

Paper II **Sawangproh, W.**, Hedenäs, L., Lang, A.S., Hansson, B. & Cronberg, N. Gene transfer across species boundaries in bryophytes: evidence from major life cycle stages in *Homalothecium lutescens* and *H. sericeum*. Manuscript.

Paper III **Sawangproh, W.** & Cronberg, N. Fitness of progeny from hybridizing populations of the bryophytes *Homalothecium lutescens* and *H. sericeum*. Manuscript.

Paper IV **Linde, A-M.**, Sawangproh, W., Cronberg, N., Szövényi, P. & Lagercrantz, U. Evolutionary history of the *Marchantia polymorpha* complex. Manuscript.

Author contributions

Paper I **WS**, NC and LH conceived the study and collected the plant material. **WS** was responsible for data collection. **WS** and ASL analysed the data. **WS** wrote the paper with input from NC, LH and ASL.

Paper II **WS**, NC and LH conceived the study. **WS** was responsible for data collection. **WS**, ASL and BH analysed the data. **WS** wrote the paper with input from NC, LH, ASL and BH.

Paper III **WS** and NC conceived the study. **WS** was responsible for data collection. **WS** analysed the data. **WS** wrote the paper with input from NC.

Paper IV AML, **WS**, NC and UL conceived the study. NC collected the plant material. AML and UL was responsible for data collection. AML, UL and **WS** analysed the data. PS provided chromosome data for *Marchantia polymorpha* subsp. *ruderalis*. AML wrote the paper with input from **WS**, NC and UL.

Authors: Weerachon Sawangproh (WS), Nils Cronberg (NC), Lars Hedenäs (LH), Annick S. Lang (ASL), Bengt Hansson (BH), Anna-Malin Linde (AML), Ulf Lagercrantz (UL), Péter Szövényi (PS).

Abstract

The role of hybridization in evolution has been debated for more than a century regarding bryophytes (mosses, liverworts, and hornworts) as well as most other organisms. Bryophytes have haplodiplontic life cycles with a dominant haploid generation. Hybridization in bryophytes involves fusion of gametes produced by haploid parental gametophytes of different species. The hybrid is thus the short-lived diploid sporophytes, which soon undergoes meiosis prior to forming a large amount of haploid recombinant spores. In this study, two moss species (*Homalothecium lutescens* and *H. sericeum*) and three subspecies of liverwort *Marchantia polymorpha* were investigated for evidence of gene transfer by hybridization.

Firstly, we compared the morphology of gametophytes and sporophytes from allopatric and sympatric populations of *H. lutescens* and *H. sericeum.* Secondly, we used species-specific SNP markers to estimate the degree of genetic mixing in three generations (i.e., haploid maternal gametophytes, diploid sporophytes, and haploid sporelings) in samples from sympatric populations of *H. lutescens* and *H. sericeum*. Thirdly, we assessed fitness traits in relation to the degree of genetic admixture in sporophytes of *H. lutescens* and *H. sericeum*, including non-admixed, mildly and strongly admixed genotypes. Finally, we investigated the genome-wide scale phylogenetic relationship between the three subspecies of *M. polymorpha* to test the hypothesis that subsp. *ruderalis* has originated as a homoploid hybrid species between subsp. *polymorpha* and subsp. *montivagans*.

Our study of *Homalothecium* shows that gametophytes from sympatric populations display intermediate morphology in a number of leaf characters, with the exception for leaf dimensions, which are strikingly smaller than those in allopatric populations. Most sporophytes with intermediate capsule inclination, initially classified as putative hybrids, did not display admixture of SNP markers. Many sporophytes appeared to be secondary hybrids by displaying asymmetrical admixture of SNP markers except five sporophytes, which were found to be primary hybrids. Admixture analyses using SNP markers identified 76 samples (17%) as mildly admixed and 17 samples (3.8%) as strongly admixed. Admixed samples represented all three generations and were found in all sympatric populations. Hybridization and introgression were bidirectional. Admixed sporophytes gave rise to viable recombinant spores and sporelings. Sporophytes with mildly admixed *H. lutescens* tended to show lower fitness, whereas sporophytes with mildly admixed *H. sericeum* showed signs of heterosis. Some strongly admixed sporophytes showed high spore counts, intermediate spore diameters and high spore germination rates.

Genomic analysis showed three distinct taxa within the *M. polymorpha* complex, coinciding with the three generally accepted subspecies. All three possible

topologies were frequent across the genome but species tree analyses using *M. paleacea* as outgroup recovered an overall branching order where subsp. *montivagans* diverged first and subsp. *ruderalis* and subsp. *polymorpha* were placed as sister species. The high degree of inconsistent gene trees suggests frequent incomplete lineage sorting (ILS) and/or recent or intermittent introgression. Evidence for recent introgression was found in two samples of *M. polymorpha.* Remarkably, pseudo-chromosome 2 in subsp. *montivagans* differed by being more diverged than other parts of the genomes. This could either be explained by specific capture of chromosome 2 from an unknown related species through hybridization or by conservation of chromosome 2 despite intermittent or ongoing introgression affecting more permeable parts of the genomes. A higher degree of chromosomal rearrangement in pseudochromosome 2 of subsp. *montivagans* provide some evidence for the latter explanation.

Our results show that gene transfer between lineages occurs in sympatric populations of both the *Marchantia polymorpha* complex and among the *Homalothecium* species. This supports the hypothesis that homoploid hybridization is more widespread among bryophytes than earlier assumed. Moreover, the population-level studies of sympatric populations of *H. lutescens* and *H. sericeum* demonstrate that they behave as true hybrid zones, where genetic material is transferred across species boundaries and secondarily backcrossed.

Presence of hybrid zones has strong evolutionary implications because genetic material transferred across species boundaries can be directly subject of natural selection in the dominant haploid generation of the bryophyte life cycle, and contribute to local adaptation, survival and speciation.

Glossary

Admixture	(also known as **genetic admixture**) The occurrence of gene transfer between two or more previously isolated and genetically differentiated populations when they begin to interbreed.
Admixed individual	Here referred to an individual showing evidence of admixture with another species of the same ploidy level, but not in itself considered as a taxonomically separate species.
Allopolyploidy	A hybrid individual with doubled ploidy level (or higher), and having resulted from interspecific crosses**.**
Antheridium (pl. **antheridia**)	The sex organ on a male gametophyte plant producing male gametes (or sperm).
Archegonium (pl. **archegonia**)	The sex organ on a female gametophyte plant producing the egg cell, and nurturing the young sporophyte.
Autopolyploid	A hybrid individual with doubled ploidy level (or higher), and having resulted from intraspecific crosses.
Backcrossing	Crossing of an interspecific hybrid with one of its parents or an individual genetically similar to its parent, will give offspring with a genetic identity, which is closer to that of the parent.
Bryophyte	An informal group of (mainly green) flowerless plants consisting of three divisions of non-vascular land plants (embryophytes) e.g. the liverworts, hornworts and mosses.
Capsule	Here referred to a 'sporophyte capsule' inside of which haploid spores are produced after meiosis.
Dioicous	(adj.) Having archegonia (female sex organ) and antheridia (male sex organ) on separate plants.
F_1 hybrid	The first "filial" generation of offspring (seedlings) following a hybridization event in vascular plants. Subsequent generations are called F_2, F_3, etc. In bryophytes the F_1 generation is the diploid sporophyte and the spores produced after meisosis in the sporophyte constitutes the haploid F_2 generation.
Gametophyte	One of two alternating phases in the life cycle of many plants and algae, producing haploid gametes (i.e., male or female gametes).
Gene flow	The movement of alleles within and between populations.
Genetic marker	A gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species.
Genome	The entire collection of genes or genetic material present in a cell or an organism. The genome includes both the genes (the coding regions) and the noncoding DNA, as well as mitochondrial and chloroplast DNA.
Genotyping	The process of determining differences in the genetic make-up or genotype of an individual by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence.
Haplotype	A group of alleles in an organism that are inherited together from a sing parent. The word is here used to represent the combination of alleles across genetic marker loci that have been found for a certain individual in the haploid phase of the life-cycle (= haploid multilocus genotype).
Heterosis	(also known as **hybrid vigour**) The superiority of hybrids compared to either parental type with respect to one or more traits.
Heterozygosity	A condition of a gene locus when the cells contain two different alleles in the diploid phase of the life cycle.
Homoploid hybridization	Here referred to the interbreeding of individuals from two distinct species by interspecific hybridization without change in chromosome number.
Homozygosity	A condition of a gene locus when the cells contain two identical alleles in the diploid phase of the life cycle.
Hybrid	An individual having resulted from hybridization**.**
Interspecific hybridization	The process of interbreeding between individuals of different species resulting in interspecific hybrid(s).
Introgression	(also known as **introgressive hybridization**) The transfer of genetic material from one species into the gene pool of another by repeated backcrossing of

	interspecific hybrids to one of its parental species.
Monoicous	(adj.) Having archegonia (female sex organ) and antheridia (male sex organ) on the same individual.
Outgroup	(in a cladistic study) A group that is likely to share ancestral traits with the ingroup (=group of taxa under study) because it shares a close common ancestor with the ingroup.
Paraphysis (pl. **paraphyses**)	Multicellular sterile hairs intermixed with the sex organs in most mosses and some liverworts.
Perichaetium (pl. **perichaetia**)	Cluster of archegonia, often with paraphyses, protected by typically modified leaves (i.e., perichaetial leaves).
Perigonium (pl. **perigonia**)	Cluster of antheridia, often with paraphyses, protected by typically modified leaves (i.e., perigonial leaves).
Peristome	A set of cells or cell parts surrounding the opening of a sporophyte capsule. In many mosses, they are sensitive to humidity, and will alter their shape to aid in spore dispersal.
Phylogeny	A hypothesis of the evolutionary history of a group of organisms, which is represented by a branching diagram showing the ancestral relations among taxa.
Pleurocarp	A moss having the archegonium or antheridium on a short side branch rather than the main stem, usually resulting in shoots with prolific branching.
Ploidy	(also known as **ploidy level**) The number of sets of chromosomes in a cell, or in the cells of an organism.
Polyploidization	Hybridization leading to formation of hybrid progeny with multiple sets of chromosomes.
Seta (pl. **setae**)	Stalk of the sporophyte terminated by the capsule/sporangium in liverworts and mosses.
SNP	A single nucleotide polymorphism (often abbreviated to SNP) is a variation in a single nucleotide that occurs at a specific position in the genome.
Spore	Haploid cell produced by meiotic division of sporangial cells.
Sporeling	(In seedless vascular plants) A young plant produced by a germinated spore.
Sporophyte	One of two alternating phases in the life cycle of many plants and algae, initiated after a fertilization event and therefore being diploid.
Transcriptome	The collective mRNA transcripts from all expressed genes and their relative expression levels in a particular cell or tissue of an organism at a particular time point.

Introduction

Interspecific hybridization

It has long been known that interspecific hybridization is an important process in plant evolution (Burke and Arnold 2001), and it is estimated that more than 50 % of the vascular plant species have some form of hybrid origin (Stebbins 1950). Processes such as allopolyploid speciation (genomes of two species combined at doubled ploidy level) and homoploid hybridization (combination of two genomes at the original ploidy level) have long been generally recognized (Reviewed in Rieseberg 1997). In nature, some interspecific hybrids are soon lost by natural selection but others may survive beyond the initial generation by interbreeding with other hybrids forming, "hybrid zones", and sometimes backcrossing to the parental species forming "hybrid swarms (Harrison 1993; Rhymer and Simberloff 1996). Repeated backcrossing of an interspecific hybrid with one of its parental species (introgression) may transfer genes between different evolutionary units, e.g. species, and contribute to increased genetic variation and new adaptive genotypes in populations.

Bryophytes, non-vascular plants, is the second most diverse group of higher plants followed only after flowering plants in species number and more diverse than the nonflowering vascular plants, with some 15,000-20,000 species in more than 1,200 genera worldwide (Gradstein *et al*. 2001; Shaw *et al*. 2011). The bryophytes consist of three remotely related major groups; mosses (Bryophyta), liverworts (Marchantiophyta) and hornworts (Anthocerotophyta) (Nickrent *et al*. 2000; Kugita *et al*. 2003). It is still under debate whether the bryophyte lineages are paraphyletic or form a monophyletic group. The relevance to bring them together in the context of hybridization is that they share a similar life cycle with a dominant haploid gametophytic phase and a short-lived diploid sporophytic phase which is physically attached to the female parent. Bryophytes can reproduce both sexually and vegetatively. Sexual reproduction of bryophytes is dependent on water, by which mobile male gametes (released from antheridia) can swim to fertilize sessile female gametes (egg-cells inside archegonia) (Gradstein *et al*. 2001). Successful fertilization of bryophytes is dependent on both water availability and the distances separating the males and females. Therefore, in order for hybridization to occur, the two species must grow in close vicinity, which is sometimes the case in intermediate or mosaic habitats.

Hybridization has largely been ignored by bryologists as an evolutionary process although bryophyte hybrids were identified based on morphological characters already in the mid 1800s (reviewed in Natcheva and Cronberg 2004). The dominant generation in bryophytes is haploid, and interspecific hybridization in bryophytes means that a diploid hybrid sporophyte is formed after fertilization. The hybrid sporophyte is physically connected to the female plant and short-lived (Nicholson 1931; Anderson 1980) (**Figure 1**). The two parental genomes are recombined during meiosis, which takes place in the sporophyte prior to the formation of spores. Thus, the true hybrid is the sporophyte and the spores produced in the sporophyte are recombinants that have a mix of genes from both parents and can be referred to as "hybrid segregates" comparable to the F_2 generation of angiosperms with hybrid origin. In most of the documented cases, which include fairly distantly related species, hybrid breakdown takes place during sporophyte initiation or spore formation. Few examples of hybrid sporophytes generated from crossing of closely related species are known, probably because the sporophytes are similar so it is impossible to point out hybrid sporophytes by traditional morphological methods. Consequently, few hybrids have been given formal taxonomic recognition (Natcheva and Cronberg 2004).

