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Assortative mating in sympatric ascomycete fungi revealed by experimental fertilizations

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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. As an experimental system, we used two interfertile species of the *Epichloë typhina* complex (*Clavicipitaceae, Ascomycota*), which are host-specific endophytes of sympatrically occurring grasses, *Dactylis glomerata* (infected *by E. typhina*) and *Holcus lanatus* (infected by *E. clarkii*). 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 in various proportions and examined their reproductive success on the stroma. 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 offspring was assessed, by determining germination rates of ascospores produced by experimental matings. 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 compared to treatments with conspecific spermatia. The preferential mating between conspecific genotypes and reduced hybrid viability may represent important reproductive barriers at the pre- and postzygotic stage that have not been documented before in sexual *Epichloë* species. Insights from fungal systems will deepen our understanding of the evolutionary mechanisms leading to reproductive isolation and speciation.

Keywords: *Epichloë*, fungi, reproductive barriers, spermatia competition

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

51 resulting in assortative mating constitute an effective reproductive barrier at the prezygotic
52 stage (Martin et al., 2013; Reynolds and Fitzpatrick, 2007; Snow and Spira, 1991) . However,
53 for positive assortative mating to occur, individuals or gametes need to be able to
54 discriminate among mating partners belonging to the same or a different species. This
55 preference for the more similar (i.e. conspecific) partner can be based on mate choice or may
56 occur as a byproduct of habitat choice, but can also originate from genes pleiotropically
57 affecting (host-) adaptation and mating or varying competitiveness of mating partners
58 (Giraud et al., 2010).

59 For the plant and animal kingdom it has been shown in numerous cases that assortative
60 mating by mate choice or gamete interaction play crucial roles in promoting prezygotic
61 reproductive isolation and thus the maintenance of species (Aldridge and Campbell, 2006;
62 Snowberg and Bolnick, 2012; Weis and Kossler, 2004). On the other hand, very few studies
63 have used fungal model systems to investigate the significance of these factors for speciation
64 processes, essentially including species of *Saccharomyces* (Murphy et al., 2006), *Neurospora*
65 (Karlsson et al., 2008; Turner et al., 2011) and *Microbotryum* (Büker et al., 2013; Le Gac et al.,
66 2007; van Putten et al., 2003). Moreover, mating systems and modes of reproduction that may
67 be relevant for emergence of reproductive isolation in nature have been poorly studied in
68 fungi, which include selfing and outcrossing, and for most fungi both sexual and asexual
69 reproduction (Billiard et al., 2012). This range in reproductive strategies makes fungi
70 attractive model systems to study fundamental processes of speciation.

71 The sexual species of the ascomycete genus *Epichloë* provide a particularly intriguing and
72 powerful model (Gladieux, 2018; Leuchtmann, 2003; Schirrmann et al., 2015, 2018). These
73 species exhibit a variety of different life styles throughout the sexual and asexual phases of
74 their life cycle and often form species complexes that include interfertile, yet reproductively
75 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 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 subpecies, 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

Dactylis glomerata (orchard grass) and *Holcus 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

150 introduced to other countries, where it may be considered a noxious weed (USDA-ARS, 2013).
151 Both species are continuously outcrossing by wind-pollination, have overlapping flowering
152 time, typically May to June, and often occur in sympatry.

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

158 Infected host plants used as stromal partners in this study originated from four naturally
159 infected grass accessions collected in Switzerland and France (Table 1). Single grass tillers
160 (and usually the whole plant) are infected by only one genotype and this genotype is
161 systemically propagated to all newly formed tillers (Leuchtmann and Clay, 1997). Accessions
162 originating from single tillers were cloned yielding 25 *D. glomerata* plants infected with *E.*
163 *typhina* and 11 *H. lanatus* plants infected with *E. clarkii*. Clones were kept in single pots in two
164 climate chambers, separated according to the mating type of infecting endophytes, at
165 22 °C/15h day (25kLux) and 15 °C/9h night, and 50 % humidity. Host grasses were fertilized
166 with solid lawn fertilizer (Mioplant, Migros, Switzerland) and received treatment against
167 gnats (Solbac 0.25 %, Andermatt Biocontrol, Switzerland) and powdery mildew (on *D.*
168 *glomerata*, Femicur 0.5 %). Climate chambers were fitted with sticky traps and ant traps to
169 prevent movement of insects among different plants.

