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Assortative mating in sympatric ascomycete fungi revealed by experimental fertilizations

Artemis D. Treindl, Adrian Leuchtmann*

Plant Ecological Genetics, Institute of Integrative Biology, ETH Zurich, Zürich, Switzerland

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ABSTRACT

Mate recognition mechanisms resulting in assortative mating constitute an effective reproductive barrier that may promote sexual isolation and speciation. While such mechanisms are widely documented for animals and plants, they remain poorly studied in fungi. We used two interfertile species of *Epichloë* (*Clavicipitaceae*, *Ascomycota*), *E. typhina* and *E. clarkii*, which are host-specific endophytes of two sympatrically occurring grasses. The life cycle of these obligatory outcrossing fungi entails dispersal of gametes by a fly vector among external fungal structures (stromata). To test for assortative mating, we mimicked the natural fertilization process by applying mixtures of spermatia from both species and examined their reproductive success. Our trials revealed that fertilization is non-random and preferentially takes place between conspecific mating partners, which is indicative of assortative mating. Additionally, the viability of hybrid and non-hybrid ascospore offspring was assessed. Germination rates were lower in *E. clarkii* than in *E. typhina* and were reduced in ascospore progeny from treatments with high proportions of heterospecific spermatia. The preferential mating between conspecific genotypes and reduced hybrid viability represent important reproductive barriers that have not been documented before in *Epichloë*. Insights from fungal systems will deepen our understanding of the evolutionary mechanisms leading to reproductive isolation and speciation.

1. Introduction

Speciation is the process by which ancestral species of sexual organisms diverge and form new species by means of adaptation, genomic change or hybridization (Coyne and Orr, 2004). Of central importance to the initial divergence and maintenance of biological species are reproductive barriers that impede gene flow and thus preserve genetic differences between species (Kohn, 2005; Seehausen et al., 2014). These barriers can be extrinsic and originate from geographic barriers, or environmental factors separating two populations. The incipient species then evolve in allopatry adapting to their local environment, and intrinsic reproductive barriers are expected to build up gradually as a result of mutation, natural selection and genetic drift. Thus, in allopatric speciation, intersterility can solely arise as a side effect of genetic divergence and selection may only act indirectly on isolating traits (Mayr, 1963). By contrast, when species emerge in sympatry from conjunct and potentially interbreeding populations, reproductive isolation is expected to constitute the critical stage in the speciation process. For sympatric speciation to occur we expect the production of viable and fertile hybrids to be absent, since even minimal gene flow would prevent differentiation (Slatkin, 1987; Butlin et al., 2008). In this context, a distinction is made between prezygotic and postzygotic reproductive barriers. Prezygotic mechanisms such as mating preference (Fernandez-Meirama et al., 2017) or sexual incompatibility (Leuchtmann and Schardl, 1998) prevent interspecific mating itself or the formation of hybrid zygotes. Postzygotic barriers may be either intrinsic, causing hybrids to exhibit reduced fitness, or extrinsic and thus linked to ecological factors (Giraud et al., 2008). Extrinsic limitations can be indicated when hybrids are viable *in vitro*, but perform poorly compared to parents in a natural environment.

In a sympatric setting, mating compatibility and thus the mechanisms that infer mating (i.e. mate recognition) are expected to be of particular importance to the development of sexual isolation and speciation (Büker et al., 2013). Indeed, mate recognition mechanisms resulting in assortative mating constitute an effective reproductive barrier at the prezygotic stage (Martin et al.,

* Corresponding author. Plant Ecological Genetics, Institute of Integrative Biology, ETH Zurich, Universitätstrasse 16, CH-8092 Zürich, Switzerland. Fax: +41 44 632 14 63.
E-mail address: adrian.leuchtmann@env.ethz.ch (A. Leuchtmann).

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2013; Reynolds and Fitzpatrick, 2007; Snow and Spira, 1991). However, for positive assortative mating to occur, individuals or gametes need to be able to discriminate among mating partners belonging to the same or a different species. This preference for the more similar (i.e. conspecific) partner can be based on mate choice or may occur as a byproduct of habitat choice, but can also originate from genes pleiotropically affecting (host-) adaptation and mating or varying competitiveness of mating partners (Giraud et al., 2010).

For the plant and animal kingdom it has been shown in numerous cases that assortative mating by mate choice or gamete interaction play crucial roles in promoting prezygotic reproductive isolation and thus the maintenance of species (Aldridge and Campbell, 2006; Snowberg and Bolnick, 2012; Weis and Kossler, 2004). On the other hand, very few studies have used fungal model systems to investigate the significance of these factors for speciation processes, essentially including species of *Saccharomyces* (Murphy et al., 2006), *Neurospora* (Karlsson et al., 2008; Turner et al., 2011) and *Microbotryum* (Büker et al., 2013; Le Gac et al., 2007; van Putten et al., 2003). Moreover, mating systems and modes of reproduction that may be relevant for emergence of reproductive isolation in nature have been poorly studied in fungi, which include selfing and outcrossing, and for most fungi both sexual and asexual reproduction (Billiard et al., 2012). This range in reproductive strategies makes fungi attractive model systems to study fundamental processes of speciation.

The sexual species of the ascomycete genus *Epichloë* provide a particularly intriguing and powerful model (Gladieux, 2018; Leuchtmann, 2003; Schirrmann et al., 2015, 2018). These species exhibit a variety of different life styles throughout the sexual and asexual phases of their life cycle and often form species complexes that include interfertile, yet reproductively isolated species on different hosts. Moreover, host strains can be maintained in culture and manipulated for experimental crossing and host inoculation, and thus represent an ideal study system.

Epichloë species (*Ascomycota*, *Clavicipitaceae*) are endophytes of cool-season grasses that establish a systemic symbiosis with the host during their entire life span (Leuchtmann and Clay, 1997). The endophytes grow in the intercellular space of host plant tissues and remain asymptomatic to the point of host flowering. Then, the fungi form external fruiting bodies (stromata) for sexual reproduction, which enclose undeveloped inflorescences and inhibit flowering and seed production (Leuchtmann, 2003; White et al., 1991). This phenomenon is commonly referred to as choke or cattail disease (Western and Cavett, 1959; White, 1997). Asexual spores are produced on the entire surface of young stromata, which are dispersed to stromata on other plants and act as male gametes (spermatia) for fertilization (White and Bultman, 1987). The dispersal of spermatia is typically mediated by flies of the genus *Botanophila* (*Anthomyiidae*) that are specialized to *Epichloë*, ecologically analogous to the process of insect pollination in angiosperms (Bultman and Leuchtmann, 2008; Bultman and White, 1988; Leuchtmann and