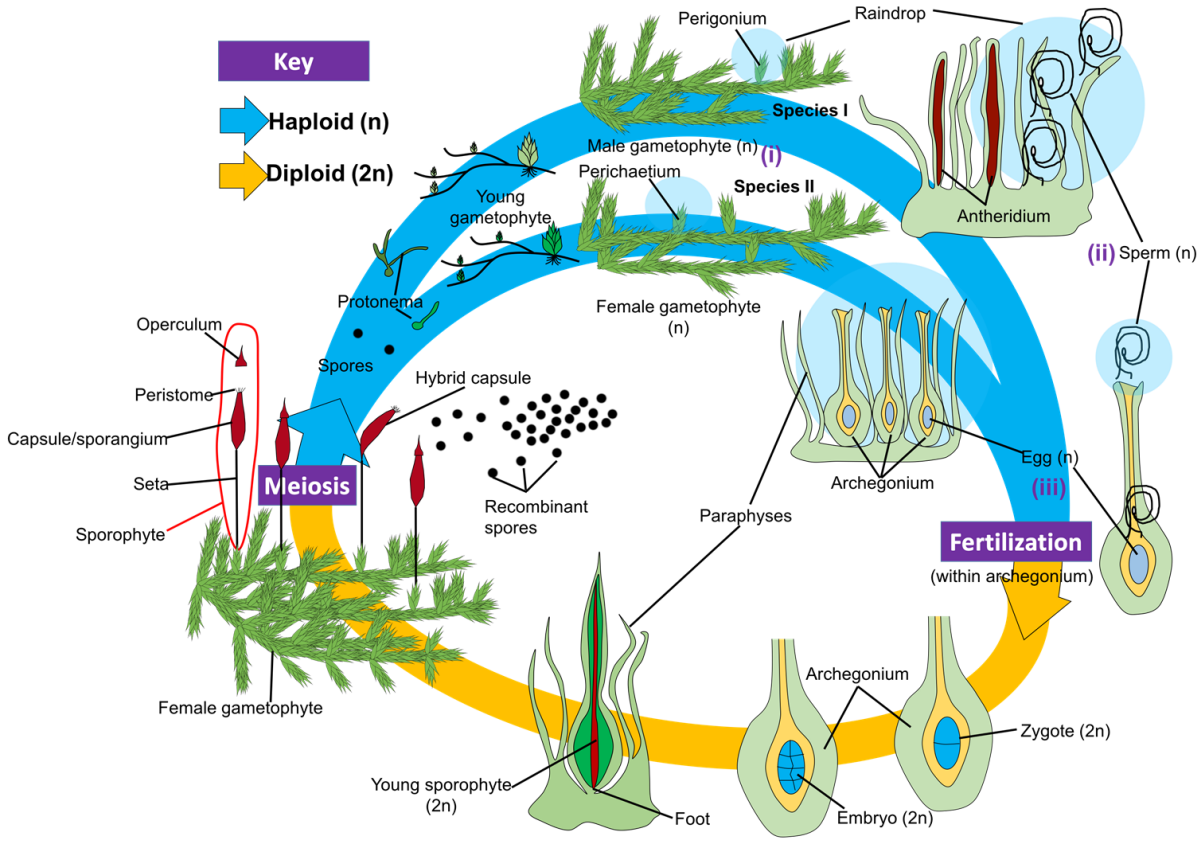


Figure 1.
Interspecific hybridization in bryophytes. A hybridization event in bryophytes requires three factors i) a close vicinity of two parental species ii) water as a medium to bring sperm into contact with egg iii) incomplete reproductive isolation between two parental species.

Evidence of Hybridization in Bryophytes

Hybridization may take place if there are no strong genetic barriers preventing crosses between two species that share the same habitats and grow in intimate contact (Anderson 1980; Cronberg 1989). There are indications that reproductive isolation is sometimes weak or insignificant between bryophytes species. For example, some geographic races of *Sphaerocarpos texanus* (Allen 1937), *Phaeoceros laevis* (Proskauer 1969), *Sphagnum capillifolium* (Ehrh.) Hedw. and *S. rubellum* Wils (Anderson 1980), *S. balticum* (Russow) C.E.O. Jensen and *S. tenellum* (Brid.) Brid. (Såstad *et al*. 2001) can intercross under certain circumstances. Cronberg (1989) was able to demonstrate that putative hybrids were present in intermediate habitats where the peat mosses *S. rubellum* and *S. capillifolium* occurred in sympatry. How well does reproductive isolation in bryophytes work during fertilization? The fertilization of female archegonia with foreign sperm was tested by Showalter (1926). He observed that sperm cells of liverworts from the genera *Aneura*, *Sphaerocarpos*, *Asterella* and *Funaria* can penetrate egg cells belonging to the genus *Fossombronia*, suggesting a weak specialization or "gametic isolation" during the initial stage of fertilization among liverworts. Parihar (1965) and Watson (1971) assumed that the chemical attractants released by fertile archegonia to attract sperm are simple sugars, proteins or inorganic salts, which are not species-specific. Most evidence suggests that fertilization involving remotely related species results in abortion of the zygotes or early embryos of sporophytes. For example, considerable sporophyte abortion occurred during development of hybrid embryos formed after cross-species fertilization between the two moss species *Polytrichum commune* Hedw. and *Polytrichum uliginosum* (Wallr.) Schriebl. (Van Der Velde and Bijlsma 2004).

I carried out online literature search to find articles that dealt with hybridization in bryophytes from the databases Web of Science® and Google Scholar. I also included references cited by various authors in the retrieved articles that did not appear in the online searches. Keywords included "Bryophyte + Hybrid"; "Bryophyte + Hybrid + Hybridization"; "Bryophyte + Interspecific Hybridization"; "Bryophyte + Allopolyploid"; "Bryophyte + Homoploid". The time span for searches was set from early 1900's until May 2016. I found 194 articles dealing with hybrid origins in bryophytes, including observations in nature, hybrid specimens from museum vouchers, experimental cross fertilization, spontaneous fertilization and hybrid detection by various molecular methods. The literature search revealed that records of hybridization are not evenly distributed among taxonomic groups – a large proportion of (putative) hybrids and their (putative) parental species belong to the acrocarpous moss groups (106 of 194 records), peat mosses (48 of 194 records) and thallose liverworts (26 of 194 records) (**Figure 2**). The three most frequent families with records of hybrids throughout the whole search result are Sphagnaceae, Funariaceae and Pottiaceae

(**Figure 3**). In the earliest articles, hybrid identification was based solely on morphological studies (i.e., morphological intermediacy of sporophytes or gametophytes) or a combination of morphology and spore germination/spore characters (i.e., spore size and shape), subsequently followed by articles employing cytological ploidy level determination and analyses of secondary chemical substances (i.e., flavonoids and oil bodies) and more recently by genetic markers (i.e., isozymes or allozymes, RFLPs, RAPDs, Microsatellites, ISSR, PCR-RFLP, QTL-associated markers, and DNA sequence markers) at the turn of the 20th century (**Figure 4**).

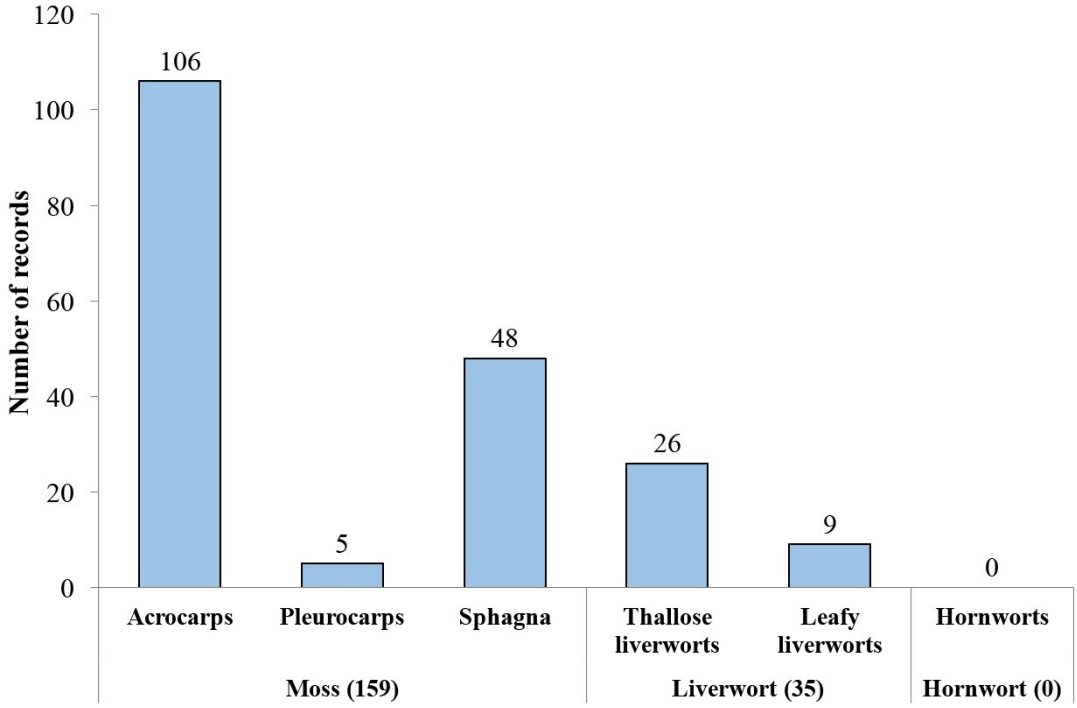


Figure 2.
Number of literature records of interspecific hybridization in bryophytes categorized by major taxonomic groups.

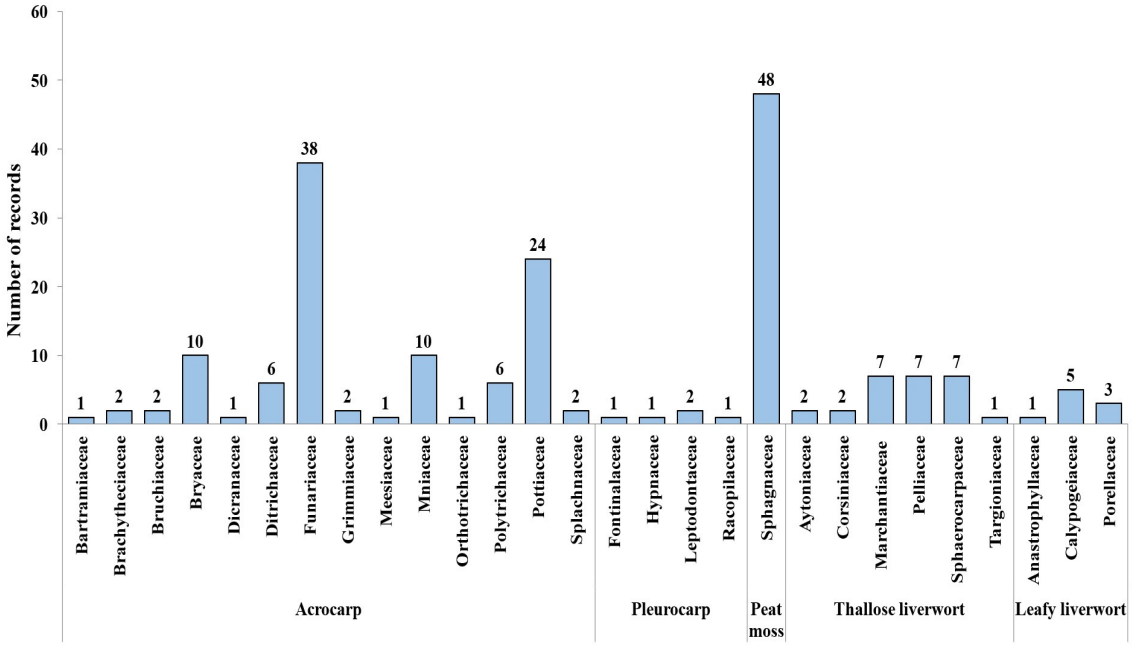


Figure 3.
Number of literature records of interspecific hybridization in bryophytes categorized by family.

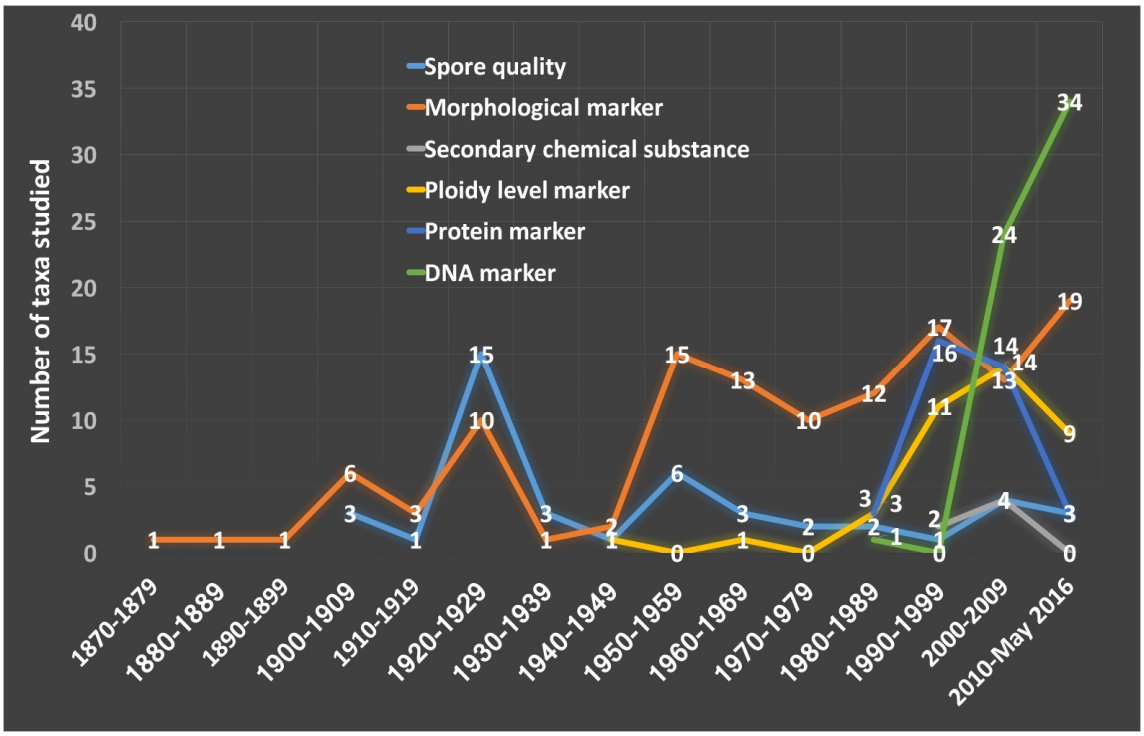


Figure 4.
Number of literature records of hybrid progeny in bryophytes recognized by each type of character (marker).

Records on hybridization and modes of hybridization in the pre-molecular (1870-1988) and molecular periods (1988-present)

The three most studied bryophyte families before the molecular era are Funariaceae, Pottiaceae, and Bryaceae, whereas the three most studied bryophyte families in the molecular era are Sphagnaceae, Pottiaceae and Mniaceae (**Figure 5**). Prior to the molecular era, studies of hybridization events were mainly based on a combination of intermediate morphological characters allowing bryologists to detect possible parental progenitors, although it was usually difficult to determine the actual hybridization process. Out of the 84 cases of putative hybridization among bryophytes, only 13 succeeded to reveal the evolutionary processes underlying the hybridization events. More or less well-documented modes of hybrid speciation include allopolyploidy (nine records), homoploid speciation (three records) and autopolyploidy (one records) (**Figure 6)**. During the molecular era, the modes of hybridization were investigated by clearly more reliable and sophisticated cytological and molecular studies – revealing more information about mechanisms of hybrid speciation in bryophytes. Cytological and molecular techniques made it possible to characterize the mode of hybridization in 87 out of 110 records of assumed hybridization. The most common was allopolyploidy (71 records), followed by (assumed) homoploid hybridization (nine records), autopolyploidy (five records), one record each of allopolyploidy mixed with autopolyploidy and allopolyploidy mixed with interploid origins (**Figure 6**).