170

171 *2.2*. Epichloë *species*

172 The sexual species of genus *Epichloë* are characterized based on their morphology, phylogeny
173 and mating compatibility. As *Epichloë* species are heterothallic, gametes carrying the opposite
174 mating type are required for cell fusion and successful mating (Schardl et al., 2014; White and

175 Bultman, 1987). The heterothallic mating system is controlled by a single genetic locus
176 discriminating two mating types (bipolar), which enforces mating between distinct haploid
177 genotypes and has fostered implementation of a biological species concept (Leuchtmann,
178 2003). In the most recent taxonomic treatment of the genus, *E. typhina* infecting *D. glomerata*
179 (and other hosts) and *E. clarkii* infecting *H. lanatus* were given the rank of subspecies because
180 of their interfertility (Leuchtmann et al., 2014). These taxa are considered part of a species
181 complex, together with additional closely related species and subspecies. The two species can
182 be easily distinguished by their ascospore morphologies. *E. typhina* has filiform, multiseptate
183 ascospores (176 ± 34 × 1.6 ± 0.2 μm) that remain unfragmented, whereas *E. clarkii* produces
184 spear-shaped part-spores (46 ± 16 × 2.3 ± 0.3 μm) resulting from fragmentation of filiform
185 spores at maturity (White, 1993).
186
187 *2.3. Natural fertilization and mating*
188 Fertilization of *Epichloë* in natural populations mostly depends on symbiotic *Botanophila* flies
189 that serve as vectors of spermatia (Leuchtmann and Michelsen, 2016) although cases are
190 known, where mating occurred in the absence of flies (Górzyńska et al., 2011; Hoffman and
191 Rao, 2014; Rao and Baumann, 2004). These flies are attracted to stromata by a set of specific
192 fungal volatiles that differ in their profile among fungal species (Schiestl et al., 2006;
193 Steinebrunner et al., 2008). Flies feed on spermatia, transport them within their gut and
194 actively deposit them on the stroma surface with their feces (Bultman et al., 1998). In a
195 mutualistic interaction, *Epichloë* species benefit from the spermatization service provided by
196 flies, whereas flies deposit their eggs on the stromata and larvae feed and develop on fungal
197 mycelium (Bultman et al., 1998). As a *Botanophila* fly visits several stromata, spermatia of
198 different species (if present) and different mating types are ingested and accumulate in its
199 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 isolates are maintained at the culture collection of the Plant Ecologial Genetics Group at ETH and can be obtained on request. A subets of these cultures are deposited at CBS-KNAW culture collection (Utrecht, The Netherlands).

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 days old colony. The mycelium was added to 500µl sterile H_2O in a 1.5ml 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

225 performed. Harvest was done after adding 5ml sterile H_2O containing 0.1 % Tween 20
226 (AppliChem, Darmstadt, Germany) by carefully scraping the surface with a sterilized spreader
227 rod and removing the suspension with a pipette. Spermatia were counted using a Neubauer
228 counting chamber and the suspensions diluted with sterile H_2O containing Tween 20 as above
229 to concentrations of 100 spermatia/µl. Mixtures and pure suspensions of spermatia for the
230 fertilization experiment were prepared from freshly harvested spermatia every week.
231
232 *2.5. Experimental fertilization*
233 Fertilizations were made on stromata of known mating type shortly after they had emerged
234 and were fully exposed. Eight µl of spermatia suspensions (containing 100 spermatia per µl)
235 were applied to stromata in 4 droplets of 2µl each with a pipette. This amount of spermatia
236 was considered ecologically relevant for fertilization and is estimated to be in the range of
237 what is naturally deposited by *Botanophila* flies within their feces on the stroma surface
238 (Bultman and Leuchtmann, 2003). Applied droplets stay in place on the stroma, as the
239 hydrophobic surface absorbs the aqueous solution very slowly (Fig. 2a). In order to mimic the
240 natural fertilizations performed by the *Botanophila* flies, the spermatia suspensions were
241 carefully spread over the surface in a linear fashion on one side of the stroma using the tip of
242 the pipette sideward. This procedure also simulates feeding behavior of *Botanophila* flies,
243 which is slightly destructive when mycelium and spermatia are ingested. As control, stromata
244 were treated with 0.1 % Tween 20 solutions not containing spermatia.
245 The experiments included seven crosses with different genotype combinations and for
246 each treatment, separate stromata were used with three to five replicate stromata per
247 treatment (depending on the number of stromata available) (Table 2). Treatments included
248 conspecific spermatia (CON), mixtures of conspecific and heterospecific spermatia in different
249 ratios (1:1, 1:10, 1:100) and heterospecifc spermatia (HET). This resulted in a total of 139