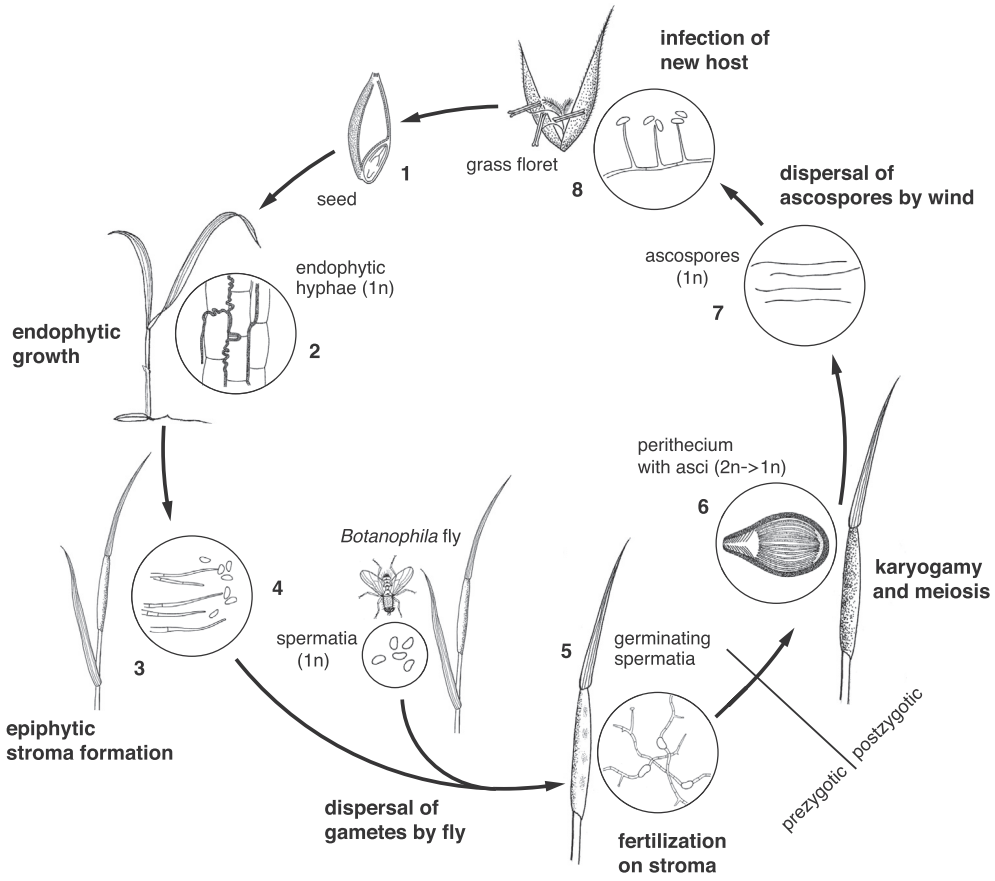

Fig. 1. Life cycle of *Epichloë* fungi. After systemic growth of haploid hyphae within seed (1) and vegetative plant tissues (2) sexual reproduction is initiated by forming an external fruiting body (stroma) around developing host inflorescences (3). On stroma surface, spermatia (male gametes) are produced (4) that are dispersed to stromata on other plants by *Botanophila* flies. Following deposition of spermatia, a mycelium spreads over the surface of the stroma from the initial point of inoculation (5). Heterothallic mating takes place between individuals of opposite mating type through fusion of germinating spermatia or hyphae growing from them and female receptive hyphae to form the dikaryon, upon which perithecia with asci develop within this mycelium (6). After karyogamy and meiosis, eight haploid ascospores are formed that are wind-dispersed (7) and mediate horizontal transmission to new hosts by infecting grass florets and then seeds (8). Figure modified from Leuchtmann and Schardl (1998).

Michelsen, 2016), but may also involve other vectors such as slugs (Hoffman and Rao, 2014). After deposition of spermatia, cell fusion between spermatia and female receptive structures takes place on the stroma surface, followed by nuclear fusion and meiosis resulting in the production of eight haploid ascospores. For details on the life cycle see Fig. 1.

In this study, we focus on two taxa of the *Epichloë typhina* species complex, *E. typhina* infecting *Dactylis glomerata* and *E. clarkii* infecting *Holcus lanatus* (Craven et al., 2001). This complex includes several genetically closely related, host-specific taxa that have been given the rank of subspecies, because they are sexually compatible and able to produce viable offspring in experimental crosses (Leuchtmann and Schardl, 1998; Leuchtmann et al., 2014). Here, we treat *E. typhina* from *D. glomerata* and *E. clarkii* from *H. lanatus* as species since they appear to be reproductively isolated and genetically sufficiently distinct (Schirrmann et al., 2015). Experimental hybrid progeny between *E. typhina* and *E. clarkii* show the normal phenotype in culture and do not exhibit reduced growth compared to parental strains (Leuchtmann and Steinebrunner, 2012; Schirrmann and Leuchtmann, 2015). Moreover, hybrid ascospores have also been detected on naturally fertilized stromata collected from sympatric populations of the two hosts (Bultman et al., 2011; personal observations), but evidence for hybrid strains infecting either *D. glomerata* or *H. lanatus* have so far never been found in natural populations. These observations suggest that *E. typhina* and *E. clarkii* are reproductively isolated and that pre- and/or postzygotic reproductive barriers exist that maintain the genetic and ecological integrity of the two species.

Previous studies have looked at the role of the spermatia transferring flies for reproductive isolation. Although some preference of flies for stromata of *E. typhina* over stromata of *E. clarkii* has been observed in field tests, different fly species laid eggs on stromata of both hosts suggesting that visitation behavior of flies is not monolectic (Bultman and Leuchtmann, 2003; Bultman et al., 2011). Thus, the *Botanophila* fly vector is likely not an important source of isolation in *Epichloë* species. Furthermore, isolation could act after mating on meiotically formed ascospore progeny. However, *in vitro* studies did not show reduced mycelial growth of experimentally produced hybrid ascospores compared to parental strains suggesting that growth performance does not present an obvious isolation barrier for hybrids (Schirrmann and Leuchtmann, 2015). Lastly, infection ability and persistence could impose a strong reproductive barrier, if hybrid progeny is impeded in the colonization of the parental hosts. Indeed, inoculation tests with seedlings revealed that species of the *E. typhina* complex, including *E. typhina* and *E. clarkii*, are host-specific and that experimentally generated hybrids infect seedlings at much lower frequencies compared to parental strains (Leuchtmann and Steinebrunner, 2012). Moreover, successful hybrid infections were only transient and systemic long-term infections were not observed. This suggests that host-specialization of *E. typhina* and *E. clarkii* contributes to reproductive isolation at the postzygotic stage.