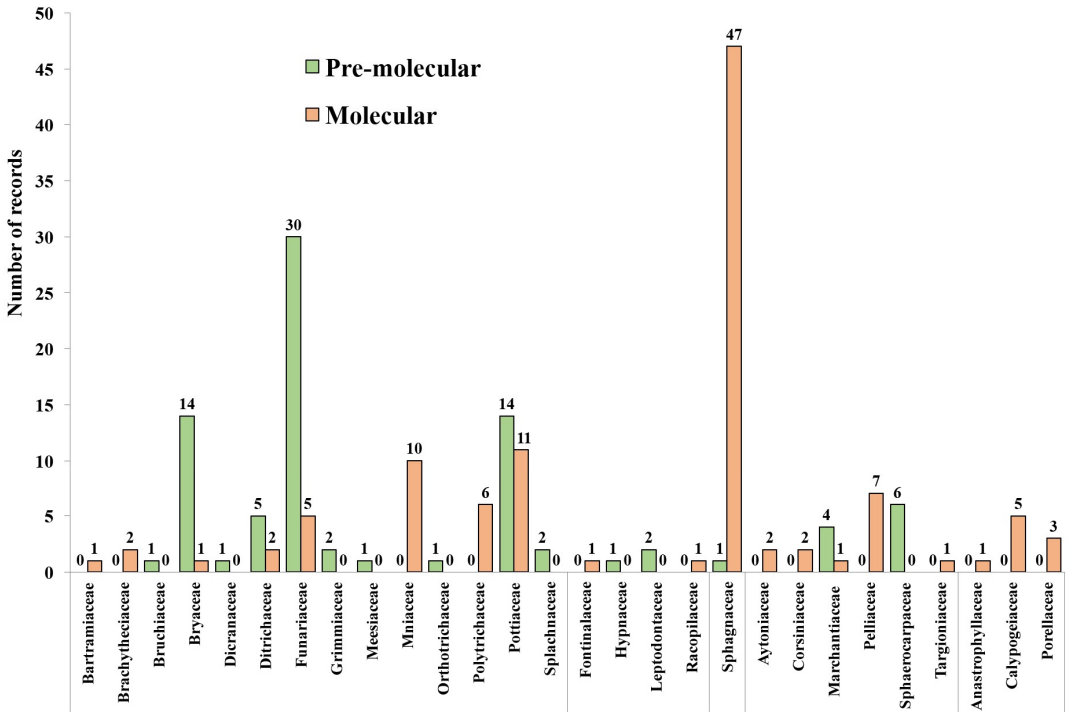


Figure 5.
Comparison of the number of literature records of interspecific hybridization in bryophytes during the pre-molecular and molecular periods, categorized by family.

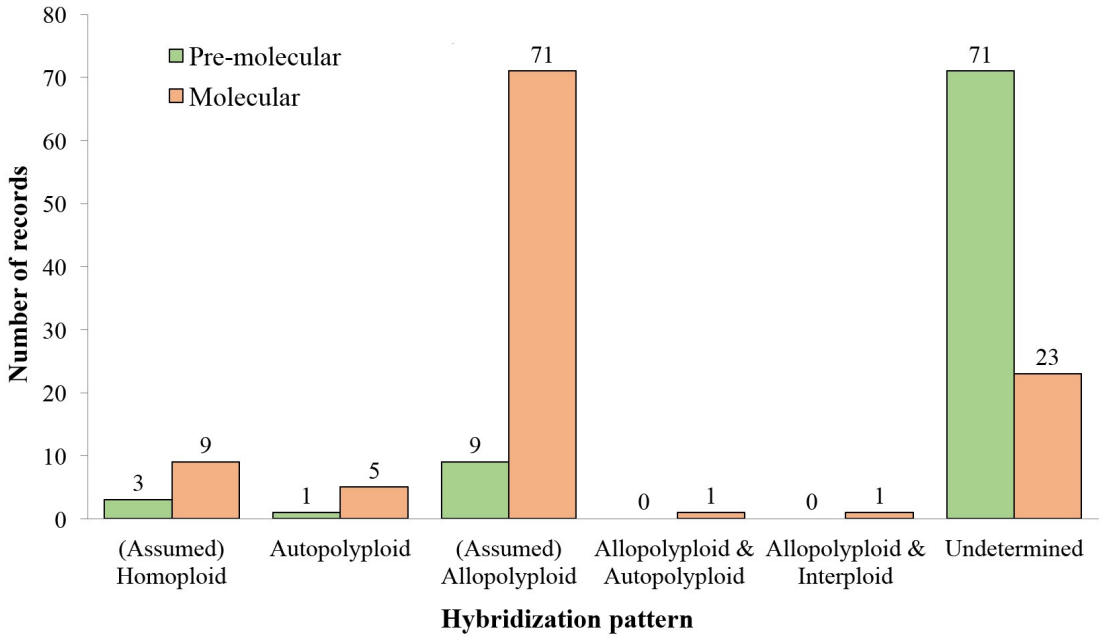


Figure 6.
Comparison of literature records with respect to modes of hybridization in bryophytes during the pre-molecular and molecular periods.

Increased number of observations of putative hybridization during the molecular period

Before the molecular era, bryophytes were interpreted as groups with low evolutionary potential since the fossil record suggested morphological stasis (Anderson 1963; Crum 1972; Schofield 1985; reviewed in Shaw 2009). Recent molecular studies show that mosses have relatively low nucleotide substitution rates in nuclear (18S rDNA), chloroplast (rbcL) and mitochondrial (nad5) genes compared to seed plants, supporting morphological data on slow evolutionary change (Stenøien 2008). Stenøien and Såstad (1999) explained slow evolution (i.e., lack of genetic differentiation between intercontinental populations of *Sphagnum angustifolium*) by large effective population sizes and thus insignificant genetic drift. Opposing this view, Wyatt *et al*. (1988) argued that bryophytes undergo both homoploid hybridization and allopolyploidization, much like vascular plants. After 1988, the number of reports claiming speciation via hybridization in bryophytes has dramatically increased as a consequence of data generated by molecular studies. Consequently, especially allopolyploidization, has been increasingly accepted as important for bryophyte evolution.

Studies on co-dominant molecular markers such as allozymes and microsatellites have proved to be very useful for detecting allopolyploid and possibly homoploid hybrid origins among closely or even more remotely related congeneric species of bryophytes (Shaw and Goffinet 2000). Most studies of hybridization have dealt with the genus *Sphagnum* (peat mosses) because of their frequent hybridization,

recent diversification, phenotypic diversity, ecological importance, and ancient history (Meleshko *et al*. 2018). Meleshko *et al*. (2018) pointed out that 82% of the recorded hybrids in *Sphagnum* are allopolyploids. Currently the most detailed study of homoploid hybridization comes from the thesis of Rayna Natcheva (2006). In her thesis, Natcheva was able to identify hybrid sporophytes involving *S. capillifolium* and *S. quinquefarium* as parents. Her results revealed (1) that only a small fraction of spores from hybrid sporophytes were viable, (2) that the viable spores had a chloroplast genome that came from the maternal parent (thus demonstrating maternal inheritance for the first time in bryophytes), (3) that the nuclear genome came mostly from the paternal parent, and (4) that this skewed pattern of inheritance matched the genomes of observed putative hybrid gametophyte which were never completely intermediate, but rather had occasional traits that seemed to be misplaced. The interpretation was that only the small fraction of spores, which by chance had inherited genomes that came almost completely from one of the parents, was viable – incompatibility between the two parental genomes appear to prohibit stronger degrees of genomic mixing. On the other hand, this means that genes that are favored by selection could be transferred from one species to the other through hybridization. Clearly this result has important implication for the evolution and speciation of bryophytes, if the mixed populations behave as true hybrid zones, which has, as far as we know, never been shown for bryophytes. It remains to be proven that admixed individuals are fertile and able to transmit introgressed genes to future generation through back-crossing. However, until now there are no comparable studies involving bryophytes outside *Sphagnum* and we therefore do not know how widespread admixture at homoploid level is and what it would mean for the evolution of other groups of bryophytes.

Why hybridization in bryophytes is interesting in science?

As mentioned above, hybridization in bryophytes appear to be difficult to detect, for three major reasons: (1) sporophytes of closely related species are similar, so it is difficult to recognize sporophytes of intermediate morphology; (2) gametophytes formed by spores from hybrid sporophytes are unlikely to show complete intermediacy; (3) aberrant morphotypes may simply be explained by phenotypic plasticity. Despite this, there is now good molecular evidence for hybridization in *Sphagnum*, although homoploid hybridization has not been accepted as evolutionary significant in studies of other bryophytes. This lack of acceptance could primarily be explained by a general paucity of molecular studies assessing putative examples of hybridization.

Most studies of hybridization concern diploid organisms. Data about hybridization in organisms with a dominant haploid generation is scarce, which means that the consequences of hybridization in such organisms are poorly understood. In particular, life-cycles in which a **single fertilization** leads to a more or less short-

lived diploid phase followed by a restoration of a dominant haploid phase through **numerous meiotic events** prior to spore production is a shared phenomenon among many different kinds of organisms. This means that an enormous number of individuals with different degrees of recombination of the parental genomes are immediately exposed to selection in the haploid phase.

Aims of Thesis

In this study, I am going to focus on molecular studies to detect primarily homoploid hybridization, and hybridization history among bryophytes. The project includes morphological studies, experimental culturing of spores and molecular analyses. Data from earlier studies of *Sphagnum* peat mosses by Cronberg and Natcheva (2002), showed that only a small fraction of spores formed by meiotic segregation in hybrid sporophytes involving relatively closely related species were viable. Such sporelings carried highly biased genomes, in which genes from one or the other of the parental species were strongly over-represented.

The aims for this thesis are:

I. To test the hypothesis that *H. lutescens* and *H. sericeum* hybridize when they grow in mixed (sympatric) populations based on morphological comparison (e.g. leaves and sporophyte capsules) and SNP markers (**Paper I**).

II. To investigate the degree of interspecific genetic mixing based on data from SNP markers in *Homalothecium* plants from sympatric populations compared to those from allopatric populations (**Paper II**).

III. To identify genetic admixture and analyse patterns of allelic SNP variation among samples in three generations - haploid (female) gametophytes (parental generation), diploid sporophyte and setae (F_1 generation) and sporelings/recombinants (F_2 generation) (**Paper II**).

IV. To test if genetic admixture is symmetrical between the two parental species in sporophytes (F_1 generation) and recombinants (F_2 generation) (**Paper II**).

V. To test if admixed genes are transmitted between maternal gametophytes and sporophytes and between sporophytes and progeny (**Paper II**). In other words, to test if the sympatric populations behave as true introgression zones.

VI. To compare the fitness of hybrid sporophytes and sporophytes produced by “pure species” in sympatric populations (**Paper III**).

VII. To analyze the degree of differentiation and hybridization between the genomes of the three subspecies of the liverwort *Marchantia polymorpha* (in collaboration with Prof. Ulf Lagercrantz and PhD student Anna-Malin Linde from Uppsala University (**Paper IV**)

Methodology

Study species

A. Pleurocarpous mosses

Homalothecium sericeum and *H. lutescens* are clonal and mat-forming pleurocarpous mosses (Rosengren 2015). They are categorized as perennial stayer species (Papp *et al.* 2005; Kürschner *et al*. 2007), which typically have a long life span, low to absent sexual and asexual reproductive effort, variable age of first reproduction, production of small spores with variable life span and variable growth forms i.e. wefts, dendroids, mats and large cushions (During 1979). Normally, the two species are ecologically separated, forming allopatric populations (Hofmann 1998; Lieske 2010). Both occur in habitats with a high pH; *H. sericeum* grows epiphytically on trunks of broad-leaved trees or epilithically on calcareous stone or mortar in forest or parks, whereas *H. lutescens* grows on calcareous soil, usually in more open habitats such as pastures. In dry condition *H. sericeum* is easily recognized by its short secondary branches, which form curled shoots with a typical golden lustre (**Figure 7**). *Homalothecium sericeum* and *H. lutescens* have similar triangular and transversally folded leaves (Hofmann 1998). Stem and branch leaves of *H. sericeum* are appressed when dry but erect when moist, whereas those of *H. lutescens* are erect both when dry and moist (Lieske 2010). The branches of *H. lutescens* are straight when dry and less densely packed. *H. lutescens* grows loosely in ascending, irregularly branched wefts, whereas *H. sericeum* grows tightly connected to its substrate and closely pinnately branched. Capsules of *H. sericeum* are erect, cylindrical, straight or rarely slightly curved below mouth, whereas those of *H. lutescens* are inclined, more or less cylindrical to asymmetrical, straight or slightly curved (Lieske 2010). In addition, there are differences in various peristomal characters, in particular the endostome segments are as long as the exostome teeth and perforated in *H. lutescens*, whereas they are shorter, without perforations in *H. sericeum* (see e.g. Hofmann 1998).

