Selective spore germination on shoots of *Homalothecium lutescens*, a moss with dwarf males

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Spores from three bryophyte species with dwarf males (*Homalothecium lutescens*, *Homalothecium sericeum* and *Isothecium alopecuroides*) were sown on shoots of *H. lutescens* in vitro. After 10 months, presence and fertility of dwarf plants were scored. Spores of the more distantly related *I. alopecuroides* were unable to develop into dwarf plants on *H. lutescens*. Spores of both *H. lutescens* and *H. sericeum* developed into dwarf plants. In fact, dwarf plants of *H. sericeum* were both more abundant and more often fertile than those of *H. lutescens*. The ability of *H. sericeum* spores to develop into dwarf males on shoots of *H. lutescens* suggests a possible pathway for hybridization between the two species. On the other hand, the inability of *I. alopecuroides* to develop into dwarf males on shoots of *H. lutescens* suggests that regulation of spore germination and dwarf male development on host shoots is associated with the degree of relatedness between species.

1. Background

Theoretical models of speciation processes often begin with ecological niche separation, followed by genetic differentiation and partial genetic incompatibility. When hybrids start to become less fit than the parental types, traits that reinforce reproductive isolation may evolve [1,2]. One source of such reinforcement is pre-zygotic recognition mechanisms. Preventing fertilization by another species may under certain circumstances be less costly than aborting unfit hybrid zygotes.

Fertilization in bryophytes typically occurs over short distances via swimming spermatozoids. One way to reduce the risk of hybrid fertilization (as well as competition) in bryophytes is spatial separation [3], for example by chemically preventing the germination and growth of another species within the colony [4,5]. On the other hand, there are a number of bryophyte species in which mechanisms that actually promote spore germination on shoots have evolved. Roughly 60% of mosses are unisexual (i.e. have male and female gametophytes) [6] and sex separation is generally assumed to be determined by sex chromosomes [7]. In so-called ‘nannandrous’ species, a portion of the males grow as tiny epiphytes on the females. These ‘dwarf males’ originate from spores that are deposited and germinate on the female shoots, where their final size is restricted to a few millimetres. Dwarf males are found in approximately 60 genera and 22 families, and it is estimated that they occur in 10–20% of all moss species [6]. Local differences in dwarf male density have previously been shown to be positively associated with female fertility [8,9].

There are, we believe, no observations in the literature of dwarf males belonging to alien species growing on female shoots. The only study that has specifically investigated the species identity of dwarf males found that the dwarf males were conspecific with their host shoot [10]. The apparent absence of alien dwarf males suggests the presence of a germination-inhibiting substance (cf. [4,5]) in nannandrous species, specifically targeted at alien spores.
The capacity for vegetative growth of most perennial bryophytes means that the female can postpone sexual reproduction until conspecific spores are deposited.

To assess the question of whether or not female shoots have developed a recognition mechanism to prevent germination of alien (heterospecific) spores, we have sown spores from three different nannan nandrous species (Homalothecium lutescens, Homalothecium sericeum and Isoleucium alopecuroides) on shoots of H. lutescens. We want to test how well shoots of H. lutescens can solve the conflict between promoting germination of conspecific spores and inhibiting germination of alien spores. More specifically, in this study, we try to address the following questions: (i) How well do spores from two different species germinate on shoots of H. lutescens, relative to conspecific spores? (ii) Is there any difference in fertility between the resulting dwarf plants from the three different spore sources? and (iii) Is spore germination related to host shoot fertility?

2. Material and methods

Homalothecium lutescens, H. sericeum and I. alopecuroides are nannan nandrous [6], perennial pleurocarpous mosses that disperse spores in spring, between February and April [11,12]. They are widespread in southern Sweden and it is possible to find all three in semi-open pastures on calcareous ground. Fertilization in I. alopecuroides occurs just before that in the other two species (January–April compared with May–June in H. lutescens and H. sericeum ([11,12]; F. Rosengren, personal observations, 2010–2014).