Here, we investigated mechanisms of assortative mating that may act as reproductive barriers between *E. typhina* and *E. clarkii* at the prezygotic stage. We performed experimental mating trials with mixtures of spermatia from both species in various proportions and examined their competitiveness and reproductive success on the stroma. By mimicking the natural fertilization process vectored by *Botanophila* flies, our trials revealed that sexual reproduction preferentially takes place between conspecific mating partners, which is indicative of assortative mating (Le Gac et al., 2007), and thus far undescribed in sexual *Epichloë* species. Additionally, viability of hybrid offspring was assessed by determining germination rates of ascospores that resulted from the experimental matings. Reduced germination of hybrids may represent another possible postzygotic isolation mechanism and has not been investigated until now. The research performed here aims to expand our knowledge of reproductive barriers within the *E. typhina* species complex and explore the evolutionary mechanisms that promote reproductive isolation and speciation in fungi.

2. Materials and methods

2.1. Host plants

D. glomerata (orchard grass) and *H. lanatus* (Yorkshire fog) are long-lived, perennial clump-forming grasses that commonly occur in pastures or other nutrient rich grassland. Originally native to Europe, temperate Asia and Northern Africa, *D. glomerata* has reached world-wide distribution as an important forage grass (Hess et al., 1976). The more narrowly distributed *H. lanatus* is native to Europe only and has been unintentionally introduced to other countries, where it may be considered a noxious weed (USDA-ARS, 2013). Both species are continuously outcrossing by wind-pollination, have overlapping flowering time, typically May to June, and often occur in sympatry.

Incidence of *Epichloë* infection is generally low throughout the distribution range of the host grasses, although infection can be common locally. Furthermore, infections in *H. lanatus* are much less frequent than in *D. glomerata*, and at all sites in central Europe where *H. lanatus* is found to be infected, *D. glomerata* plants are also infected suggesting that the *Epichloë* species infecting *D. glomerata* may represent the ancestral lineage.

Infected host plants used as stromal partners in this study originated from four naturally infected grass accessions collected in Switzerland and France (Table 1). Single grass tillers (and usually the whole plant) are infected by only one genotype and this genotype is systemically propagated to all newly formed tillers (Leuchtmann and Clay, 1997). Accessions originating from single tillers were cloned yielding 25 *D. glomerata* plants infected with *E. typhina* and 11 *H. lanatus* plants infected with *E. clarkii*. Clones were kept in single pots in two climate chambers, separated according to the mating type of infecting endophytes, at 22 °C/15 h day (25 kLux) and 15 °C/9 h night, and 50 % humidity. Host grasses were fertilized with solid lawn fertilizer (Mioplant, Migros, Switzerland) and received treatment against gnats (Solbac 0.25 %, Andermatt Biocontrol, Switzerland) and powdery mildew (on *D. glomerata*, Femicur 0.5 %). Climate chambers were fitted with sticky traps and ant traps to prevent movement of insects among different plants.

2.2. Epichloë species

The sexual species of genus *Epichloë* are characterized based on their morphology, phylogeny and mating compatibility. As *Epichloë* species are heterothallic, gametes carrying the opposite mating type are required for cell fusion and successful mating (Schardl et al., 2014; White and Bultman, 1987). The heterothallic mating system is controlled by a single genetic locus discriminating two mating types (bipolar), which enforces mating between distinct haploid genotypes and has fostered implementation of a biological species concept (Leuchtmann, 2003). In the most recent taxonomic treatment of the genus, *E. typhina* infecting *D. glomerata* (and other hosts) and *E. clarkii* infecting *H. lanatus* were given the rank of subspecies because of their interfertility (Leuchtmann et al., 2014). These taxa are considered part of a species complex, together with additional closely related species and subspecies. The two species can be easily distinguished by their ascospore morphologies. *E. typhina* has filiform, multiseptate ascospores (176 ± 34 × 1.6 ± 0.2 μm)

Table 1
Strains of *Epichloë typhina* (Et) and *E. clarkii* (Ec) used in this study with isolate number, endophyte species, mating type, host species, place of origin, and type of experimental use. Infected plants originated from places in Switzerland and France (F).

Isolate no.	Culture collection no.	Endophyte	Mating type[a]	Host species	Origin	Experimental use
Et1203	CBS 145506	*E. typhina*	mat-1	*D. glomerata*	Vesancy (F)	stromata & spermatia
Et1306	CBS 145507	*E. typhina*	mat-1	*D. glomerata*	Aubonne	spermatia
Et1204	CBS 145508	*E. typhina*	mat-2	*D. glomerata*	Vesancy (F)	stromata & spermatia
Et1305	CBS 145512	*E. typhina*	mat-2	*D. glomerata*	Changins	stromata & spermatia
Et1217	CBS 145509	*E. typhina*	mat-2	*D. glomerata*	Merishausen	spermatia
Ec1205		*E. clarkii*	mat-1	*H. lanatus*	Aubonne	stromata
Ec1401	CBS 145510	*E. clarkii*	mat-1	*H. lanatus*	Aubonne	spermatia
Ec1402		*E. clarkii*	mat-1	*H. lanatus*	Aubonne	spermatia
Ec1403		*E. clarkii*	mat-2	*H. lanatus*	Aubonne	spermatia
Ec1206	CBS 145511	*E. clarkii*	mat-2	*H. lanatus*	La Rippe	spermatia

[a] Determined by mating tests with reference strain (Leuchtmann et al., 1994).

that remain unfragmented, whereas *E. clarkii* produces spear-shaped part-spores (46 ± 16 × 2.3 ± 0.3 μm) resulting from fragmentation of filiform spores at maturity (White, 1993).

2.3. Natural fertilization and mating

Fertilization of *Epichloë* in natural populations mostly depends on symbiotic *Botanophila* flies that serve as vectors of spermatia (Leuchtmann and Michelsen, 2016) although cases are known, where mating occurred in the absence of flies (Górzyńska et al., 2011; Hoffman and Rao, 2014; Rao and Baumann, 2004). These flies are attracted to stromata by a set of specific fungal volatiles that differ in their profile among fungal species (Schiestl et al., 2006; Steinebrunner et al., 2008). Flies feed on spermatia, transport them within their gut and actively deposit them on the stroma surface with their feces (Bultman et al., 1998). In a mutualistic interaction, *Epichloë* species benefit from the spermatization service provided by flies, whereas flies deposit their eggs on the stromata and larvae feed and develop on fungal mycelium (Bultman et al., 1998). As a *Botanophila* fly visits several stromata, spermatia of different species (if present) and different mating types are ingested and accumulate in its digestive tract (Bultman and Leuchtmann, 2003). Spermatia pass through the gut intact and viable, which allows effective cross-fertilization when the fly defecates on the stroma surface. Fertilization involves a distinct behavior: following egg-laying flies traverse the length of the stroma in a straight line and then back in a spiraling fashion, while dragging the tip of their abdomen over the stroma surface and defecating ingested spermatia (Bultman et al., 1998). After hatching, fly larvae feed on fungal hyphae and developing perithecia, thus imposing a cost for the fungus. However, the interaction appears stable resulting from density dependent mortality of the fly larvae (Bultman et al., 2000).