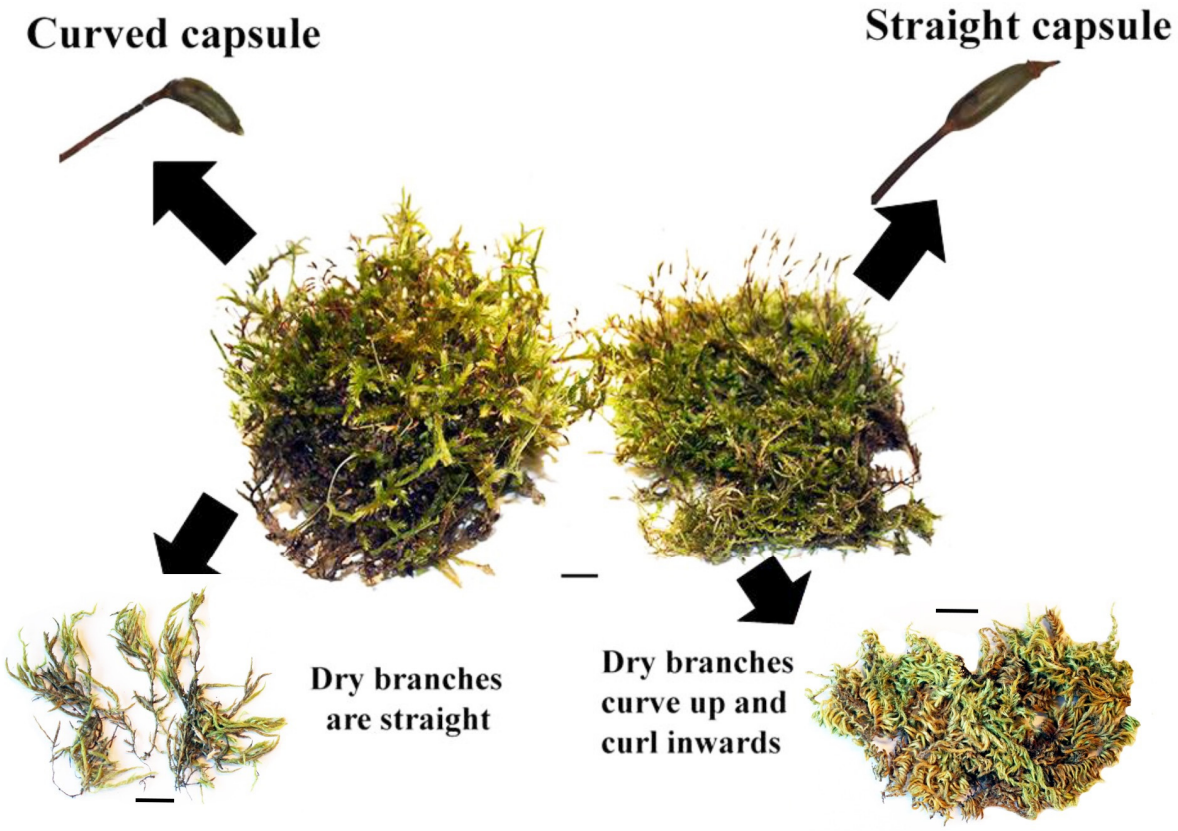


Figure 7.
Diagnostic morphological characters of *Homalothecium lutescens* and *H. sericeum*. [Scale bar = 1 cm]

A less common form of *H. lutescens* which grows closely attached to limestone rocks and boulders, is sometimes confused with *H. sericeum* according to Atherton *et al*. (2010), but may be of hybrid origin. Specimens with intermediate gametophyte morphology have been recognized as *H. lutescens* var. *fallax* by Hofmann (1998), although hybrid sporophytes have not been documented. Both species can form dwarf males as a means for reducing the fertilization distance, and Rosengren and Cronberg (2015) could experimentally show that spores of *H. sericeum* germinated well on females of *H. lutescens* and developed into fertile dwarf males, suggesting an obvious route for hybridization. Putative hybrids between *H. lutescens* and *H. sericeum* have been observed by Lars Hedenäs (pers. comm. 4 November 2014) (**Figure 8**) in Mittlandsskogen, Öland, where the two parental species occur in sympatry (mixed populations) along limestone walls.

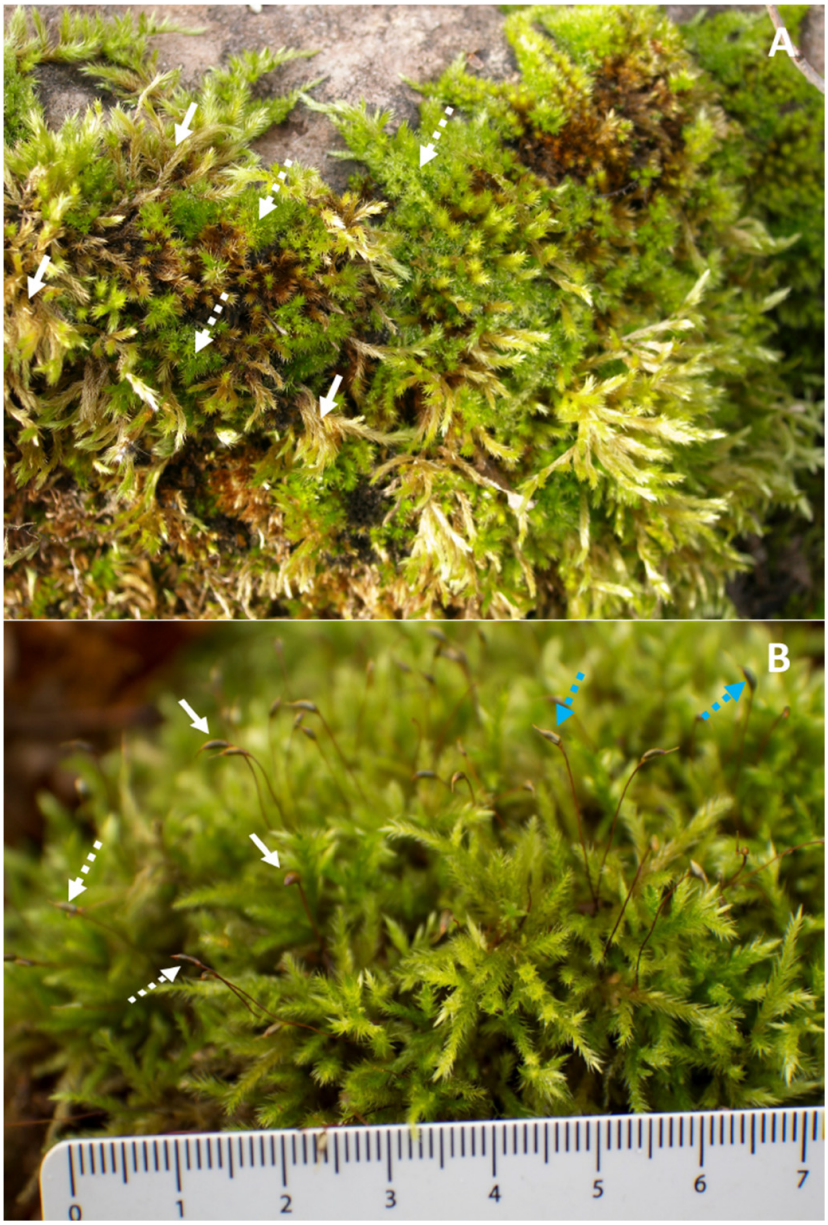


Figure 8.
A. Shoots of *H. lutescens* (white solid arrows) and *H. sericeum* (white broken arrors) grow in close vicinity in one sympatric population on the Baltic island of Öland (Photo: Nils Cronberg). B. Aberrant capsule shapes (blue broken arrows) were frequently found where *H. lutescens* with sharply curved capsules (white solid arrows) and *H. sericeum* with straight capsules (white broken arrows) were growing together.

B. Liverwort species

Marchantia polymorpha L. is a dioicous thalloid liverwort found worldwide in temperate and tropical regions (Bischler-Causse 1989; Bishler-Causse and Boisselier-Dubayle 1991). It is easily recognized by its large thallus with air chambers extending over the entire ventral surface, reaching the thallus margin (Siregar *et al*. 2013). At present, three subspecies of *M. polymorpha* L. are recognized on the basis of morphological characters, isozyme patterns and

ecological preferences: *M. polymorpha* L. subsp. *polymorpha*, *M. polymorpha* L. subsp. *montivagans* and *M. polymorpha* L. subsp. *ruderalis*. The subsp. *polymorpha* is characterized by continuous black median lines present on the dorsal side of thallus and growing in natural riparian habitats. The subsp. *montivagans* is lacking blackish median lines and growing in base-rich waterside habitats in higher elevations. The subsp. *ruderalis* has a discontinuous blackish median lines on the thallus and grown in anthropogenic or disturbed sites (review in Shimamura 2016). In theory, *Marchantia* is relatively easy to cross experimentally – because of its short life cycles, unisexual shoots and easy culturing in greenhouse environment. Burgeff (1943) presented data, which indicated that some crosses resulted in hybrid sporophytes with more or less fertile spores, while other crosses failed completely. He also saw strong differences between reciprocal crosses. Burgeff drew the conclusion that the taxa should be recognized at the species level, and postulated that the ruderal species (now: subsp. *ruderalis*) (**Figures 9** and **10**) is a stabilized hybrid involving the two other species (now: subsp. *montivagans* and subsp. *polymorpha*). Our aim was to repeat crosses between the subspecies, in order to analyse the progeny with molecular methods. This failed, primarily because it turned out to be difficult to bring the subspecies into fertile condition simultaneously under artificial light. To answer whether hybrids exist between the taxa or not, the three subspecies of *M. polymorpha* L. were instead compared by different analytical methods at the whole-genome level.

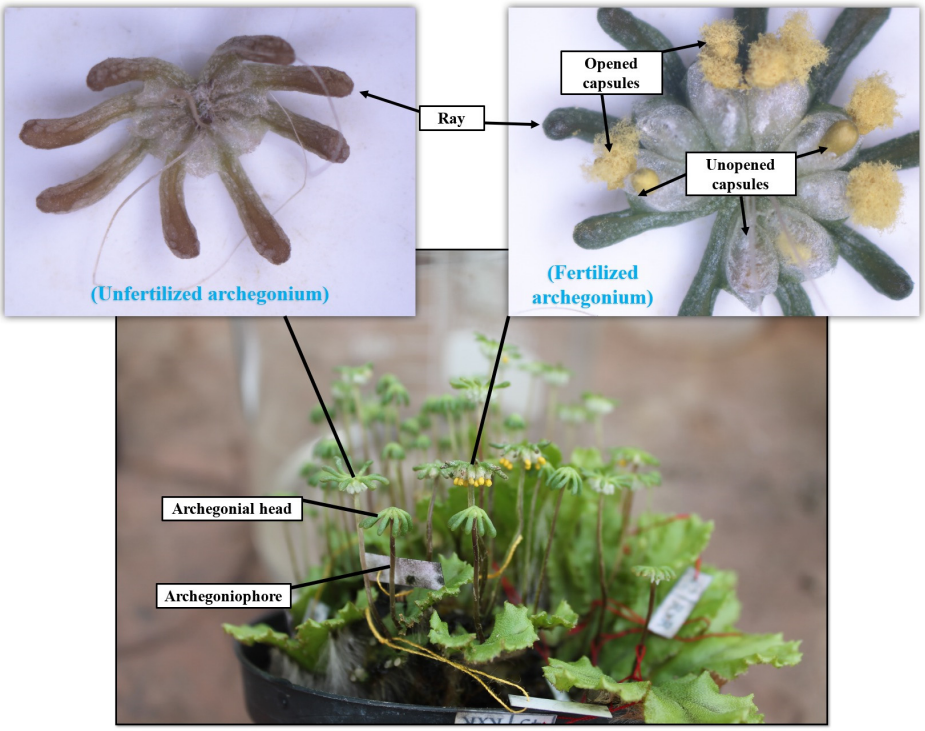


Figure 9.
Female gametophyte of *Marchantia polymorpha* L. subsp. *ruderalis* during reproductive stage. The plant collected from a locality at Brösarp (Tomelilla Municipality, Skåne).

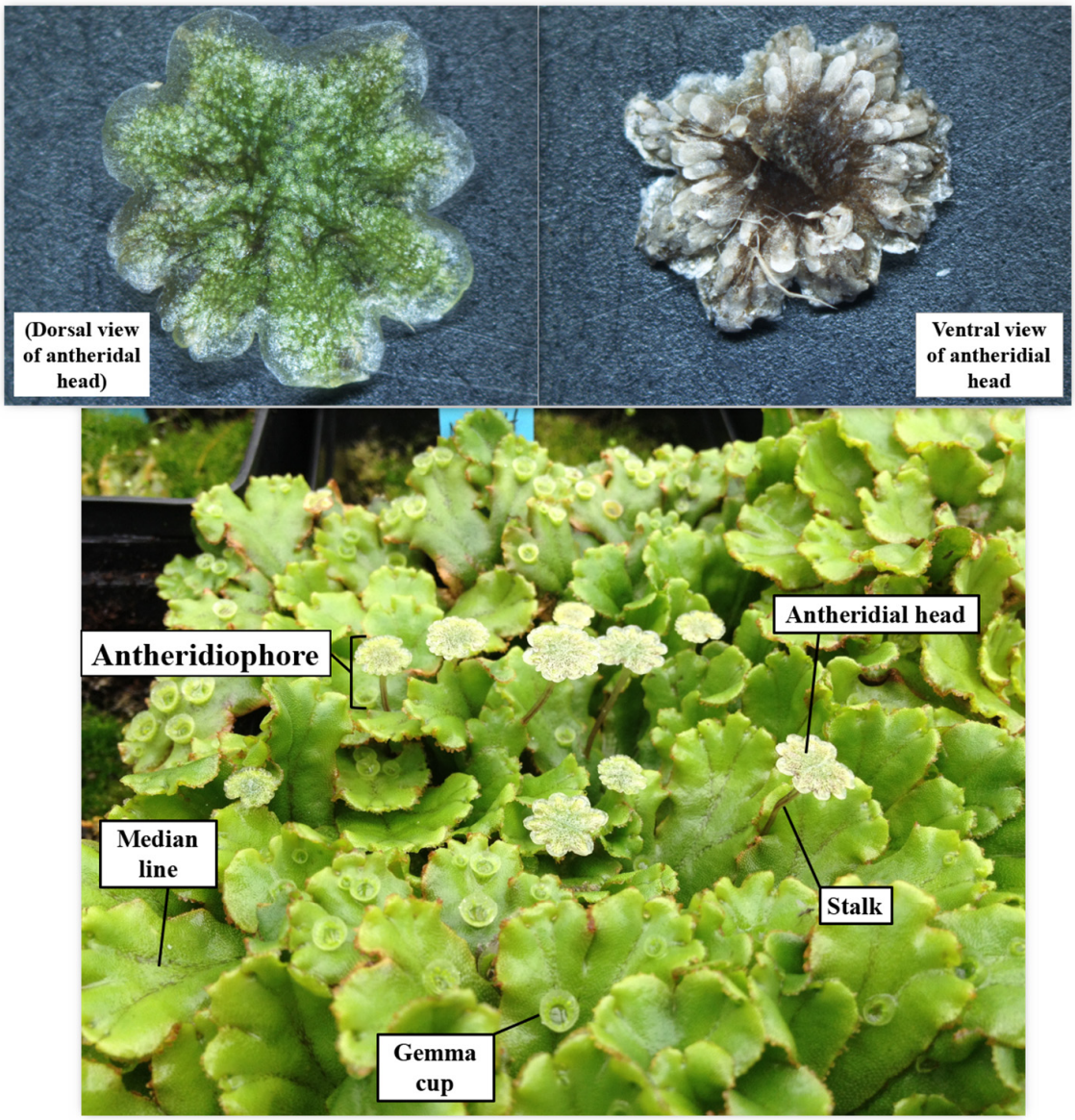


Figure 10.
Male gametophyte of *Marchantia polymorpha* L. subsp. *ruderalis* during reproductive stage. The plant collected from a locality at Brösarp (Tomelilla Municipality, Skåne).