250 stromata that were fertilized. All treated stromata were tagged with color-coded and labeled
251 plastic tubes. After fertilization, host plants were enclosed by Plexiglas cylinders (25cm in
252 diameter, 50cm high) to prevent unwanted contact with other plants and to avoid
253 contamination.
254

255 *2.6. Microscopic analysis*

256 Mating success was evaluated 5-6 weeks after fertilization when perithecia containing
257 ascospores had developed on the stromata. From each stroma, three separate probes
258 (considered replicates) comprising several perithecia were taken along the length of the
259 stroma with tweezers and carefully squashed in a drop of water on a microscope slide.
260 Ascospore morphology was then examined using a light microscope at 400× magnification
261 and probes were categorized as 'typhina' (ascospores exhibiting the morphology of *E.*
262 *typhina*), 'clarkii' (ascospores exhibiting the morphology of *E. clarkii*), 'hybrid' (asci containing
263 both spore-morphotypes) or 'barren' (not containing any developed ascospores). Ascospores
264 of the two species are easily distinguishable, with *E. clarkii* producing short, spear-shaped
265 spores resulting from fragmentation at maturity, and *E. typhina* producing filiform,
266 unfragmented spores (White, 1993). Hybrid asci usually contain four filiform spores of the *E.*
267 *typhina*-type and four of the *E. clarkii*-type that typically disarticulate into four part-spores
268 each. However, in crosses where *E. clarkii* was the maternal (stromal) parent, besides normal
269 *E. clarkii*-type spores some ascospores of intermediate length were observed in asci, which
270 looked more similar to *E. typhina*-type spores but then disarticulated into two fragments. This
271 could indicate a predominantly maternal inheritance of spore morphology in *E. clarkii,* which
272 is manifested in hybrid progeny. From all probes containing mature ascospores, a minimum of
273 five single spores were isolated using a micromanipulator equipped with a micro dissecting
274 needle and plated on SMA plates (Supplementary Table S1). Germinating ascospores typically

275 produce phialids and conidia first (iterative germination), before colonies with vegetative
276 hyphae are formed (Bacon and Hinton, 1988). To assess germination of single spore isolates,
277 plates were checked for colony growth every other day for 2 weeks following isolation, and, if
278 necessary, contaminations were removed.
279
280 *2.7. Genetic identification of progeny*
281 Since only a limited number of isolates could be genotyped, we focused on one cross (cross 4)
282 analyzing the majority of isolates from this cross (main sample) and then selected a
283 subsample from other crosses (cross 1, 2 and 7) (for identity of crosses see Table 2). This
284 approach made it possible (1) to test for contamination with other genotypes than the ones
285 applied, (2) to verify conformity with the microscopic evaluation and (3) to assess the rate of
286 hybridization based on genetic markers by deducing from the main sample.
287 For the extraction of DNA, mycelium was harvested from colonies actively growing on
288 SMA medium. Strips of mycelium (appr. 120mg fresh weight) were placed in sterile plastic
289 tubes with metal beads, freeze dried for 24h and then ground using a mixer mill (Qiagen
290 Retsch M301). DNA was extracted using the NucleoSpin® Plant II Core Kit (Macherey-Nagel,
291 Düren, Germany) following the manufacturer's protocol based on the Cetyl Trimethyl
292 Ammonium Bromide (CTAB) lysis method. The DNA concentration of extracted samples was
293 quantified using a Quantus™ Fluorometer (Promega Corporation, Wisconsin, USA).
294 Genotypes of selected fungal isolates were determined by PCR amplification of 15
295 microsatellite markers using a multiplex PCR approach as previously described (Schirrmann
296 et al., 2015). Markers were arranged in four multiplex sets, and each set was amplified using a
297 PCR volume of 10µl containing approximately 1ng of genomic DNA in 5× PCR Buffer
298 (Promega, Madison, WI, U.S.A.), 5 U/µl goTaq Polymerase (Promega, Madison, WI, U.S.A.), 25
299 mM $MgCl_2$, 2.5 mM of each dNTP and 10× Primermix (2µM). The amplification conditions were