2.4. Fungal isolates and harvest of spermatia

Endophyte isolates were obtained from surface sterilized stems and leaf-sheaths of infected *H. lanatus* and *D. glomerata* plants as described previously (Leuchtmann and Clay, 1988) (Table 1). Fungal cultures were grown on supplemented malt extract-agar (SMA) containing 1 % malt extract, 1 % glucose, 0.25 % bacto peptone, 0.25 % yeast extract, 1.5 % bacto agar and 0.005 % oxytetracycline (Pfizer, New York, NY, U.S.A.). Identity of mating types was verified by confronting spermatia to stromata of reference strains with known mating type (Leuchtmann et al., 1994). Cultures of representative isolates are deposited at CBS-KNAW culture collection (Utrecht, The Netherlands) (Table 1).

In order to prepare suspensions of spermatia for fertilization, fresh cultures were established from a small piece of mycelium removed from the margin of a 14 d old colony. The mycelium was added to 500 μl sterile H_2O in a 1.5 ml Eppendorf tube and homogenized using a sterile micropestle before plating the suspension on a fresh SMA plate with a spreader rod. This technique leads to even growth of mycelium on the entire surface of the plate and stimulates formation of spermatia. The spermatia were harvested every week from freshly prepared seven days old cultures over the course of four weeks while fertilizations were performed. Harvest was done after adding 5 ml sterile H_2O containing 0.1 % Tween 20 (AppliChem, Darmstadt, Germany) by carefully scraping the surface with a sterilized spreader rod and removing the suspension with a pipette. Spermatia were counted using a Neubauer counting chamber and the suspensions diluted with sterile H_2O containing Tween 20 as above to concentrations of 100 spermatia/μl. Mixtures and pure suspensions of spermatia for the fertilization experiment were prepared from freshly harvested spermatia every week.

2.5. Experimental fertilization

Fertilizations were made on stromata of known mating type shortly after they had emerged and were fully exposed. Eight μl of spermatia suspensions (containing 100 spermatia per μl) were applied to stromata in 4 droplets of 2 μl each with a pipette. This amount of spermatia was considered ecologically relevant for fertilization and is estimated to be in the range of what is naturally deposited by *Botanophila* flies within their feces on the stroma surface (Bultman and Leuchtmann, 2003). Applied droplets stay in place on the stroma, as the hydrophobic surface absorbs the aqueous solution very slowly (Fig. 2A). In order to mimic the natural fertilizations performed by the *Botanophila* flies, the spermatia suspensions were carefully spread over the surface in a linear fashion on one side of the stroma using the tip of the pipette sideward. This procedure also simulates feeding behavior of *Botanophila* flies, which is slightly destructive when mycelium and spermatia are ingested. As control, stromata were treated with 0.1 % Tween 20 solutions not containing spermatia.

The experiments included seven crosses with different genotype combinations and for each treatment, separate stromata were used with three to five replicate stromata per treatment (depending on the number of stromata available) (Table 2). Treatments included conspecific spermatia (CON), mixtures of conspecific and heterospecific spermatia in different ratios (1:1, 1:10, 1:100) and heterospecifc spermatia (HET). This resulted in a total of 139 stromata that were fertilized. All treated stromata were tagged with color-coded and labeled plastic tubes. After fertilization, host plants were enclosed by Plexiglas cylinders (25 cm in diameter, 50 cm high) to prevent unwanted contact with other plants and to avoid contamination.

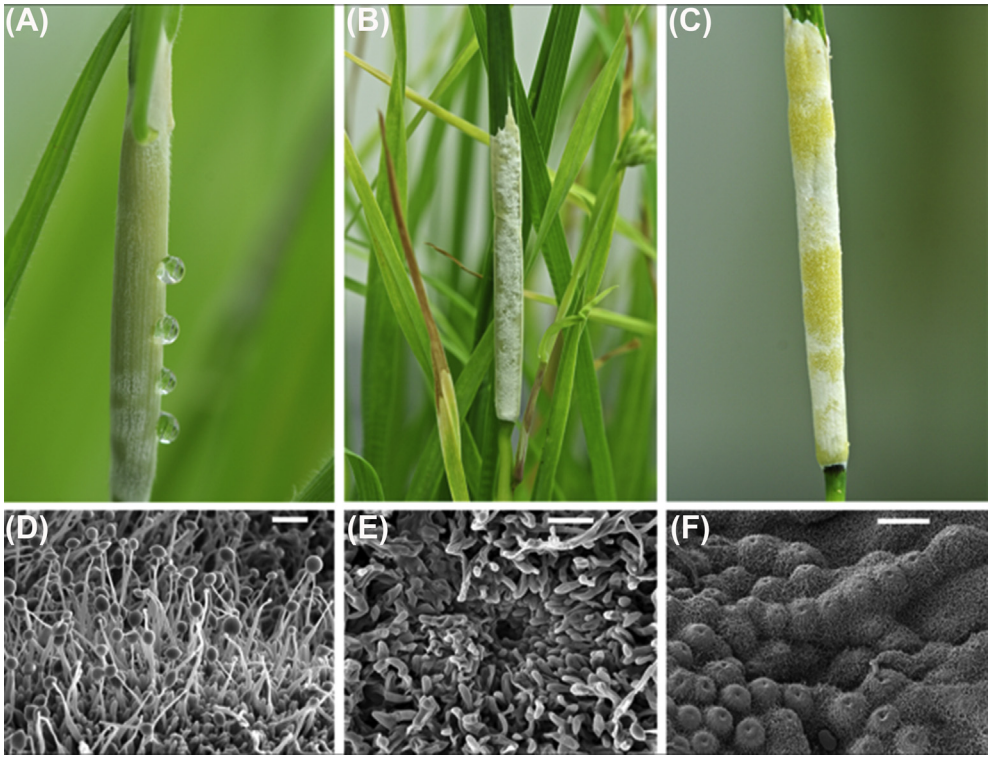

Fig. 2. Experimental fertilization of an *Epichloë* stroma and different stages of development: (A) application of spermatia suspension in small droplets of 2 µl, (B) germinated spermatia produce a proliferating mycelium on stroma surface after two weeks, (C) perithecia (yellow) developing after four weeks. Scanning electron microscope (SEM) images of platinum coated stroma surfaces at different stages of development: (D) young unfertilized stroma with spermatia, scale bar = 10 µm, (E) initial stages of perithecial development from proliferating mycelium after fertilization, scale bar = 10 µm, (F) developing perithecia, scale bar = 200 µm.

Table 2
Crosses performed between *Epichloë typhina* (Et) and *E. clarkii* (Ec) strains with number of ascospore probes evaluated per treatment. Each treatment was made using separate stromata (3–5 per treatment and cross) and 1–3 probes per stroma were taken. Treatments were conspecific spermatia (CON), mixtures of conspecific and heterospecific spermatia in different ratios (1:1, 1:10, 1:100), and heterospecifc spermatia (HET).