Field work

Sexual reproduction of *Homalothecium* spp. occurs in Sweden in May/June, and the spores are dispersed around February/March the following year (Arnell 1875). In this study, material (representing allopatric and sympatric populations) used in **Paper I**, **II** and **III** were collected from different localities in southern Sweden, including Öland and the mainland region of Skåne (**Table 1**). The first sample set (HB1-7, HL1-2, and HS1-3) was used for DNA extraction of the gametohytes and, when present, separate extraction of associated sporophytes (seta + capsule)

(**Paper II**). Shoots with unopened ripe capsules of hybrid populations were sampled at points located every two meters along limestone walls (as line transects) at seven sites (HB1-7) in dry, semi-natural grassland on the Baltic island of Öland, where the two parental species, *H. lutescens* and *H. sericeum* grow sympatrically (shown as blue line transects in **Figure 11**). The length of line transects varied depending on the presence of sympatric populations of the two parental species. Shoots of allopatric populations representing the parental species were sampled from Öland: 2 localities for *H. lutescens* (HL1-2) and 3 localities for *H. sericeum* (HS1-3) (**Figure 11**). The second sample set was used for morphogical studies and spore germination. Sporulating gametophyte colonies with aberrant growth form such as irregular and asymmetric branching pattern different from both *H. lutescens* and *H. sericeum* were collected separately adjacent to the transect lines from the sympatric populations on Öland (shown as green circles in **Figure 11**). The gametophytes and associated sporophytes in this dataset were used for morphogical studies (**Paper I**) and spore germination (**Paper I**, **II** and **III**). The remaining setae from spore germination were use individual DNA extraction for identifying hybrid sporophytes (**Paper I** and **III**). The third sample set was collected from allopatric populations of *H. lutescens* (HL3-6) and *H. sericeum* (HS4-5) from the mainland region of Skåne. Shoots of these populations represented reference populations (**Paper I**) in which hybridization is less likely since sympatric populations are rare. Sampes 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 (HS4) and various kinds of stone walls (HS5), whereas *H. lutescens* came from the ground in former limestone quarries gravel pits (HL3-6).

Table 1.
The study sites of allopatric and sympatric populations of *H. lutescens* and *H. sericeum*.

Population	Locality	Geographic coordinates	Habitat	Sample size	Date of sampling
Sympatric population: *H. lutescens* and *H. sericeum*					
HB1	Next to Station Linné (Öland)	N 56º 37′ 08′′ E 16º 29′ 52′′	Limestone wall	24	03-Nov-14
HB2	Near Arontorp Nature Reserve (Öland)	N 56º 38′ 42′′ E 16º 33′ 22′′	Limestone wall	38	04-Nov-14
HB3	Near Arontorp Nature Reserve (Öland)	N 56º 38′ 37′′ E 16º 36′ 02′′	Limestone wall	19	04-Nov-14
HB4	Near Bostorp (Öland)	N 56º 38′ 16′′ E 16º 35′ 03′′	Limestone wall	15	04-Nov-14
HB5	Near Kåtorp (Öland)	N 56º 36′ 48′′ E 16º 33′ 60′′	Limestone wall	11	05-Nov-14
HB6	Near Lenstad (Öland)	N 56º 36′ 40′′ E 16º 34′ 59′′	Limestone wall	12	05-Nov-14
HB7	Near Lenstad (Öland)	N 56º 36′ 44′′ E 16º 34′ 27′′	Limestone wall	2	05-Nov-14
			Total	121	
Allopatric population: *H. lutescens*					
HL1	Gårdby Sandhed (Öland)	N 56º 37′ 02′′ E 16º 38′ 46′′	Calcareous grassland	10	04-Nov-14
HL2	Near Lenstad (Öland)	N 56º 36′ 42′′ E 16º 34′ 26′′	Calcareous grassland	10	05-Nov-14
HL3	Bjärsjölagård (Skåne)	N 55º 43′ 33′′ E 13º 42′ 17′′	Limestone quarry	10	10-Sep-14
HL4	The Klagshamn peninsula (Skåne)	N 55º 31′ 18′′ E 12º 54′ 10′′	Calcareous grassland	10	10-Sep-14
HL5	Käglinge (Skåne)	N 55º 32′ 06′′ E 13º 04′ 19′′	Calcareous grassland	10	10-Sep-14
HL6	Arrie (Skåne)	N 55º 31′ 21′′ E 13º 06′ 07′′	Calcareous grassland	10	10-Sep-14
			Total	60	
Allopatric population: *H. sericeum*					
HS1	Torslunda (Öland)	N 56º 37′ 59′′ E 16º 30′ 53′′	Limestone wall	14	04-Nov-14
HS2	N. Möckleby (Öland)	N 56º 38′ 51′′ E 16º 40′ 48′′	Limestone wall	14	04-Nov-14
HS3	Gårdby kyrka (Öland)	N 56º 36′ 00′′ E 16º 38′ 12′′	Limestone wall	7	04-Nov-14
HS4	Dalby (Skåne)	N 55º 40′ 29′′ E 13º 19′ 53′′	Tree trunk	13	21-Feb-15
HS5	Övedskloster (Skåne)	N 55º 68′ 74′′ E 13º 63′ 00′′	Limestone wall	11	21-Feb-15
			Total	59	
			Grand total	**240**	

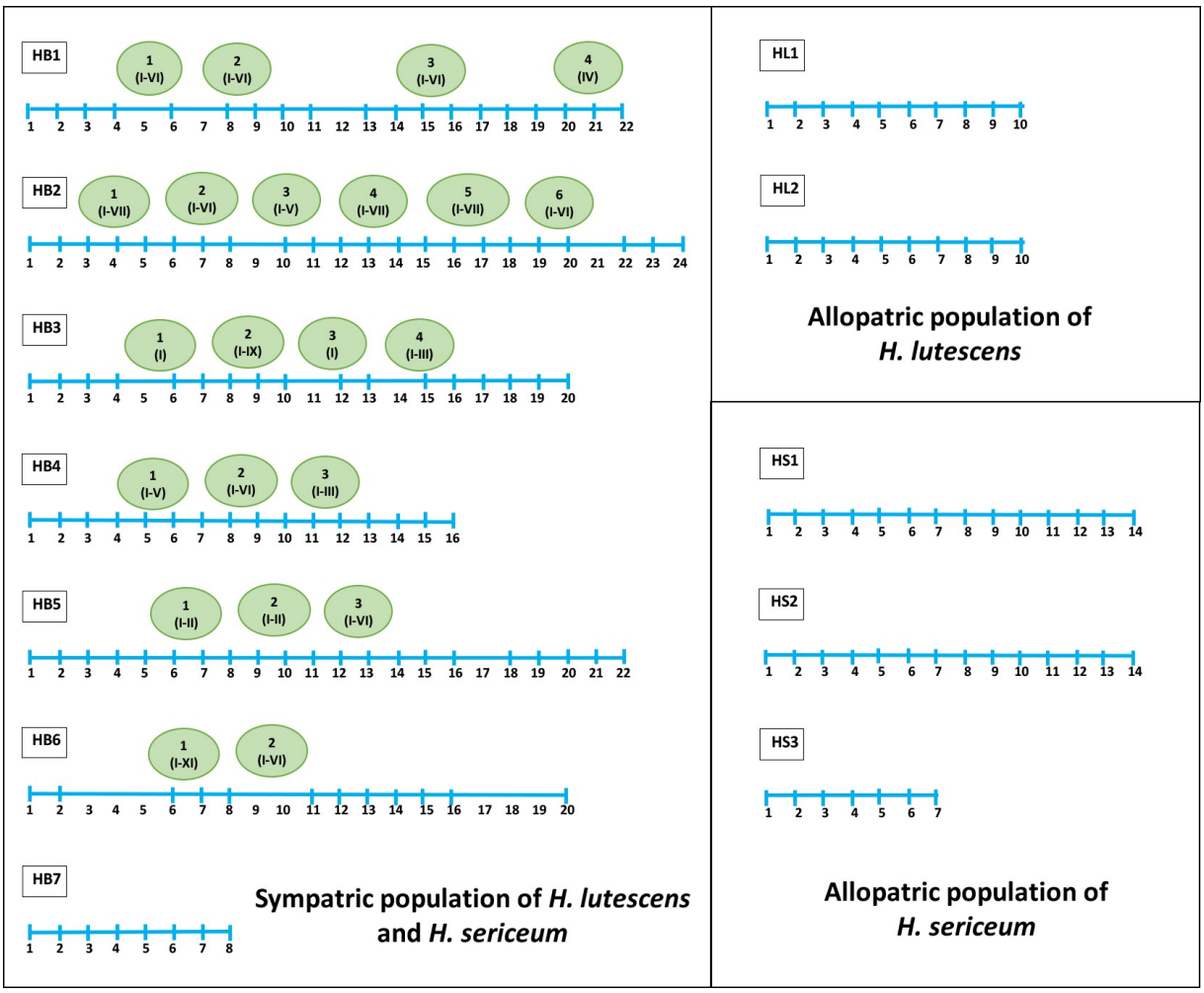


Figure 11.
Gametophyte and sporophyte samples for the first sample set were collected along blue-colored line transects from allopatric populations of *H. lutescens* (HL1-2) and *H. sericeum* (HS1-3) and from sympatric populations (HB1-7). Additional sporulating gametophyte colonies with aberrant growth form adjacent to transect lines included in the second sample set were collected separately (green colored circles). Numbers of putative hybrid sporophytes from such colonies are indicated by roman numerals.

Laboratory work

Shoots sampled from pure populations of parental species and mixed, putatively hybridizing populations were brought back to the laboratory for morphological studies and spore germination analysis. The objectives were i) to investigate morphological variation of branch leaves and capsule inclination in relation to the degree of admixture as indicated SNP markers (**Paper I**), ii) to detect gene transfer between *H. lutescens* and *H. sericeum* in samples collected from sympatric populations in three major life cycle stages (i.e., mother gametophytes, attached sporophytes, and sporelings) using SNP markers (**Paper II**) and iii) to investigate fitness parameters such as total spore count, frequency of aborted spores, spore size and germination frequency in relation to the degree of admixture (**Paper III**).

A. Morphological studies

Leaf morphometric analysis

Gametophyte shoots with capsules from the study sites (**Table 1**) were used in this analysis. To keep samples fresh prior to leaf measurement, gametophyte shoots with young capsules were kept in the growth chamber room with regular water spraying. For leaf measurement, mature branch leaves positioned about 2.5 cm below stem apices were removed and put on a microscopic slide for taking digital photos (Nikon DS-2Mv and Nikon DS-Fi1). All measurements were done using the Imaging Software NIS-Elements AR 3.0. Leaf characters included 14 direct measurements and 4 derived measurements. The characters were analysed separately by ANOVAs and the total variation was summarized in a PCA analysis in RStudio version 3.3.1 (RStudio Team 2016). In total, 240 samples were measured: 59 samples of *H. sericeum*, 60 samples of *H. lutescens*, and 121 samples from the putatively hybridizing sympatric populations (**Paper I**).

Inclination of capsules

Sporophytes from the same individuals (if sporophytes were present) as in the leaf morphology study were chosen for the study of capsule inclination. Unopened ripe capsules were characterized by dark green to brown colored capsules with opercula but no calyptra. The capsules including setae were gently removed from the maternal shoots, photographed by a digital microscope camera (Nikon-DS 2Mv). Then the inclination of capsules was measured using the free imaging software ImageJ (https://imagej.nih.gov/ij/) (Schneider *et al*. 2012) (**Figure 12**). In total, 101 sporophytes with ripe capsules from 101 shoot samples from mixed (sympatric) populations were analyzed. The capsules from the sympatric populations was compared with those from pure populations of the two parental species – *H. lutescens* (N=19) and *H. sericeum* (N=18) **(Paper I)**.

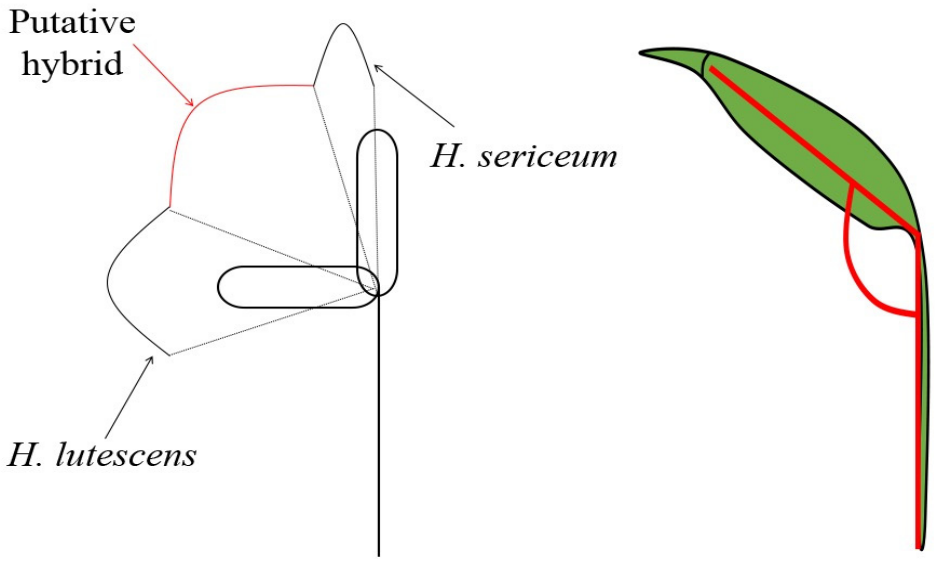


Figure 12.
Measurement of capsule inclination in *Homalothecium* specimens (in degrees) between the seta and spore capsule. The capsules with inclination between 159 and 168 degrees were considered to have putative hybrid origins. The inclination of capsules collected from pure populations of each parental species were measured to represent typical capsule curvature of the species.

B. Spore germination analysis

For each sample from the morphological study the seta and the capsule were detached from each other and put in separate 1.5 ml microtubes for DNA extraction and spore germination tests, respectively. Capsules were sterilized before spore germination, by dipping into 25 ml 1% sodium hypochlorite solution for 1 min followed by separate rinsing in sterile deionized water two times during one minute. Spore capsules were then transferred into a new microtube with 1 ml of deionized water to be opened using a sterile pestle to squeeze the capsule against the microtube wall. Spore suspensions of 10 µl was transferred by a micropipette and spread over nutrient agar in a sterile plastic Petri dish (size: 85 mm diameter x 15 mm high). New liquid nutrient solution was added onto the sporelings in the Petri dishes, when necessary, to avoid nutrient limitation. Because phosphate is a growth-limiting factor (Rudolph *et al*. 1988), we enhanced the amount of KH_2PO_4 and used a nutrient solution of double-strength as compared to the original nutrient concentration described by Simon (1988). The Petri dishes were of ventilated type and sealed by micropore tape to allow gas exchange. The spore cultures were maintained at constant temperature of c. 22º C with a photoperiod of 14:10 (Light:Dark) hrs, as most spores require > 5 hrs of light to germinate (Kinugawa and Nakao 1965), Light source was a light fixture with two Philips Master TL-D Super 80 18W/840 1SL fluorescent tubes; photon flux 39-50 µmol m^{-2} s^{-1}.

The individual Petri dishes were observed daily under a compound microscope until spores started to germinate. Once germinated, sporeling and protonemal development was captured with a DS-Fi1 digital microscope camera by placing the Petri dish upside-down for convenient observation. Spore germination frequencies (in %) were determined from photographs taken after day 7 at 25x magnification by counting germinated/ non-germinated spores. Sporelings were maintained in culture until the gametophytic stage was well developed (**Figure 13**). All gametophytes were then sampled to extract DNA for genotyping (see below) in **Paper II** and **III**.