Shoots of H. lutescens were collected from the site ‘Hammars bakkar’ (55°23.834’ N, 14°1.543’ E), 4 April 2013. Dwarf males or sporophytes had not been detected in this site before collection, nor had plants of I. alopecuroides or H. sericeum. Sporophytes from H. lutescens, H. sericeum and I. alopecuroides were gathered in February/March 2013 (H. lutescens approx. three weeks before the other two), from three different localities: H. lutescens in ‘Arrie ponds’ (55°31.357’ N, 13°6.028’ E), H. sericeum in ‘Frua-liid’ (55°41.495’ N, 13°39.128’ E) and I. alopecuroides in ‘Dalby soderskog’ (55°40.453’ N, 13°19.840’ E). Homalothecium lutescens was not observed in the surroundings at the two latter localities. The sporophytes were stored in a freezer until 11 April, when spor solutions were made with distilled water (around 20 sporophytes for each solution). The three spor solutions were diluted to approx. 135 green spores µl⁻¹.

Sporophytes were sown on shoots of H. lutescens 25 April with a Pasteur pipette (three drops per shoot, approx. 150 µl and 20,000 spores). Each sporophyte treatment (+ control, i.e. no spores) consisted of 46 shoots. Spore germination was confirmed in the spore solutions 1 day after sowing (gern tube formation in between 42 and 51% of all spores) and later also on agarose plates. On 7 May, an additional three drops of spor solution were sown on each shoot. Shoots were kept in plastic test tubes in a growth chamber (12 C, light period as outside, 55°42’ N). The light period was increased in November to 11 h and again in January to 13 h, to accelerate antheridial development. The test tubes were open and shoots were regularly sprayed with distilled water to prevent desiccation. The position of all tubes was randomized once a week until September when they remained in the last randomized position.

Number of perichaetia on the host shoots was counted in September 2013. Dwarf plants were counted in February 2014. On each shoot, the 20 dwarf plants with the most pronounced buds (i.e. likely to bear mature gametangia) were examined for sexual organs. Approximately 100 dwarf plants on host shoots sown with I. alopecuroides spores were transplanted onto agarose plates cast with a nutrient solution [13], with the aim of species determination. In addition, development of dwarf plants from I. alopecuroides spores on conspecific shoots in vitro was tested and confirmed in spring 2015 with the same sowing procedure as described above.

The number of dwarf plants in the four different treatments was compared in a generalized linear model (R v. 3.1.1) with quasi-Poisson error distribution to correct for over-dispersion [14]. Host shoot fertility (number of perichaetia) and spore treatment were treated as fixed effects. Because there was no significant difference between the models including or excluding the interaction term between fertility and treatment (F₂,₇₉ = 0.394, p = 0.775), it was excluded in the final model. Tukey post hoc pairwise comparisons were made with the function glht (R package multcomp; http://cran.r-project.org/web/packages/multcomp/index.html).

3. Results

The number of dwarf plants differed between the treatments (F₃,₁₅₀ = 115.4, p < 0.001) (figure 1). Shoots sown with spores from H. sericeum had the highest number of dwarf plants (mean 70, z = 7.851 against control, z = 9.285 against I. alopecuroides, z = 6.104 against H. lutescens, p < 0.001 for all comparisons). Shoots sown with spores from H. lutescens had the second highest number of dwarf plants (mean 34, z = 6.215 against control and z = 6.872 against I. alopecuroides, p < 0.001 for both comparisons). The number of dwarf plants on shoots sown with spores from I. alopecuroides did not differ from the control shoots (mean 34 and 34, respectively, z = 1.305, p = 0.524). There was a weak positive association between the number of dwarf plants and host shoot fertility as evinced from the number of perichaetia (F₁,₁₇ₙ = 5.827, p = 0.0168, estimate = 0.026).

No dwarf plants with archegonia (female sexual organs) were found on any of the shoots. Furthermore, no dwarf plants with antheridia (male sexual organs) could be found on the control shoots or the shoots sown with spores from I. alopecuroides. For the shoots sown with spores from H. lutescens, 14 had 10 or more dwarf plants with antheridia; for the shoots sown with spores from H. sericeum, 32 had 10 or more dwarf plants with antheridia (figure 2).