Cross		Treatment				
	Stroma × Spermatia	CON	1:1	1:10	1:100	HET
1	Et1305 × Et1203 | Ec1401	15	15	12	15	10
2	Et1305 × Et1203 | Ec1402	15	12	11	15	15
3	Et1203 × Et1217 | Ec1403	10	6	9	5	4
4	Et1204 × Et1306 | Ec1401	9	6	6	6	9
5	Et1203 × Et1217 | Ec1206	10	9	3	4	8
6	Ec1205 × Ec1403 | Et1305	28	12	7	7	np[a]
7	Ec1205 × Ec1403 | Et1217	28	10	6	6	9

[a] np = not performed.

2.6. Microscopic analysis

Mating success was evaluated 5–6 weeks after fertilization when perithecia containing ascospores had developed on the stromata. From each stroma, three separate probes (considered replicates) comprising several perithecia were taken along the length of the stroma with tweezers and carefully squashed in a drop of water on a microscope slide. Ascospore morphology was then examined using a light microscope at 400× magnification and probes were categorized as 'typhina' (ascospores exhibiting the morphology of *E. typhina*), 'clarkii' (ascospores exhibiting the morphology of *E. clarkii*), 'hybrid' (asci containing both spore-morphotypes) or 'barren' (not containing any developed ascospores). Ascospores of the two species are easily distinguishable, with *E. clarkii* producing short, spear-shaped spores resulting from fragmentation at maturity, and *E. typhina* producing filiform, unfragmented spores (White, 1993). Hybrid asci usually contain four filiform spores of the *E. typhina*-type and four of the *E. clarkii*-type that typically disarticulate into four part-spores each. However, in crosses where *E. clarkii* was the maternal (stromal) parent, besides normal *E. clarkii*-type spores some ascospores of intermediate length were observed in asci, which looked more similar to *E. typhina*-type spores but then disarticulated into two fragments. This could indicate a predominantly maternal inheritance of spore morphology in *E. clarkii*, which is manifested in hybrid progeny. From all probes containing mature ascospores, a minimum of five single spores were isolated using a micromanipulator equipped with a micro dissecting needle and plated on SMA plates (Supplementary Table S1). Germinating ascospores typically produce phialids and conidia first (iterative germination), before colonies with vegetative hyphae are formed (Bacon and Hinton, 1988). To assess germination of single spore isolates, plates were checked for colony growth every other day for 2 weeks following isolation, and, if necessary, contaminations were removed.

2.7. Genetic identification of progeny

Since only a limited number of isolates could be genotyped, we focused on one cross (cross 4) analyzing the majority of isolates from this cross (main sample) and then selected a subsample from other crosses (cross 1, 2 and 7) (for identity of crosses see Table 2). This approach made it possible (1) to test for contamination with other genotypes than the ones applied, (2) to verify conformity with the microscopic evaluation and (3) to assess the rate of hybridization based on genetic markers by deducing from the main sample.

For the extraction of DNA, mycelium was harvested from colonies actively growing on SMA medium. Strips of mycelium (appr. 120 mg fresh weight) were placed in sterile plastic tubes with metal beads, freeze dried for 24 h and then ground using a mixer mill (Qiagen Retsch M301). DNA was extracted using the NucleoSpin® Plant II Core Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol based on the Cetyl Trimethyl Ammonium Bromide (CTAB) lysis method. The DNA concentration of extracted samples was quantified using a Quantus™ Fluorometer (Promega Corporation, Wisconsin, USA).

Genotypes of selected fungal isolates were determined by PCR amplification of 15 microsatellite markers using a multiplex PCR approach as previously described (Schirrmann et al., 2015). Markers were arranged in four multiplex sets, and each set was amplified using a PCR volume of 10 µl containing approximately 1 ng of genomic DNA in 5× PCR Buffer (Promega, Madison, WI, U.S.A.), 5 U/µl goTaq Polymerase (Promega, Madison, WI, U.S.A.), 25 mM $MgCl_2$, 2.5 mM of each dNTP and 10× Primermix (2 µM). The amplification conditions were as follows: initial denaturation of dsDNA at 94 °C for 3 min, 30 cycles of 30s denaturation at 94 °C with 1 min annealing at 56 °C and 30s extension at 72 °C, and final elongation at 72 °C for 5 min. Signals of the PCR products were detected on a 3130xl DNA Analyzer (Applied Biosystems, Foster City, California, U.S.A.) with GeneScan-500 LIZ as size standard. Electropherograms were analyzed using Geneious 6.1.8 (Drummond et al., 2013). Genotypes of offspring were compared and assigned to their respective parents based on lengths of alleles at microsatellite loci.

2.8. Statistical analyses

We analyzed whether proportions of hybrid offspring in the 1:1, 1:10 and 1:100 treatments differed from the expected 50 %, 90 % and 99 % under the assumption of random mating (see Supplementary Table S2). To test for deviation from random mating, the exact binomial test was used. Analyses were first performed separately for each cross and subsequently for crosses pooled by maternal genotype (stromatal partner). Results of the statistical tests were consistent across 1:1 and 1:10 treatments (exact binomial tests, $p < 0.001$) and 1:100 treatments (exact binomial tests, $p > 0.5$) with the exception of one cross also showing a significant result in the 1:100 treatment (exact binomial test, $p < 0.01$), independent of the genotypes involved or the level at which the tests were performed. Therefore, data for all replicates of a particular treatment were pooled, and analyses repeated for all crosses combined and for crosses separated by identity of the maternal species (*E. typhina* or *E. clarkii*). To address pseudoreplication issues when pooling data from crosses that differed only in the genotypes of the spermatial partner, we run a linear mixed model on the pooled data using genotypes as a random effect with the package lme4 (Bates et al., 2015), showing that mating outcome is predominantly dependent on treatments with some variation among genotypes, but without affecting significance of the results of pooled data. The statistical significance of differences in germination rates between *E. typhina* and *E. clarkii* crosses, and within the crosses for different treatments, was analyzed using Student's *t*-test on data pooled according to identity of the maternal species. To analyze differences between treatments within each cross, and additionally for pooled data since results were consistent, a binomial analysis of variance (ANOVA) was performed, followed by post-hoc analysis using Tukey's HSD test. All statistical analyses were conducted with R (version 3.3; R Development Core Team).

3. Results

3.1. Assortative mating

Following experimental fertilization of young emerging stromata of *Epichloë typhina* and *E. clarkii* mimicking natural fertilization by *Botanophila* flies, 78 % of fertilized stromata (109 of 139) survived until evaluation. The relatively high death rate of stromata may be attributed to mildew infestation of *D. glomerata* resulting in drying out of some tillers, and contamination with *Penicillium* spp. in *H. lanatus* due to very dense growth of tillers creating an extremely humid environment. In all control treatments with no spermatia applied, stromata were free from perithecia and mostly dried out, since no fertilization had taken place.