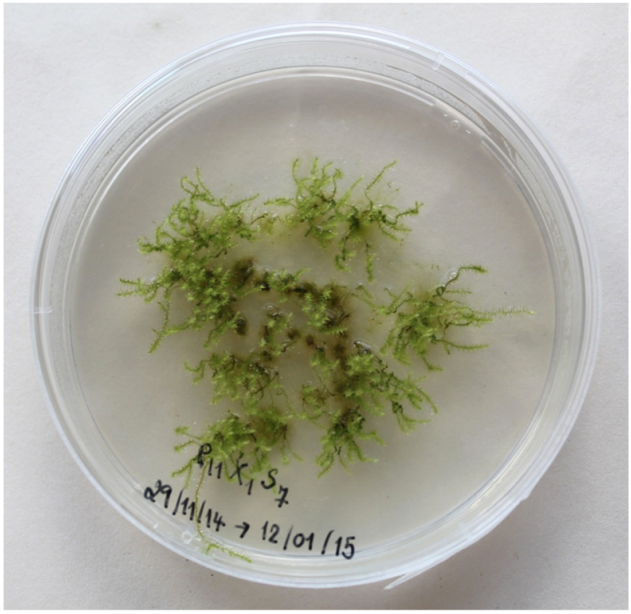


Figure 13.
Example of gametophore stage (at day 44) cultured from a hybrid capsule of one sympatric population. This stage was used for extracting DNA and molecular analysis.

C. Spore sizes and spore count

To study spore sizes and spore counts, we used parts of the remaining spore suspension prepared for the spore germination test. We captured three pictures of each spore suspension with a DS-Fi1 digital microscope camera at a 312.5x magnification. 1-32 individual intact spores without spore rupture (i.e., both viable and non-viable spores) were chosen to measure the spore size at the maximal diameter. The measurements of spore sizes were performed by ImageJ software version 1.48 (http://rsb.info.nih.gov/ij/) (Schneider *et al*. 2012). For spore count, we used a hemocytometer (Bürker-Türk) to count spores in a given volume of spore suspension (= 0.075 mm^3) under a compound microscope (at 312.5 x total magnification). All spores were counted and grouped into two categories – aborted

or viable spores. Spores that were green, papillose and circular in shape were counted as viable spores, otherwise, as aborted spores (**Paper III**).

D. SNP selection from transcriptomes

To assess the genetic relationships and degrees of genomic intermixing of the gametophytes, sporophytes and sporelings we employd SNP markers (= single nucleotide polymorphisms) developed from transcriptomes (RNA sequences). The scheme for SNP marker development is shown in **Figure 14**. SNP markers are highly efficient molecular markers because they are specifically designed to target variable DNA bases in multiple loci. SNPs refer to single base pair positions along a DNA sequence that vary between individuals (most SNPs have only two alternative states, i.e., each individual has one of two possible nucleotides at a given SNP locus; Freeland *et al*. 2011).

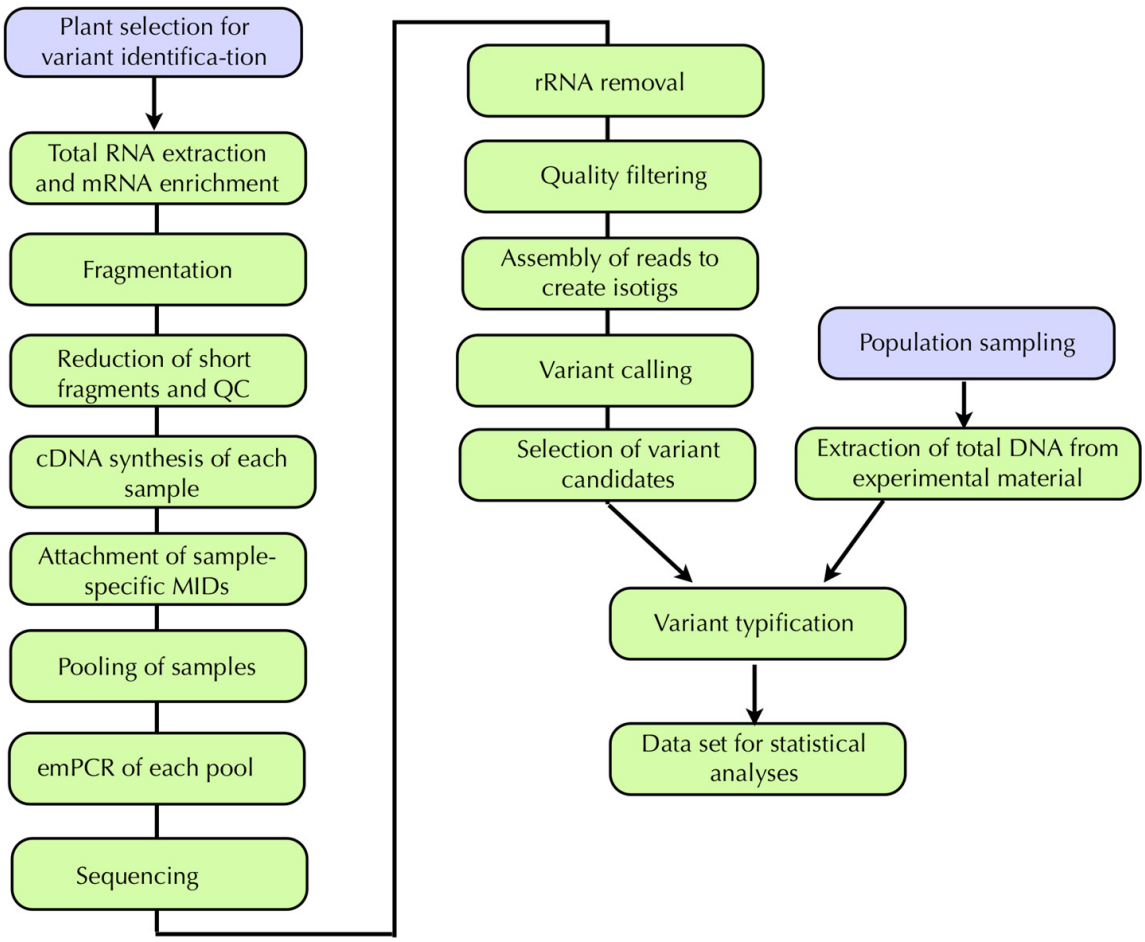


Figure 14.
A flow diagram summarizing the strategy for the SNP (variant) selection procedure and how it connects to the DNA sampling and variant typification (= SNP haplotype determination). Abbreviations: mRNA = messenger RNA, QC = quality control, cDNA = complementary DNA, MIDs = molecular identifiers, emPCR = emulsion polymerase chain reaction, rRNA = ribosomal RNA.

Samples from pure (allopatric) populations of *H. lutescens* and *H. sericeum* were collected around the province of Scania, southern Sweden (**Table 2**). Before RNA extraction, samples were acclimatized by placing them onto a moist sandy substrate in a plastic box of H18 x W33 x D40 cm for 5 days in a culture room with a full light illumination and a constant temperature of ca. 12 ºC. The boxes were aerated. All shoots with young green capsules were selected for extraction. The upper 25 mm of gametophyte apices and associated young green sporophytes (capsule + seta) of each specimen were separated for separate extraction. We extracted total RNA by using Qiagen RNeasy Plant Total RNA Kit for transcriptome analysis, following the manufacturers protocol, with the following modification: sterile sand was added to the samples to assist sample grinding and homogenization. Gametophyte and sporophyte samples were separately pooled for later construction of cDNA libraries for each of *H. lutescens* and *H. sericeum*. Six paired-end libraries were sequenced on an Illumina MiSeq Platform at the Sequencing Facility at the Department of Biology, Lund University.

In total, 111 SNP markers, expressed in both the gametophyte and sporophytes generations, were selected with species-specifc alleles for the two parental species *H. lutescens* and *H. sericeum*. The SNP markers were used to determine the degree of genomic admixture for individual samples in **Papers I**, **II**, and **III**.

Table 2.
The localities of allopatric populations of *H. lutescens* and *H. sericeum* used for total RNA extraction.

Species	Locality	Geographic coordinates (WGS84)	Habitat	Shoot samples
H. lutescens (allopatric population)	Bjärsjölagård (Skåne)	N 55º 43′ 33′′ E 13º 42′ 17′′	Limestone quarry	10
	The peninsula Klagshamn (Skåne)	N 55º 31′ 18′′ E 12º 54′ 10′′	Calcareous grassland	10
	Käglinge (Skåne)	N 55º 32′ 06′′ E 13º 04′ 19′′	Calcareous grassland	10
	Arrie (Skåne)	N 55º 31′ 21′′ E 13º 06′ 07′′	Calcareous grassland	10
			Total	**40**
H. sericeum (allopatric population)	Dalby (Skåne)	N 55º 40′ 29′′ E 13º 19′ 53′′	Tree trunk	13
	Övedskloster (Skåne)	N 55º 68′ 74′′ E 13º 63′ 00′′	Limestone wall	11
			Total	**24**

E. DNA extraction

DNA was extracted from mature gametophytes and sporophytes (only seta) (**Paper I**, **II** and **III**) and the complete sporophytes and sporelings (**Paper II**) using a standard DNA extraction kit (Qiagen DNeasy Plant MiniKit). The DNA extracts were subsequently screened for SNP variation to obtain individual haplotypes (see above). For **Paper II** about 25 sporelings (young gametophytes) per sporophyte were randomly sampled and merged into an aggregated DNA extract. If the germination rate of a capsule was lower than 30%, all sporelings were aggregated for the DNA extract.

F. SNP genotyping

SciLifeLab in Uppsala (Sweden) did the SNP genotyping of the DNA extracts. The resulting mulitilocus SNP genotypes were analyzed to assess the degree of genomic admixture in individual samples (**Paper I**, **II**, and **III**).

G. Genetic variation analysis of *Marchantia polymorpha* L.

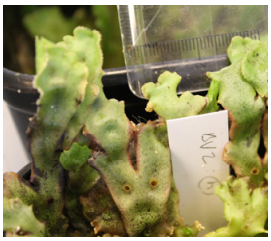
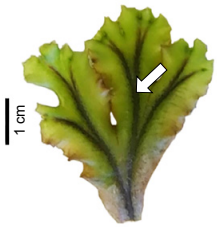
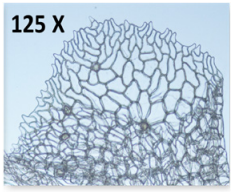
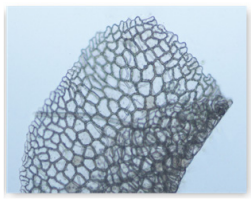
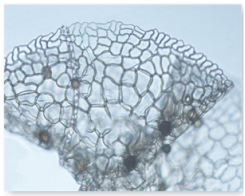
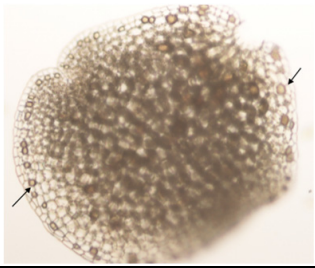
In **Paper IV**, representatives of three subspecies of *Marchantia polymorpha* i.e., *M. p. polymorpha*, *M. p. montivagen*, *M. p. ruderalis*, from various localities in Sweden and Bulgaria, were cultured and sent for DNA sequencing. The samples were determined to subspecies according to diagnostic characters pointed out by Paton (1999) and Damsholt (2002), such as thallus structure, bifurcation, median line, cells at thallus margin, median ventral scale appendages, gemma cups, marginal oil cells in gemma, archegoniophore, antheridiophores, perichaetium and perichaetium mouth (**Table 3).**

Two sample IDs (one of subsp. *polymorpha* and one of subsp. *montivagans*) were sequenced using the Single-molecule real-time (SMRT) sequencing technology developed by Pacific BioSciences (Roberts *et al*. 2013), nine sample IDs (five of subsp. *ruderalis*, two of subsp. *polymorpha*, and two of subsp. *montivagans*) were sequenced using the Illumina HiSeq X sequencing platform with paired-end reads of 2x150 bp. DNA sequencing was performed at SciLife Lab in Uppsala. We used one publicly available genome of *M. polymorpha ruderalis* from Bowman *et al*. (2017) as reference genome and used one genome of *M. paleacea* var. *diptera* as the outgroup in the analyses. The reads were assembled using HGAP (Chin *et al*. 2013). Data preparation such as alignment of genomic fragments (GFs), RNA extraction and genome annotation, alignment of coding sequences, repeat annotation, assembly of scafford into chromosomes are described in **Paper IV**.

Phylogenetic trees were reconstructed from nuclear, mitochondrial and chloroplast DNA using three different phylogenetic methods, i.e., MrBayes, neighbor joining (NJ), and RaxML methods. Introgression statistics (i.e., Pattersons D statistic,

Martins f statistic and Bd fraction) and chromosome-level comparisons (i.e., pairwise nucleotide diversity between subspecies) were calculated using the R package "PopGenome" (see more details in **Paper IV**).

Table 3.
Morphological characters of three subspecies of *Marchantia polymorpha* L. *Martantia polymorpha* L. subsp. *ruderalis* (M.p.r.) is a presumably homoploid hybrid species between *Marchantia polymorpha* L. subsp. *montivagens* (M.p.m.) and *Marchantia polymorpha* L. subsp. *polymorpha* (M.p.p.).

Character	M.p.m	M.p.r.	M.p.p.
Thallus structure	Thick, leathery, rigid, greasy green	Not leathery, green to dark green, verruculose surface	Not leathery, deep translucent green smooth surface
Bifurcation	Infrequent, with short branches	Frequent, branches less than 2 cm apart	Infrequent, branches more than 2 cm apart, sometimes monopodial
Median line	Lacking 	Present, i.e., a darker central discontinuous line where air chambers are lacking 	Present, i.e., a darker central continuous line where air chambers are lacking 
Thallus margin	Unistratose 3-5(-6) cells wide marginal 1-celled teeth	Air chambers almost to margin almost lacking entire or denticulate	Unistratose 3-7 cells wide entire margin
Median ventral scale appendages	Dentate, thick-walled marginal cells, reniform-ovate-triangularly ovate 	Dentate, reniform-widely orbicular 	Entire or crenulate, thin-walled marginal cells, reniform-widely orbicular 
Gemma cups	With 7-9 gemmae	With 7-13 gemmae	With 22-25 gemmae
Gemmae	With 50-55 marginal oil cells	With 50-60 marginal oil cells 	With 70-75 marginal oil cells
Archegoniophore	Short and stout stalks 11-13 slightly clavate rays	Relatively long stalks (8-)9(-11) terete rays	Long and slender, dark stalks 8-9 long and slender rays
Antheridiophores	Short and stout stalks	Relatively long stalks	Long and slender, dark stalks
Perichaetium	Long; longer than half the distance from the centre of the disc to the ray apex	Shorter; 0.35 of the distance from the centre of the disc to the ray apex	Shorter; rarely longer than 0.35 of the distance from the centre of the disc to the ray apex

Results and discussion

Morphometric analyses of branch leaves

The principal components analysis (PCA) based on 18 leaf morphology traits show that samples from allopatric populations of *H. lutescens* and *H. sericeum* (**Figure 15**) are morphologically well differentiated, which confirms the characters that have traditionally been used for species identification by Hofmann (1998). For instance, *H. lutescens* has longer and wider branch leaves, and denticulations at the basal leaf margin, whereas *H. sericeum* has shorter and narrower branch leaves, and conspicuous teeth at the basal leaf margin. Once the sympatric putatively hybridizing populations are included in the analysis, leaves from the sympatric populations tend to be separated from the allopatric populations of the two parental species (**Paper I**). Sympatric populations showed a high variability of quantitative leaf characters. Several leaf characters were in average intermediate as compared to sympatric populations of either species, 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. However, other characters fell out of range of both species, in particular the ratio between leaf length and width. In general, leaf specimens from allopatric populations of *H. lutescens* had the longest and widest leaf dimensions, followed in size by specimens from allopatric populations of *H. sericeum*, whereas specimens from the sympatric populations show the shortest and narrowest branch leaves (**Paper I**). The PCA revealed that 48.54% of the observed variation is explained by the first three principal components. The traits that contribute most to PC1 (explaining 19.56% of variation) are width of basal leaf cell, ratio of basal leaf cell length to width and width of medial leaf cell; the traits that contribute most to PC2 (16.45%) are width of leaf lamina, length of leaf lamina and leaf surface area. PC3 also explains a fairly high proportion of the variation (12.53%) but does not separate the pure species and the putative hybrids and may represent plasticity to environmental variation.