300 as follows: initial denaturation of dsDNA at 94 °C for 3 min, 30 cycles of 30s denaturation at
301 94 °C with 1 min annealing at 56 °C and 30s extension at 72 °C, and final elongation at 72 °C
302 for 5 min. Signals of the PCR products were detected on a 3130xl DNA Analyzer (Applied
303 Biosystems, Foster City, California, U.S.A.) with GeneScan-500 LIZ as size standard.
304 Electropherograms were analyzed using Geneious 6.1.8 (Drummond et al., 2013). Genotypes
305 of offspring were compared and assigned to their respective parents based on lengths of
306 alleles at microsatellite loci.
307
308 *2.8. Statistical analyses*
309 We analyzed whether proportions of hybrid offspring in the 1:1, 1:10 and 1:100 treatments
310 differed from the expected 50 %, 90 % and 99 % under the assumption of random mating (see
311 Supplementary Table S2). To test for deviation from random mating, the exact binomial test
312 was used. Analyses were first performed separately for each cross and subsequently for
313 crosses pooled by maternal genotype (stromatal partner). Results of the statistical tests were
314 consistent across 1:1 and 1:10 treatments (exact binomial tests, $p < 0.001$) and 1:100
315 treatments (exact binomial tests, $p > 0.5$) with the exception of one cross also showing a
316 significant result in the 1:100 treatment (exact binomial test, $p < 0.01$), independent of the
317 genotypes involved or the level at which the tests were performed. Therefore, data for all
318 replicates of a particular treatment were pooled, and analyses repeated for all crosses
319 combined and for crosses separated by identity of the maternal species (*E. typhina* or *E.*
320 *clarkii*). To address pseudoreplication issues when pooling data from crosses that differed
321 only in the genotypes of the spermatial partner, we run a linear mixed model on the pooled
322 data using genotypes as a random effect with the package lme4 (Bates et al., 2015), showing
323 that mating outcome is predominantly dependent on treatments with some variation among
324 genotypes, but without affecting significance of the results of pooled data.The statistical

325 significance of differences in germination rates between *E. typhina* and *E. clarkii* crosses, and
326 within the crosses for different treatments, was analyzed using Student's t-test on data pooled
327 according to identity of the maternal species. To analyze differences between treatments
328 within each cross, and additionally for pooled data since results were consistent, a binomial
329 analysis of variance (ANOVA) was performed, followed by post-hoc analysis using Tukey's
330 HSD test. All statistical analyses were conducted with R (version 3.3; R Development Core
331 Team).
332

333 **3. Results**

334 *3.1 Assortative mating*

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

342 Results reported here are based on microscopic evaluation of hybrid or non-hybrid
343 progeny. As expected, stromata that received only conspecific spermatia (CON) produced 100
344 % non-hybrid offspring and stromata that received only heterospecific spermatia (HET)
345 produced 100 % hybrid offspring based on the ascospore probes examined (Fig. 3).
346 Microsatellite genotyping of ascospore progeny confirmed that assignment as hybrid or non-
347 hybrid based on morphological observation was correct. Every single ascospore isolate of the
348 selected subsample could be assigned to the parental genotypes of stromatal and spermatial

349 partners used for mating. Representative results from cross 4 are depicted in Supplementary
350 Table S3.

351 When spermatia were applied in equal concentrations (1:1 treatments), proportions of
352 hybrid offspring were significantly lower (exact binomial test, $p < 0.001$) than expected under
353 random mating (50 %). In five out of seven crosses of this treatment, no hybrid offspring was
354 found at all and the remaining two crosses produced 13 % and 33 % hybrids, respectively.
355 Likewise, in 1:10 treatments results deviated significantly from expectations, whereas in
356 1:100 treatments hybrid offspring was still diminished although not significantly (mean 89 %,
357 exact binomial test, $p > 0.5$) (Fig. 3). Hybridization rates in 1:10 treatments varied
358 considerably among different crosses ranging from 0 to 43 % (mean 25.5 %) (Supplementary
359 Table S2).