Results reported here are based on microscopic evaluation of hybrid or non-hybrid progeny. As expected, stromata that received only conspecific spermatia (CON) produced 100 % non-hybrid offspring and stromata that received only heterospecific spermatia (HET) produced 100 % hybrid offspring based on the ascospore probes examined (Fig. 3). Microsatellite genotyping of ascospore progeny confirmed that assignment as hybrid or non-hybrid based on morphological observation was correct. Every single ascospore isolate of the selected subsample could be assigned to the parental genotypes of stromatal and spermatial partners used for mating. Representative results from cross 4 are depicted in Supplementary Table S3.

When spermatia were applied in equal concentrations (1:1 treatments), proportions of hybrid offspring were significantly lower (exact binomial test, $p < 0.001$) than expected under random mating (50 %). In five out of seven crosses of this treatment, no hybrid offspring was found at all and the remaining two crosses produced 13 % and 33 % hybrids, respectively. Likewise, in 1:10 treatments results deviated significantly from expectations, whereas in 1:100 treatments hybrid offspring was still diminished although not significantly (mean 89 %, exact binomial test, $p > 0.5$) (Fig. 3). Hybridization rates in 1:10 treatments varied considerably among different crosses ranging from 0 to 43 % (mean 25.5 %) (Supplementary Table S2).

3.2. Germination rates of ascospores

Germination of viable ascospores usually occurred within 24 h after ejection from asci, typically producing phialids and conidia first (iterative germination), before colonies with vegetative hyphae were formed. Ascospores that had not germinated within two weeks after isolation were considered to be non-viable. Overall, germination rates varied significantly depending on treatments ($F_4 = 9.35$, $p < 0.001$). Germination rates were similarly high for spore isolates from conspecific, 1:1 and 1:10 treatments (HSD p = nonsignificant), but lower for 1:100 treatments and for heterospecific crosses (HSD $p < 0.01$), with one exception where 1:10 treatments also yielded significantly lower germination rates (cross 4, $p < 0.01$) (Fig. 4). Generally, ascospores originating from treatments with pure or high proportions of heterospecific spermatia took longer to mature and appeared to be more fragile. Furthermore, isolates from *E. clarkii*

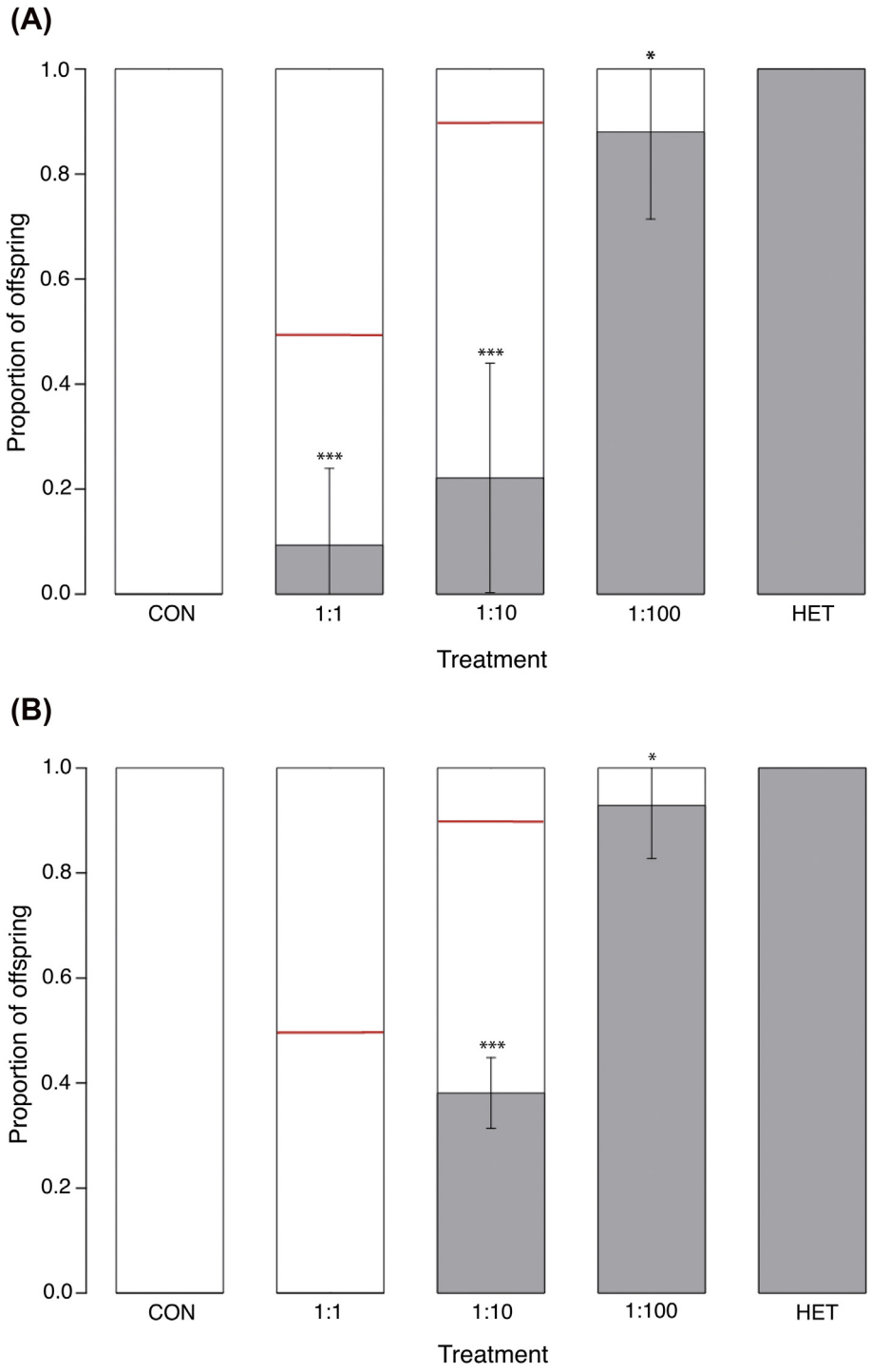

Fig. 3. Proportions of hybrid (gray) and non-hybrid offspring (white) resulting from fertilizations of *Epichloë typhina* (A) and *E. clarkii* (B) with spermatia mixtures of the two species at different ratios (1:1, 1:10, 1:100), or treatments with only conspecific spermatia (CON) or heterospecific spermatia (HET). Red reference lines indicate expected hybrid proportion at random-mating conditions (0.5 and 0.9). Asterisks indicate significant differences between expected and observed values indicative of assortative mating (***$p < 0.001$, * < 0.5). Mean errors are given by error bars.

stromata had lower germination rates than isolates from *E. typhina* (Student's *t*-test $t_{1,27} = 3.22$, $p < 0.003$). Many ascospores germinated and formed phialides with conidia, however they failed to establish a mycelium. Therefore, the observed germination rates overestimate the rate of spores that actually establish a viable colony. The amount of failure to establish colonies depended on the parental genotypes and treatments applied.