The term “hybrid” in bryophytes is restricted to the sporophyte (= F_1 generation) as it contains a diploid (or polyploid) genome from two different species (Shaw 2000). The progeny produced by such a hybrid sporophyte forms the F_2 generation and individuals are referred to “recombinants” (Shaw 1994). Gametophytes that are morphologically intermediate between two species have been described having hybrid origin (=being recombinants) in a number of genera (reviewed by Natcheva

and Cronberg 2004). The intermediacy of leaf morphology in our study suggests that the hybridization is introgressive between *H. lutescens* and *H. sericeum* when growing in close proximity. Introgressive hybridization may be bidirectional but admixture is more frequently biased in the direction of *H. sericeum* according to the leaf character analysis. (**Figure 16**). Because species of the genus *Homalothecium* sometimes grow sympatrically, Hofmann (1998) and Hedenäs *et al*. (2009) speculated that hybridization might play an evolutionary role within the genus. Variable gametophyte characters could suggest multiple origins and interspecific crossing in some species; 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. Hofmann (1997) made a biometrical analysis and found that branch leaves of *H. aeneum* are intermediate between *H. aureum* and *H. nevadense*, in accordance with the theory of a hybrid origin in our results from the sympatric populations of *H. lutescens* and *H. sericeum*.

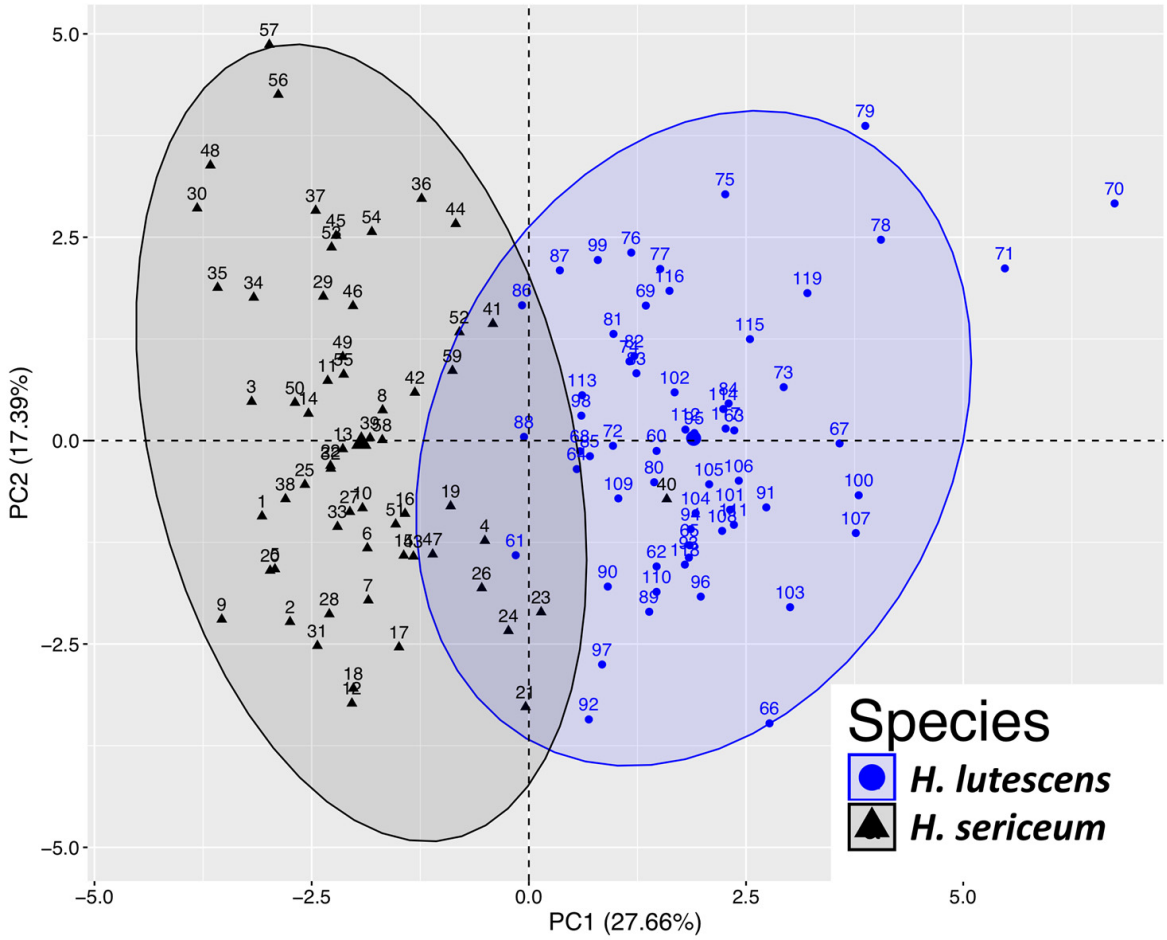


Figure 15.
Principal component analysis of 18 leaf characters from 119 specimens representing allopatric populations of *Homalothecium lutescens* and *H. sericeum*. The first two axes (PC1 and PC2) representing together 45 % of the variation. The colours and shapes of data points correspond to the population of the specimens. The encircled surfaces represent 95% confidence level of the sample means with the centre of each cluster marked by a larger symbol.

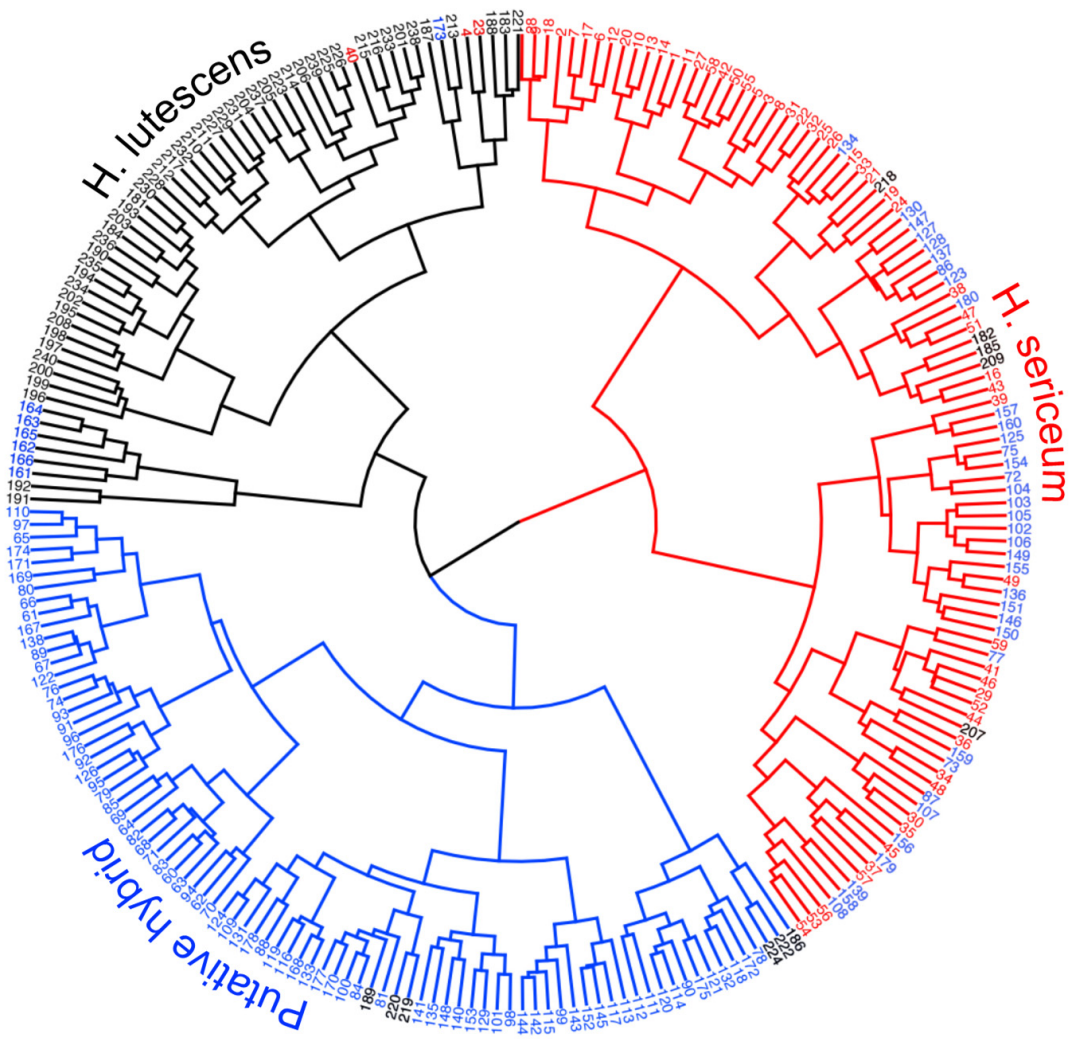


Figure 16.
Tree-based representation of the leaf morphology data created using hierarchical clustering methods implemented in the Factoextra and Dendextend packages in R Studio (Galili 2015). The three major clusters correspond to allopatric population of *H. lutescens* (black), allopatric population of *H. sericeum* (red), and putative hybrid sympatric populations (blue). Numbers are sample ID. Some individuals from the sympatric populations appear to be pure species according to leaf morphology and turns up together with allopatric *H. lutescens* or *H. sericeum*.

Morphological and genetic analyses of sporophyte capsules

Morphological analysis of capsule inclination from allopatric populations show that capsules of *H. lutescens* are orthogonal (horizontal) to homotropous (inclined) (125-154 degrees) whereas the capsules of *H. sericeum* are almost orthotropous (erect) (152-180 degrees), in accordance with the generally accepted species characteristics (see Hofmann 1998). However, in the sympatric populations, there is more variation in capsule inclination ranging from strongly homotropous to

orthotropous (122-180 degrees). The intermediate capsule inclination (c. 150-160 degrees) is out of the capsule inclination range of the allopatric populations of both species (**Paper I**). This suggests that there is hybridization and maybe also introgression occurring between taxa of *H. lutescens* and *H. sericeum,* when growing in close proximity.

We confirmed admixture in sporophytes collected from sympatric populations by using the 85 species-specific nuclear SNP markers (**Paper I**). The SNP markers in 100 capsules samples indicated 53 as pure *H. lutescens*, 14 sporophytes as pure *H. sericeum* and 33 as admixted with most of the SNP alleles from one species and a limited number of alleles from the other species. Overall, admixed sporophytes showed biased SNP markers towards either *H. lutescens* (22 sporophytes = 67%) or *H. sericeum* (11 sporophytes = 33%). However, we found five sporophytes to be primary hybrids based on the SNPs from the progeny (after finishing **Paper I**) and capsule inclination of such sporophytes was typical for *H. lutescens* (**Paper II** and **III**). Observation of hybrid sporophytes produced in hybrid zones seems to be rare, even in zones where hybrids are abundant (cited by Shaw in Shaw 2000). Similarly, molecular studies of hybrid zones in angiosperms also indicate that the actual formation of F_1 hybrids seems to be a rare event, even in areas where hybrids are abundant (Arnold 1997). In primary hybrids complete heterozygotic expression is expected, which means that the sporophyte should display intermediate character states compared to the parental species, especially if the sporophyte traits are controlled by few genes with co-dominant expression. However, intermediate inclination was common among individuals in the sympatric populations, even among those that did not show admixture in their SNP genotype. Therefore, intermediate capsule inclination in *Homalothecium* is an indication of diffuse introgression rather than primary hybridization. Largely non-overlapping indications of admixture from leaf morphology, sporophyte inclination and SNP markers suggest that the populations are subject to extensive introgression affecting different parts of the genome in different individuals.

DNA analyses of specimens from allopatric and sympatric populations

Whether or not hybridization is common, the ability of gene regions to pass across species bourdaries is often determined by many factors including genetic architecture, the fitness value of particular gene regions, and genotype-evironment interactions (Freeland *et al*. 2011). We compared multilocus SNP genotypes to estimate the degree of genetic mixing in 449 plants collected from both allopatric and sympatric populations on Öland, using 1) STRUCTURE 2) Principal Coordinate Analysis (PCoA), and 3) a simple hybrid index **(Paper II)**. The plant

material represented three generations of *Homalothecium* mosses: haploid maternal gametophytes, diploid sporophytes, and haploid sporelings. A majority of plant specimens were identified as pure *H. lutescens* or pure *H. sericeum*, but 93 samples (out of 449; 21%) were identified as admixed. Although mildly admixed samples were more abundant (76 out of 93; 82%), strongly admixed samples (17 out of 93; 18%) occurred in all generations and in most of the sympatric populations. Surprisingly, mildly admixed genotypes were also detected in a few sporophytes from allopatric populations of *H. lutescens* and *H. sericeum*. This confirmed that hybridization and introgression commonly occurs in mixed populations and sometimes also in pure populations. Hybridization and introgression is bidirectional – meaning that either *H. lutescens* or *H. sericeum* act as both maternal and paternal plants. We prove that admixed genomes can be transferred between the generations, so that the populations behave as true hybrid zones. The majority of admixed specimens are mildly admixed in our study, which is similar to the two previous studies in the hybrid zones of mosses (Shaw 1998; Natcheva and Cronber 2007). For example, the isozyme study of hybrid zones of copper mosses, *Mielichhoferia elongata* and *M. mielichhoferiana* showed that recombinant gametophytes, growing in mixed populations, are on average skewed in the direction of the pure *M. mielichhoferiana* parental type and that sporophytes appear to belong exclusively to gametophytes of *M. elongata* (Shaw 1998). The other population level study of bryophyte hybrid zones, involving the peat mosses *Sphagnum capillifolium* and *S. quinquefarium,* found that progenies from hybrid sporophytes were mainly mildly admixed, skewed in the direction of one of the parental species (Natcheva and Cronberg 2007).