360

361 *3.2. Germination rates of ascospores*

362 Germination of viable ascospores usually occurred within 24h after ejection from asci,
363 typically producing phialids and conidia first (iterative germination), before colonies with
364 vegetative hyphae were formed. Ascospores that had not germinated within two weeks after
365 isolation were considered to be non-viable. Overall, germination rates varied significantly
366 depending on treatments (F_4=9.35, $p < 0.001$). Germination rates were similarly high for
367 spore isolates from conspecific, 1:1 and 1:10 treatments (HSD p=nonsignificant), but lower for
368 1:100 treatments and for heterospecific crosses (HSD $p<0.01$), with one exception where 1:10
369 treatments also yielded significantly lower germination rates (cross 4, $p < 0.01$) (Fig. 4).
370 Generally, ascospores originating from treatments with pure or high proportions of
371 heterospecific spermatia took longer to mature and appeared to be more fragile. Furthermore,
372 isolates from *E. clarkii* stromata had lower germination rates than isolates from *E. typhina*
373 (Student's t-test $t_{1,27}$=3.22, $p < 0.003$). Many ascospores germinated and formed phialides with

374 conidia, however they failed to establish a mycelium. Therefore, the observed germination
375 rates overestimate the rate of spores that actually establish a viable colony. The amount of
376 failure to establish colonies depended on the parental genotypes and treatments applied.
377

378 **4. Discussion**

379 Understanding the fundamental mechanisms leading to species divergence is one of the main
380 objectives in evolutionary biology. While the theoretical framework of speciation has been
381 established, widely discussed and tested in a range of different organisms of the plant and
382 animal kingdom, speciation theory in fungi remains enigmatic and experimental studies
383 testing specific hypotheses are mostly limited to model systems (Giraud et al., 2008). Studies
384 of sympatric, closely related taxa such as the one presented here are particularly valuable to
385 disentangle the contributions of pre- and postzygotic barriers leading to reproductive
386 isolation. Furthermore, the reproductive model of *Epichloë* involving heterothallic transfer of
387 spermatia shares common features with many other fungi including important pathogens.
388 Examples are rust fungi (Helfer, 2014), the ascomycete *Leptographium* causing root collar
389 diseases in pine (Jacobs et al., 2006) and *Botrytis cinerea* causing grey mold in numerous crop
390 plants including grape (Williamson et al., 2007). Although largely unexplored, prezygotic
391 isolation mechanisms such as assortative mating through gamete competition may play an
392 important role in evolution and speciation of fungal pathogens. Here we investigated potential
393 reproductive barriers between two species of the *E. typhina* complex (*E. typhina* and *E. clarkii*)
394 that remain morphologically and genetically distinct in sympatric populations, although they
395 are interfertile. Specifically, we tested spermatia competition leading to assortative mating in
396 experimental fertilization trials and assessed germination success of hybrid ascospores.

397 The results of this study reveal that mating on the stroma is non-random and
398 preferentially takes place between individuals of the same species when mixtures of con- and

399 heterospecific spermatia are applied to stromata. However, with increasing proportion of
400 heterospecific spermatia the prevalence of conspecific matings declines while the proportion
401 of hybrid offspring increases. We propose two different mechanisms that could lead to the
402 observed positive assortative mating. Firstly, female structures (ascogonia or receptive
403 hyphae) may exhibit preference towards conspecific spermatia (or *vice versa*) when
404 undergoing cell fusion. This may involve pheromone signaling or other interactions on a
405 molecular level, similar to what has been described for mating type recognition (Casselton,
406 2002; Karlsson et al., 2008; Kim and Borkovich, 2006). It would imply that the stromal
407 partner can differentially attract or distinguish among spermatia from different species, and
408 thus may be referred to as spermatia recognition. Secondly, spermatia or mycelia growing
409 from them may compete with one anonther when proliferating over the surface of the stroma.
410 If conspecific spermatia germinate faster or spread more vigorously than heterospecific
411 spermatia, this could result in an increased number of fertilizations between conspecific
412 partners. The underlying mechanism would be a form of spermatia competition, much like the
413 competition of pollen that is known as a common reproductive barrier among plant species
414 (Rahmé et al., 2009).

415 In our experimental matings that involved conspecific or only moderate levels of
416 spermatia of the alternate species (CON, 1:1 and 1:10 treatments) and that resulted in only or
417 mostly conspecific progeny, perithecia developed very densely on the entire stroma surface.
418 By contrast, in matings that involved high densities or only heterospecific spermatia (HET and
419 1:100 treatments) and mostly yielded hybrid progeny, perithecia were more scattered and
420 often individually embedded among a dense white mycelium. This observation could indicate
421 that fewer fertilizations involving heterospecific spermatia led to the development of
422 perithecia, supporting the spermatia recognition scenario. Proliferation of mycelia appeared

423 to be similar in all treatments of spermatia mixtures suggesting that spread of heterospecific
424 mycelia was not impaired.