4. Discussion

Understanding the fundamental mechanisms leading to species divergence is one of the main objectives in evolutionary biology. While the theoretical framework of speciation has been established, widely discussed and tested in a range of different organisms of the plant and animal kingdom, speciation theory in fungi

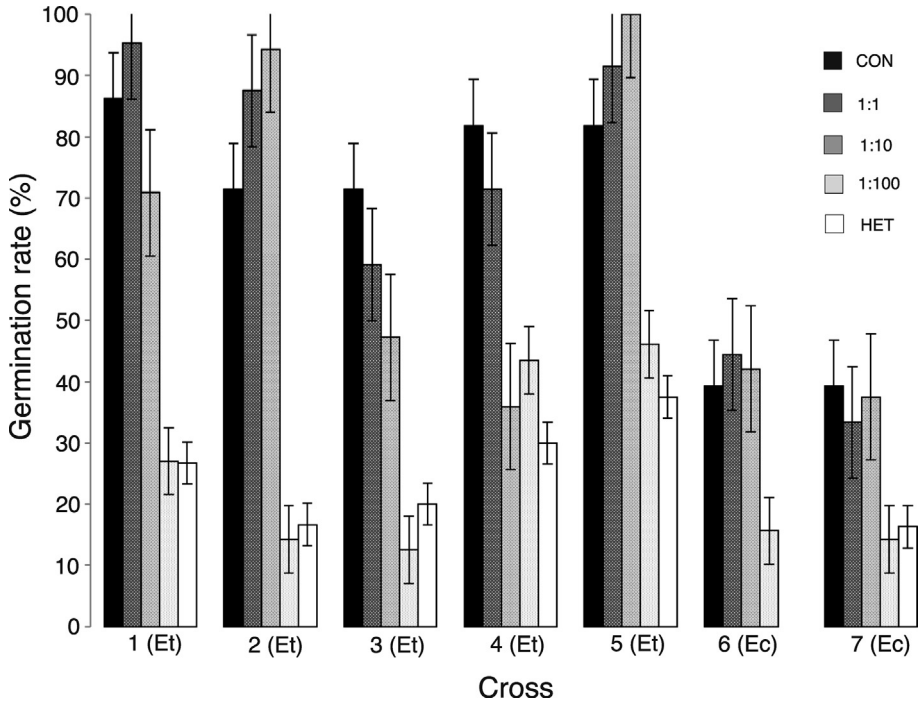

Fig. 4. Germination rates of ascospores on agar medium after single spore isolations of progeny from seven different crosses involving *Epichloë typhina* (Et) and *E. clarkii* (Ec) stromata and different spermatia treatments. Treatments were mixtures of spermatia of the two species at different ratios (1:1, 1:10, 1:100), or only conspecific spermatia (CON) or heterospecific spermatia (HET). For identity of strains used in each cross see Table 2. Standard errors are given by error bars.

remains enigmatic and experimental studies testing specific hypotheses are mostly limited to model systems (Giraud et al., 2008). Studies of sympatric, closely related taxa such as the one presented here are particularly valuable to disentangle the contributions of pre- and postzygotic barriers leading to reproductive isolation. Furthermore, the reproductive model of *Epichloë* involving heterothallic transfer of spermatia shares common features with many other fungi including important pathogens. Examples are rust fungi (Helfer, 2014), the ascomycete *Leptographium* causing root collar diseases in pine (Jacobs et al., 2006) and *Botrytis cinerea* causing gray mold in numerous crop plants including grape (Williamson et al., 2007). Although largely unexplored, prezygotic isolation mechanisms such as assortative mating through gamete competition may play an important role in evolution and speciation of fungal pathogens. Here we investigated potential reproductive barriers between two species of the *E. typhina* complex (*E. typhina* and *E. clarkii*) that remain morphologically and genetically distinct in sympatric populations, although they are interfertile. Specifically, we tested spermatia competition leading to assortative mating in experimental fertilization trials and assessed germination success of hybrid ascospores.

The results of this study reveal that mating on the stroma is non-random and preferentially takes place between individuals of the same species when mixtures of con- and heterospecific spermatia are applied to stromata. However, with increasing proportion of heterospecific spermatia the prevalence of conspecific matings declines while the proportion of hybrid offspring increases. We propose two different mechanisms that could lead to the observed positive assortative mating. Firstly, female structures (ascogonia or receptive hyphae) may exhibit preference towards conspecific spermatia (or *vice versa*) when undergoing cell fusion. This may involve pheromone signaling or other interactions on a molecular level, similar to what has been described for mating type recognition (Casselton, 2002; Karlsson et al., 2008; Kim and Borkovich, 2006). It would imply that the stromal partner can differentially attract or distinguish among spermatia from different species, and thus may be referred to as spermatia recognition. Secondly, spermatia or mycelia growing from them may compete with one another when proliferating over the surface of the stroma. If conspecific spermatia germinate faster or spread more vigorously than heterospecific spermatia, this could result in an increased number of fertilizations between conspecific partners. The underlying mechanism would be a form of spermatia competition, much like the competition of pollen that is known as a common reproductive barrier among plant species (Rahmé et al., 2009).

In our experimental matings that involved conspecific or only moderate levels of spermatia of the alternate species (CON, 1:1 and 1:10 treatments) and that resulted in only or mostly conspecific progeny, perithecia developed very densely on the entire stroma surface. By contrast, in matings that involved high densities or only heterospecific spermatia (HET and 1:100 treatments) and mostly yielded hybrid progeny, perithecia were more scattered and often individually embedded among a dense white mycelium. This observation could indicate that fewer fertilizations involving heterospecific spermatia led to the development of perithecia, supporting the spermatia recognition scenario. Proliferation of mycelia appeared to be similar in all treatments of spermatia mixtures suggesting that spread of heterospecific mycelia was not impaired.

Previous findings in *E. festucae* made by Chung and Schardl (1997) demonstrate that application of conspecific spermatia halted interspecific matings, unless heterospecific spermatia were applied 6 d in advance. This suggests that heterospecific spermatia may be less effective in triggering development of female structures that are receptive for gamete fusion, which would lead to the observed deferred mating. Alternatively, conspecific spermatia may exhibit faster growth and interfere with heterospecific spermatia, even when applied with a time delay. To test these hypotheses, intra- and interspecific mating trials should be performed and modern imaging techniques implemented to reveal the mechanisms leading to assortative mating.