Gene transfer across generations

Overall, the admixed/misplaced alleles came from a limited number of loci in the mildly admixed individuals, suggesting that gene transfer between species had occurred more readily at certain loci (genes) **(Paper II)**. It is well documented that gene exchange between species may not be uniform in space and time, across the genome. Harrison and Larson (2014) emphasize that species boundaries are semipermeable – meaning that gene exchange is a function of genome region or introgression is differential in hybrid zones. Our genetic data suggest that species boundaries in *Homalothecium* are semipermeable and this may prevent complete fusion of the species. However, it should be noted that our SNP markers are selected to be species-specific, therefore we do not know to what extent other genes are transferred between species. It is also possible that the relatively few strongly admixed specimens are subject to hybrid depression dependent on break up of adaptive gene complexes that are necessary for survival in the typical habitats of either species (reviewed in Todesco *et al*. 2016).

Genomic comparisons of maternal gametophytes and attached sporophytes

We found that 14 out of 50 sporophytes (28%) were identified as admixed in the sympatric populations (**Paper II**). All admixed sporophytes possessed heterozygotic allele(s) and/or occasional homozygotic allele(s) that differed from their maternal gametophytes and were diagnostic for the other parental species. The majority of admixed sporophytes (8 out of 14) displayed mildly admixed genomes dominated by one of the parental species, whereas the remaining 6 sporophytes displayed strongly admixed genomes, with more or less equal numbers of SNP markers from both parents. Surprisingly, we also found nine mildly admixed sporophytes in the allopatric populations (1 out of 8 sporophytes in a population of *H. lutescens* and 8 out of 35 sporophytes from three separate populations of *H. sericeum*), which displayed a 1-2 "misplaced" alleles in each sporophyte as revealed by the hybrid index analysis. None of these alleles were found in the maternal shoots from the allopatric poulations.

According to the paternity analysis, the paternal parent is in most cases inferred to be similar to the maternal parent, and thus referred to the same species, but sometimes mildly admixed, providing 1-2 alleles specific to the other species. In several of the strongly admixed sporophytes, the female parent is a pure species (either *H. lutescens* or *H. sericeum*) and the male appear to belong to the other species. The contribution to sporophyte (F_1 generation) by *H. sericeum* as the paternal plant varied from 1% in mildly admixed sporophytes to 68% in strongly admixed sporophytes, while the contribution of *H. lutescens* as the paternal plant varied from 2% in mildly admixed sporophytes to 43% in strongly admixed sporophytes.

Genomic comparison of sporophytes and recombinants/ sporelings

The analysis of genome in sporelings germinated from 81 sporophytes from sympatric populations (**Paper II**) showed that the aggregated SNP genotypes of sporelings were mainly identical to the diploid genotypes of parental sporophytes. It means that the majority of alleles in the sporophyte were transmitted to their sporelings. The admixed sporophytes/sporelings have genomes mainly biased towards one of the parental species – except five sporeling aggregates from one patch collected from one sympatric population (HB2) that displayed haplotypes homozygous for alleles typical for both *H. lutescens* and *H. sericeum* together with heterozygotes, with more or less equal represenation of both species.

Effects of breeding systems

There are three possible explanations for the presence of mildly admixed specimens, having genomes biased towards either *H. lutescens* or *H. sericeum*: Back-crossing through normal-sized males, back-crossing through repeated son-mother fertilization, and selective survival of admixed progeny (**Paper II**). In species with facultative nannandry, such as the *Homalothecium* species, fertilization is caused by either sperm from normal-sized males or that from dwarf males that growing as tiny epiphytes on normal-sized females (**Figure 17**). The normal-sized males are exceedingly rare in comparison to dwarf males in *H. lutescens* (Rosengren *et al*. 2014) and sexual reproduction involving dwarf males is probably the most common for both species. The chance for a male spore to germinate if it lands on a female is much higher than if it lands on an abiotic subtrate, and as an epiphyte on the female it is likely be buffered from some of the abiotic stress factors. On the other hand, the alleles carried by the dwarf male in a son-mother cross have the best chances to enter the population of normal-sized gametophytes by female spores, if normal-sized males are scarce. In general, bryophyte spore dispersal is known to be strongly leptokurtic, meaning that a majority of spores are deposited in close vicinity to the female (Longton 1976). This tendency may be increased by the "hygrochastic" peristome type present at least in *H. sericeum* – meaning that spores are dispersed under relatively humid conditions (Zanatta *et al*. 2018). It is not known if *H. lutescens* also has a hygrochastic peristome, but in general *H. lutescens* is relatively less specialized to dry habitats (Rosengren *et al*. 2014). Short-distance spore movement may increase the incidence of son-mother fertilizations in a nannandric system. Repeated son-mother fertilization is relatively frequent in *H. lutescens* (Rosengren *et al*. 2016). If repeated son-mother fertilization occurs, it reduces the genetic variability so that the males becomes identical or nearly identical to the females by loss of 50% of the paternal alleles for each cycle. To sum up, we propose two alternative explanations for the presence of mildly admixed specimens: 1) selective survival of mildly admixed haplotypes at sporeling stage (see genomic comparison of sporophytes and recombinants) as a consequence of genomic incompatibilities or 2) loss of paternal alleles through repeated son-mother fertilization.

Figure 17.
Dwarf male of the moss *H. sericeum* (white arrow) grows on the normal-size female shoot. **Photo**: Lars Hedenäs.

Fitness of hybrid progeny

Hybridization is frequent in many organismal groups, but its role in adaptation is poorly understood (Rieseberg *et al*. 2003). Hybridization can have a substantial impact on hybrid progeny because it involves the transfer of genes or large parts of the genome from one species to another. The genetic reshuffling can have both positive and negative consequences (Freeland *et al*. 2011). Gene flow between genomes from different species can lead to the break-up of epistatic gene complexes, which results in a decrease in fitness of progeny following a process known as "hybrid breakdown". In contrast, gene recombination can create new combination of beneficial alleles that lead to high fitness traits in hybrid individuals (Freeland *et al*. 2011). Hybrid sporophytes in bryophytes are often reported to be almost completely or completely sterile. The sterility of hybrid sporophytes may result from three different phenomena: maternal effects, segregation distortion and differential survival (Natcheva and Cronberg 2004). In **Paper III**, we compared the fitness of capsules with degrees of genetic admixture

derived from sympatric populations of *H. sericeum* and *H. lutescens*. Again, we used the 85 species-specific nuclear SNP markers to identify the degree of admixture. We also used capsule inclination score and failure rate (no detection) of SNPs in the sporophyte (seta samples) and in the progeny as alternative indicators for hybridization. This allowed us to assess fitness traits in terms of spore abortion rate, spore size, total spore count and germination frequency in the progeny in relation the indicators of hybridization. From the SNP markers, we found that the majority of capsules showed no admixture. Of the 106 sporophytes analysed, 38 were identified as admixed (33 mildly admixed and five strongly admixed). The distribution of maximal spore diameters derived from the pooled data for each capsule type (degree of admixture) is exemplified in **Figure 13**. Our analyses of fitness traits show that the mildly admixed *H. lutescens* tends to show a higher percentage of aborted spores than the others, whereas, the mildly admixed *H. sericeum* have larger spore size, and the strongly admixed sporophytes show high spore count and intermediate spore diameter. These findings suggest that hybrid progeny show differential fitness dependent on the degree of admixture and dependent on which of the parents that have contributed most of the genome to the mildly admixed sporophytes. Progeny derived from the strongly admixed sporophytes showed higher viability and higher fitness than spores from those of parental species and mildly admixed ones.

Genetic variation analysis of *Marchantia polymorpha* L.

Morphological inconsistency of the three subspecies of *Marchantia polymorpha* L. collected from a number of populations suggests hybridization and possibly introgression. Preliminary data also suggest that sympatric populations are more common than earlier assumed. In **Paper IV**, genome-wide phylogenetic reconstructions displayed in general a clear separation of the three subspecies with high support values – meaning that samples from the same subspecies according to their morphology are genetically similar to each other but are genetically different from samples from the different subspecies. The high frequency of supported trees for all three possible topologies suggested a similar divergent age of the three subspecies and frequent incomplete lineage sorting (ILS), possibly accompanied by hybridization and introgression.

The genome-wide pattern seen in the phylogenetic analysis showed that chlomosome 2 has a distinct evolutionary history for subsp. *montivagans* showing a strikingly higher nucleotide divergence than the two subspecies. The higher divergence is observed over a large section of the chromosome 2 except at one end where it is similar to the rest of the genome. This could either be explained by a hybridization event between subsp. *montivagans* and a more distantly related unknown species leading to the capture of chromosome 2 from the foreign species

or by extensive hybridization between the subspecies in combination with protection from recombination at chromosome 2. The latter explanation is supported by a somewhat higher rate of chromosomal restructuring at chromosome 2 in subsp. *montivagans*.

Our data refute the hypothesis proposed by Burgeff (1943) and Schuster (1992) that subsp. *ruderalis* is a recent homoploid hybrid between subsp. *montivagans* and subsp. *polymorpha.* At a genome-wide scale, no clear evidence of introgression was detected except for two samples; one sample of subsp. *montivagans* (MpmBU3) and another sample of subsp. *polymorpha* (MppBV1) in restricted parts of their genomes, a segment of chromosome 1. In these cases, introgression appears to involve subsp. *polymorpha* and subsp. *montivagans* in the first case, and subsp. *montivagans* and subsp. *ruderalis* the second case. At the respective sampling localities, the relevant two subspecies occurred in sympatry.

Due to the limited number of sampled individuals per subspecies, it was difficult to differentiate between ILS and recent hybridization. Probably, hybridization has happened frequently in the past with some chrosomosomal regions more likely to recombine than others across the entire genome. Studies of vascular plants suggests that foreign DNA fragments are rapidly diluted through backcrossing and recombination. Scascitelli *et al*. (2010) analysed several natural populations of the sunflowers *Helianthus anuuus* ssp. *annuus*, *H. debilis* and the hybrid subspecies, *H. annuus* spp. *texanus*, using 88 microsatellite loci distributed across the genome. Analysis of all loci showed that between 14% and 27% of individuals in each of the populations represent recent immigrants from one of the other taxa. Overall, introgression from *H. annuus* to *H. debilis* was greater than *H. debilis* to *H. annuus*. However, they found one allele at which there was no introgression from *H. annuus* to *H. debilis*, but very high levels of introgression from *H. debilis* to *H. annuus*. The authors reached conclusions that several small genomic regions show low porosity and higher than expected levels of differentiation, which means these regions are not subject to gene flow between the two species. Similarly, Tang *et al*. (2010) analysed artificial hybrids produced by backcrossing a F_1 hybrid to parental species (i.e., *Iris fulva* and *I. brevicaulis*) and found that alleles from *I. fulva* were overrepresented compared to alleles from *I brevicaulis*, indicating that gene flow is more likely to occur from *I. fulva* to *I. brevicaulis* than vice versa.

Conclusions

Almost all studies of hybrid zones deal with organisms with a diplontic life cycle (e.g. animals) or with a haplodiplontic life cycle dominated by the diploid generation (e.g. vascular plants). Our results indicate that hybridization also play an important role in the evolution of bryophytes which have a life cycle dominated by the haploid generation.

Based on the literature survey, hybridization is widespread both in diploid-dominant organisms and in haploid-dominant organisms. In bryophytes, records of putative hybridization increased considerably after the advent of molecular tools, especially in cryptic or nearly cryptic species such as peat mosses. More cases of hybridization are likely to be discovered in the future based on molecular inferences, especially when a larger number of sequences or marker genes are studied, so that reticulations are easier to observe.

Our morphological studies proved that two closely related pleurocarpous mosses *H. lutescens* and *H. sericeum* are incompletely reproductively isolated and that interspecific hybridization and introgression occur when they grow in sympatry. The sympatric populations behave as true hybrid zones. Analysis of branch leaves of gametophytes collected from sympatric populations shows intermediate morphology in a number of characters but generally smaller in size in comparison to those of either *H. lutescens* or *H. sericeum* collected from 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 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 possibly produce viable hybrid spores. Stronger morphological affinity of branch leaves to one or the other of the parents of admixed individuals and asymmetrical SNP markers in sporophytes sometimes suggest bidirectional hybridization and introgression.

Hybridization and introgression are signaled in specimens in all three generations (e.g. maternal gametophytes, sporophytes, and sporelings) in almost all of sympatric populations and some sporophyte specimens of allopatric populations. Gene transfer is more common in certain loci (genes) than the others. F_1 hybrid capsules formed after spontaneous hybridization are fit and spores derived from F_1

hybrids are capable of germination and gametophore formation. After all there were sporophytes that seemed to be aborted or had non-viable spores. Admixture in sympatric populations has previously been demonstrated in a few cases in bryophytes, but without evidence for gene transfer between life stages. Our studies are the first to prove that gene transfer between generations (e.g. maternal gametophytes to sporophytes to sporelings).

Hybrid porophytes from sympatric populations of the *Homalothecium* species expressed different degrees of genetic admixture – mainly mildly admixed and occasionally strongly admixed. Admixed sporophytes show differential fitness in terms of spore count, percentage aborted spores, percentage spore germination, and spore sizes. Most of admixed sporophytes did not show complete spore germination failure. The sporophytes of mildly admixed *H. lutescens* tend to show lower fitness (i.e., smaller spore size, fewer spores and higher number of aborted spores than those of either parental species), which can be interpreted in terms of hybrid depression. The sporophytes of mildly admixed *H. sericeum* show signs of heterosis (i.e., larger spores and higher spore germination than those of either parental species). Although sporophytes with strong admixture were rare, such sporophytes showed high fitness (i.e., fewer aborted spores, higher total spores, and higher spore germination rate than those of either parental species) and displayed intermediate spore size to the two parental species.

Finally, our large-scale phylogenetic analysis of the *M. polymorpha* species complex showed that three distinct taxa diverged independently in a short period of time in the past; subsp. *montivagans* diverged first and the other two subspecies were resolved as sister species. Our study therefore disproves the hypothesis that subsp. *ruderalis* is a recent homoploid hybrid between subsp. *montivagans* and subsp. *polymorpha*. A strong representation of all three gene tree topologies across the genomes suggests frequent incomplete lineage sorting (ILS) and/or recent hybridization and introgression in *Marchantia polymorpha* L. Future studies including (1) individuals from more populations, (2) comparison of individuals at population level (similar to our studies of *Homalothecium*) and (3) reciprocal crossing experiments at subspecies level, may shed light on the relative importance of ILS and hybridization.

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The author, Weerachon Sawangproh (WS), collecting *Homalothecium* mosses growing on a stone wall from a mixed population during fieldwork on the Baltic island of Öland (Sweden) in November 2015.
Photo: Lars Hedenäs.

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Paper III Sawangproh, W. & Cronberg, N. Fitness of progeny from hybridizing populations of the bryophytes *Homalothecium lutescens* and *H. sericeum*. Manuscript.

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