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

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

446 Hybrid ascospores showed a significantly reduced rate of germination compared to
447 ascospores from intraspecific matings. This effect was more apparent in crosses with *E.*

448 *typhina* as the stromal partner, whereas germination rates in crosses with *E. clarkii* as the
449 stromal partner were more similar for hybrid and non-hybrid ascospores (Fig. 4). This may be
450 explained by the generally lower germination rates of *E. clarkii* ascospores, which become
451 fragented within the ascus and appear to be more fragile particularly in hybrid progeny. Also,
452 there may be an experimental bias as with single spore isolations using a micromanipulator
453 turgid, mature spores may be preferred over less developed ones. Thus, actual germination
454 rates of hybrid ascospores may even be lower than reported here. Clearly, impeded hybrid
455 vigor can diminish the success of hybrid ascospores to be dispersed and transmitted to new
456 hosts.

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

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

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

523 one of few cases in fungi where assortative mating has been demonstrated experimentally
524 and the first in a non-model ascomycete system. Furthermore, germination rates of hybrid
525 ascospores were reduced indicating that hybrid offspring suffer from a competitive
526 disadvantage compared to non-hybrid offspring. Moreover, previous work suggest that the
527 host imposes selection on the ability of genotypes to infect and establish symbiosis and that
528 traits contributing to host specialization may be responsible, at least in part, for reproductive
529 isolation (Gladieux, 2018; Schirrmann et al., 2018). Coincidence of several barrier effects may
530 strengthen reproductive isolation and promote evolutionary processes that lead to speciation.
531 The *E. typhina* species complex with its mostly interfertile and host-specific taxa provides an
532 ideal system to study reproductive barriers that act in concert to build the arena for
533 speciation. Further research is needed to elucidate the origin and evolutionary significance of
534 each component contributing to reproductive isolation.
535

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543

544 **Appendix A. Supplementary data**

545 Supplementary data to this article can be found online at https://
546

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Figure legends:

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).

Fig. 2. Experimental fertilization of an *Epichloë* stroma and different stages of development: (a) application of spermatia suspension in small droplets of 2µl , (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 = 10µm, (e) initial stages of perithecial development from proliferating mycelium after fertilization, scale bar = 10µm, (f) developing perithecia, scale bar = 200µm.

Fig. 3. Proportions of hybrid (grey) 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.

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.

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).

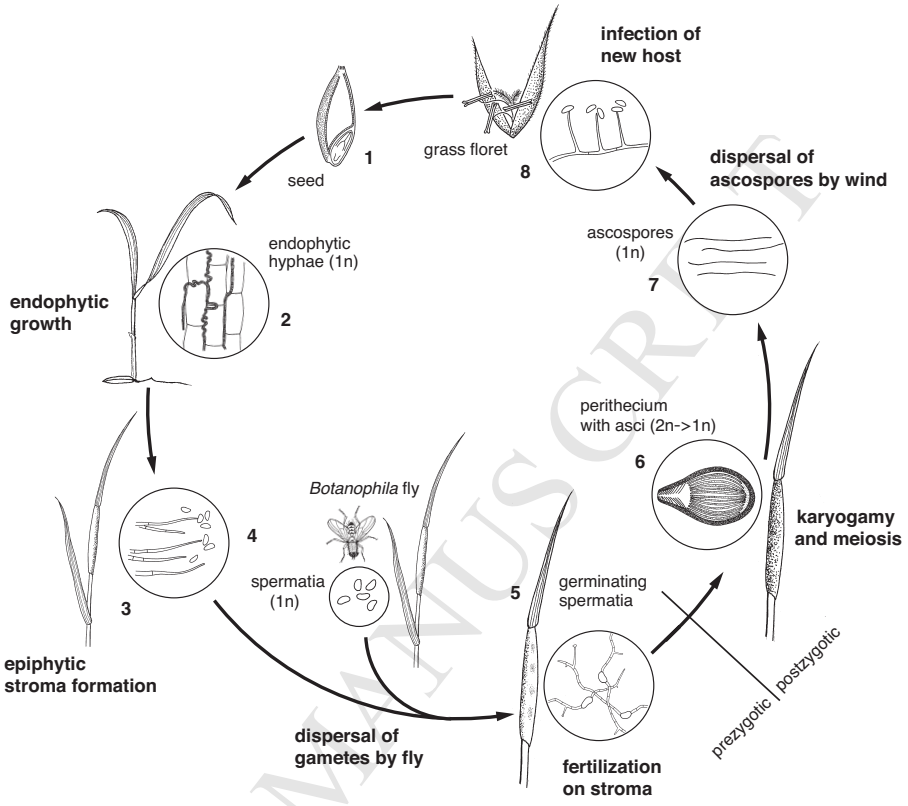
Isolate no.	Culture collection no.	Endophyte	Mating type[a]	Host species	Origin	Experimental use
Et1203	CBS 145506	*E. typhina*	mat-1	*D. glomerata*	Vesancy (F)	stromata & spermatia
Et1306	CBS 145507	*E. typhina*	mat-1	*D. glomerata*	Aubonne	spermatia
Et1204	CBS 145508	*E. typhina*	mat-2	*D. glomerata*	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