The two scenarios, spermatia recognition and spermatia competition, are not mutually exclusive and either one, or a combination of both, may lead to non-random mating. However, positive assortative mating due to recognition or increased competitiveness of conspecific spermatia does not seem to impose full reproductive isolation between *E. typhina* and *E. clarkii*. The two species were shown to be interfertile under experimental conditions and it is likely that also under natural conditions interspecific matings occur. Indeed, in a previous study (Bultman et al., 2011) approximately 9 % of the stromata collected from sympatric populations of *E. typhina* and *E. clarkii* contained hybrid ascospores. Personal observations in two additional sympatric populations confirmed this result. These findings indicate that the two species do mate regularly and that hybrid ascospores can be formed which could potentially infect new hosts, suggesting that additional factors play a role in impeding the establishment of hybrid offspring.

Hybrid ascospores showed a significantly reduced rate of germination compared to ascospores from intraspecific matings. This effect was more apparent in crosses with *E. typhina* as the stromal partner, whereas germination rates in crosses with *E. clarkii* as the stromal partner were more similar for hybrid and non-hybrid ascospores (Fig. 4). This may be explained by the generally lower germination rates of *E. clarkii* ascospores, which become fragmented within the ascus and appear to be more fragile particularly in hybrid progeny. Also, there may be an experimental bias as with single spore isolations using a micromanipulator turgid, mature spores may be preferred over less developed ones. Thus, actual germination rates of hybrid ascospores may even be lower than reported here. Clearly, impeded hybrid vigor can diminish the success of hybrid ascospores to be dispersed and transmitted to new hosts.

Although many ascospores germinated and formed mycelium on agar medium, some immediately sporulated and did not produce a mycelium. This characteristic was especially pronounced in hybrid offspring but occurred across all treatments and seemed to depend on the genotypes involved in the cross. Germination of ascospores by first producing conidiophores with conidia has been observed before in *Epichloë* infecting *Sphenopholis obtusata* and was described as iterative germination (Bacon and Hinton, 1988). Conidia produced by iterative germination may undergo up to three stages of microcyclic conidiation until mycelium development occurs (Bacon and Hinton, 1991). The role of iterative germination of the ascospores is so far unresolved. More abundant conidia could represent a repository of spermatia that allows further fertilizations when ascospores are dispersed to stromata that have been left unfertilized. In fact, it has been shown that ascospores (or the conidia produced from them) can function as spermatia for fertilization in *E. typhina* infecting cultivated *D. glomerata* (Rao et al., 2012). Furthermore, facilitating transmission of sexual *Epichloë* species, iterative conidiation of wind-dispersed ascospores can multiply the number of available spores that may infect grass florets of new hosts, or extend the period when infective spores are present.

Results of this study showed that the extent of non-random mating and ascospore viability can differ between genotypes. Such differences may also be relevant at the population level depending on structure and density of the infected grasses. For example, assortative mating is expected to be important in sympatric populations, where flies actually disperse mixtures of spermatia from different species. Moreover, if selection against hybrids is strong in these settings, prezygotic reproductive barriers are likely to be strengthened in a process of reinforcement (Servedio and Noor, 2003). Additionally, the number and size of stromata produced as well as fly visiting behavior (e.g. whether stromata are visited by only one or several flies) can affect the conditions of fertilization and thus the outcome of mating. In order to evaluate the importance of prezygotic mechanisms as potential reproductive barriers, it is crucial to understand the genetic and cellular mechanisms involved in fertilization (cell fusion), ascospore formation (karyogamy and meiosis) and subsequent infection of new host plants. Neither of these processes have been investigated in depth and will require further research.

Assortative mating clearly acts as an incomplete reproductive barrier between *E. typhina* and *E. clarkii* still leaving potential for gene flow if hybrids persist in nature. A previous study taking a population biology approach has provided evidence that the two species are in fact reproductively isolated (Schirrmann et al., 2015). Based on findings that each species is compatible only with its original host and that experimental hybrids between species have reduced infectivity in either parental host, it has been suggested that mechanisms of host-specificity may promote reproductive isolation (Schirrmann and Leuchtmann, 2015). Hybrid progeny likely display intermediate traits, which may lead to a mismatch of endophyte genotypes and host plants and could explain the lower infection success of hybrids. Thus, host-specialization appears to be able to effectively reduce gene flow between populations of *E. typhina* and *E. clarkii*. In combination with the pre- and post-zygotic mechanisms of assortative mating and reduced hybrid viability, this seems to constitute an established framework imposing reproductive isolation between the two species.

Reproductive isolation is often considered an important criterion for defining species, particularly in a sympatric context (Giraud et al., 2008). The two species *E. typhina* and *E. clarkii* appear to be reproductively isolated supported by the high measures of differentiation between sympatric populations (Schirrmann et al., 2018), although they can hybridize both in experimental settings and in nature. Since speciation usually occurs over a long period of time, intersterility of incipient species may build up gradually following genetic divergence. The members forming the *E. typhina* complex appear to be at different stages of the speciation process and exhibit different degrees of isolation. For example, *E. sylvatica* Leuchtmann and Schardl infecting *Brachypodium sylvaticum* (Huds.) P.B. is morphologically distinct and completely intersterile with other species of the *E. typhina* complex representing the most advanced stage of speciation, whereas the endophyte strains of several *Poa* hosts are morphologically indistinguishable and sexually fully compatible (Leuchtmann et al., 2014). *Epichloë* endophytes thus represent an ideal taxonomic group to shed light on mechanisms of speciation, but may also serve as a promising model system for broader research with a focus on host specialization, coevolution and symbiosis.

5. Conclusions

Our study underlines the importance of prezygotic mechanisms in limiting gene flow of a non-model organism and expands our knowledge of the mechanisms that may also be at play in evolution and speciation of many important pathogens. We demonstrate that fertilization preferentially takes place between conspecific spermatia and corresponding female structures (stromata) in the presence of sexually compatible spermatia of two *Epichloë* species suggesting that assortative mating may act as prezygotic reproductive barrier. This is one of few cases in fungi where assortative mating has been demonstrated experimentally and the first in a non-model ascomycete system. Furthermore, germination rates of hybrid ascospores were reduced indicating that hybrid offspring suffer from a competitive disadvantage compared to non-hybrid offspring. Moreover, previous work suggest that the host imposes selection on the ability of genotypes to infect and establish symbiosis and that traits contributing to host specialization may be

responsible, at least in part, for reproductive isolation (Gladieux, 2018; Schirrmann et al., 2018). Coincidence of several barrier effects may strengthen reproductive isolation and promote evolutionary processes that lead to speciation. The *E. typhina* species complex with its mostly interfertile and host-specific taxa provides an ideal system to study reproductive barriers that act in concert to build the arena for speciation. Further research is needed to elucidate the origin and evolutionary significance of each component contributing to reproductive isolation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.06.005.

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