A few dwarf plants on a majority of the shoots sown with H. sericeum spores (125 in total) were checked for protruding teeth along the basal leaf margin (separating character for

Figure 1. Total number of dwarf plants found on host shoots of H. lutescens 10 months after sowing with spores from three different sources: I. alopecuroides, H. lutescens and H. sericeum (N = 46).
Homalothecium species considered in this study. We do not know how these conditions are met for the two spores produced in hybrid spore capsules [3]. Currently, development of pre-zygotic reinforcement is an effective cause of hybridization. On the other hand, our results suggest species relatedness is an influential factor controlling dwarf male development. On the other hand, our results suggest that dwarf male development is not a determinant for species separation between 

H. sericeum and H. lutescens). In total, 93% were confirmed to belong to H. sericeum. After six months on agarose plates, the majority of dwarf plants from shoots sown with I. alopecuroides spores were morphologically similar to H. lutescens dwarf plants. Only one dwarf plant was found to have characters typical for I. alopecuroides.

Figure 2. Number of fertile dwarf males (recognized from antheridia) detected on host shoots of H. lutescens sown with spores from H. lutescens or H. sericeum. N.B. the final category on the x-axis is more than or equal to 20 (i.e. 20 dwarf plants or more).

4. Discussion

We show that spores from H. sericeum can germinate and develop into dwarf males on shoots of H. lutescens as well as spores from H. lutescens. Spores from I. alopecuroides did not develop into dwarf males on the H. lutescens shoots. Considering that H. sericeum and H. lutescens are resolved as sister taxa by molecular analysis [15], our results indicate that species relatedness is an influential factor controlling dwarf male development. On the other hand, our results suggest that dwarf male development is not a determinant for species separation between H. sericeum and H. lutescens.

By facilitating contact between sexual structures, growth of dwarf males on alien species may be an effective cause of hybridization. However, development of pre-zygotic reinforcement is not only dependent on the chances of species to meet, but also the fitness of the hybrid sporophytes and the viability of spores produced in hybrid spore capsules [3]. Currently, we do not know how these conditions are met for the two Homalothecium species considered in this study. Homalothecium lutescens and H. sericeum are ecologically separated but plants of intermediate appearance have been observed (L. Hedénäs, personal communication, 2014) in habitats where both species grow. Potential hybrids with erect, straight capsules (like H. sericeum) but closely similar shoot morphology to H. lutescens have sometimes been recognized as H. lutescens var. fallax [16].

Phylogenetic analysis of Homalothecium [15] places H. lutescens var. fallax in a basal position to H. lutescens proper, and resolves var. fallax plus H. lutescens as the sister clade to H. sericeum. This is consistent with var. fallax being a hybrid of the two species. If species recognition systems for spore germination and dwarf male formation on females are poorly developed among other closely related taxa also, there is a strong potential for hybridization in several nannandrous species where dwarf males occur.

The positive association between dwarf plant frequency and host shoot fertility is supported by previous studies in the field [8,9]. However, further studies are needed to determine whether this association has a chemical basis or is a mere consequence of fertile branches modifying the host shoot architecture so as to improve spore capture and/or spore germination.

In line with the notion that dwarf females are generally absent in bryophytes, no dwarf plants with archegonia were ever encountered in our experiments. One possible explanation for the male bias in dwarf plants is that either spore germination or sex expression is substrate-specific. Female spores may not be able to germinate on female shoots, possibly as a result of host shoot intervention, as there are no benefits from supporting dwarf females. Alternatively, it is possible that dwarf females are not able to develop archegonia, consistent with the regular occurrence of a substantial number of dwarf plants with no gametangia.

The dwarf plants found on the shoots sown with I. alopecuroides spores as well as on the control shoots may have germinated from spores already present in the field or from contamination in the cultivation chamber. Spore germination and subsequent growth are dependent on many ecophysiological factors such as moisture, pH and nutrient levels [17,18] and it is possible that H. lutescens and I. alopecuroides shoots differ in some chemical properties, making the H. lutescens shoots suboptimal for I. alopecuroides spores.

We see our results as an addition to the growing evidence that bryophytes have a chemical recognition system with the specific task of inhibiting spore germination. Such a recognition system has in some previous studies [4,5] been suggested to inhibit both conspecific and alien spores. Our results suggest that with respect to dwarf males such a hypothetical system is capable of discriminating conspecific spores from alien spores to a certain extent—it prevents spore germination of the more remotely related I. alopecuroides, but appears to promote spores of the close relative H. sericeum.

Data accessibility. Data have been deposited in the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.r6b0p).

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