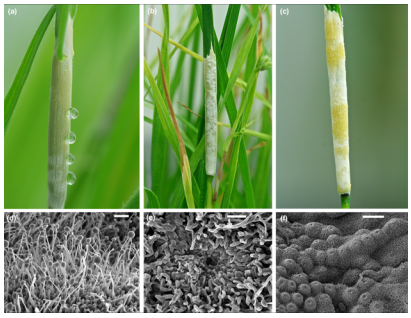
[a] Determined by mating tests with reference strain (Leuchtmann et al., 1994).

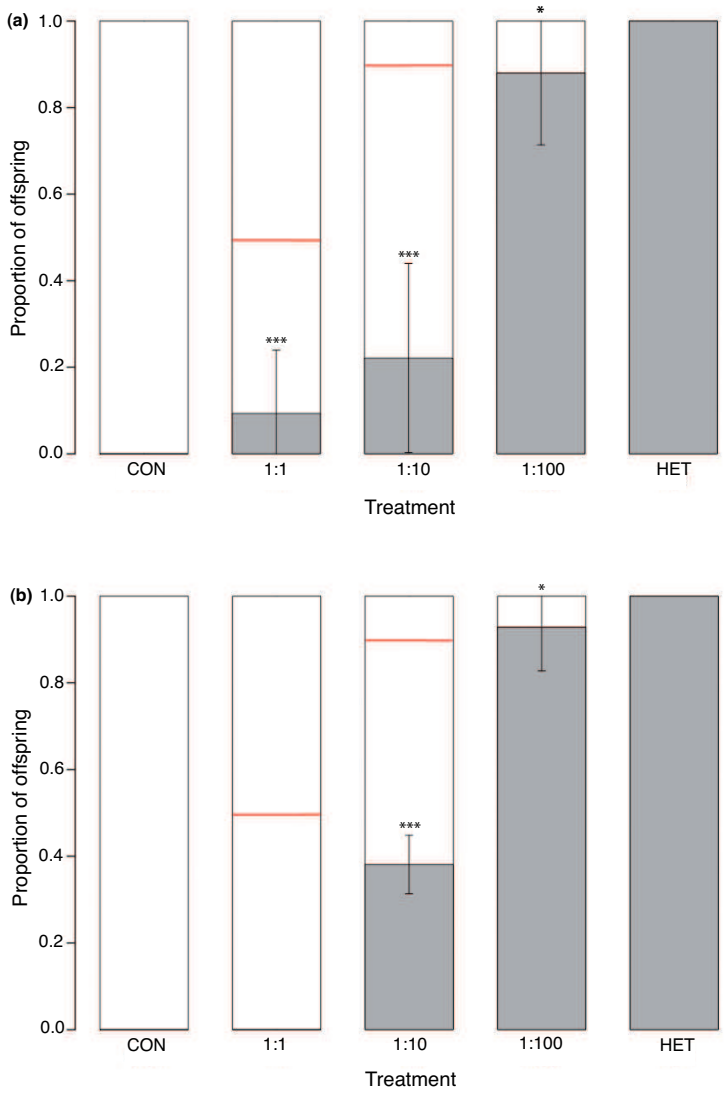
Table 2 Crosses performed between *Epichloë typhina* (Et) and *E. clarkii* (Ec) strains with number of ascospore probes evaluated per treatment. Each treatment was made using separate stromata (3-5 per treatment and cross) and 1-3 probes per stroma were taken. Treatments were conspecific spermatia (CON),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

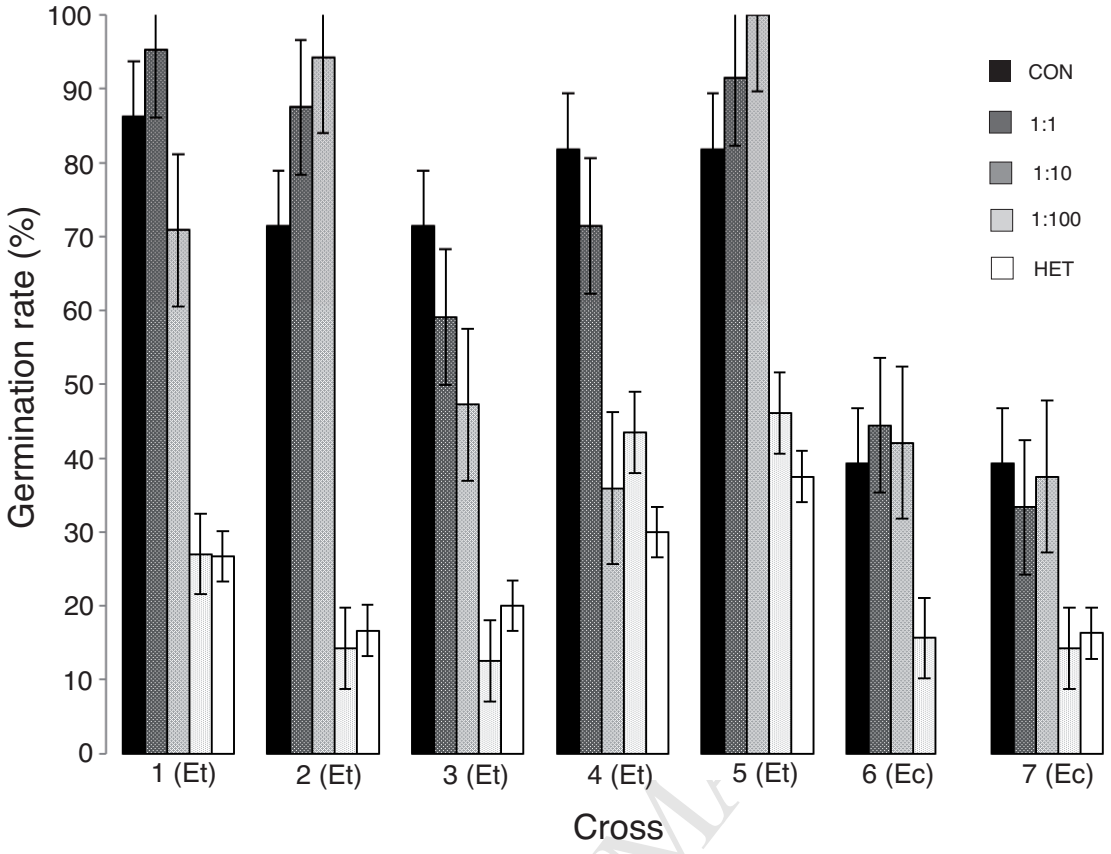
Cross				Treatment				
	Stroma		Spermatia	CON	1:1	1:10	1:100	HET
1	Et1305	×	Et1203 | Ec1401	15	15	12	15	10
2	Et1305	×	Et1203 | Ec1402	15	12	11	15	15
3	Et1203	×	Et1217 | Ec1403	10	6	9	5	4
4	Et1204	×	Et1306 | Ec1401	9	6	6	6	9
5	Et1203	×	Et1217 | Ec1206	10	9	3	4	8
6	Ec1205	×	Ec1403 | Et1305	28	12	7	7	np[a]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

[a] np = not performed.









Research highlights

- Mating preferentially takes place between conspecific individuals of *Epichloë*
- Hybrid ascospores have reduced viability
- Assortative mating and inferior hybrid vigour both act as reproductive barriers
- Pre- and postzygotic mechanisms underlie speciation processes in *Epichloë* species