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1 **Sheltered load in fungal mating-type chromosomes revealed by fitness**

2 **experiments**

3

4 Lou Guyot^{1,2}, Elizabeth Chahine¹, Christophe Lalanne³, Fanny E. Hartmann^{1*}, Tatiana

5 Giraud^{1*}

6

7 1 Ecologie, Systematique et Evolution, CNRS, Universite Paris-Saclay, AgroParisTech, Gif-
8 sur-Yvette, France

9 2 Master de Biologie, Ecole Normale Supérieure de Lyon, Universite Claude Bernard Lyon

10 1, Universite de Lyon, 69342 Lyon Cedex 07, France

11 3 Laboratoire Interdisciplinaire des Energies de Demain, Université Paris Cité, Paris, France

12

13 *FEH and TG are joint senior authors on this work.

14

15

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18

19 Corresponding author: tatiana.giraud@universite-paris-saclay.fr

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21

22

23 **Abstract**

24 Sex chromosomes and mating-type chromosomes can carry large regions with suppressed
25 recombination. In these non-recombining regions, recessive deleterious mutations are
26 expected to occur, as i) they are predicted to accumulate as a result of lower efficacy of
27 selection, and ii) they may even pre-exist and drive the evolution of recombination
28 suppression. Multiple genomic analyses have indirectly inferred the presence of deleterious
29 mutations in sex and mating-type chromosomes, but direct experimental evidence remains
30 scarce. Here, we performed fitness assays in fungi with megabase-large and young non-
31 recombining regions around the mating-type locus, using the *Schizothecium tetrasporum* and
32 *Podospora anserina* species complexes, to test whether heterokaryons (diploid-like,
33 heterozygous at the mating-type locus) exhibited a fitness advantage over homokaryons
34 (haploid-like, with a single mating-type allele), in terms of spore germination dynamics or
35 mycelium growth speed, under different conditions of light and temperature. We found a
36 faster growth of heterokaryons compared to one of the homokaryons for *P. anserina* at 18°C,
37 for *S. tetrasporum* and *S. tritetrasporum* at 22°C under light, and also at 22°C in the dark for
38 *S. tetrasporum*. These findings suggest the presence of a sheltered load, i.e. recessive
39 deleterious mutations at the heterozygous state in or near non-recombining regions, as these
40 species are highly homozygous otherwise. Leveraging on the experimental assets of fungi,
41 allowing cultivating separately haploid-like and diploid-like life stages, our experiments
42 provided one of the rare direct experimental evidence of sheltered load around mating-
43 compatibility loci, which is crucial for our understanding of sex-related chromosome
44 evolution.

45

46 **Introduction**

47

48 Even in sexual species, genomes can display large non-recombining regions (Wright et al.,
49 2016, Schwander et al., 2014, Bergero & Charlesworth, 2009). Such non-recombining
50 regions are particularly frequent around sex-determining loci and mating-type loci
51 (Charlesworth, 2017, Wright et al., 2016, Ma & Veltsos, 2021, Hartmann et al., 2021b, Jay et
52 al., 2024, Nicolas et al., 2004, Branco et al., 2017, Branco et al., 2018, Papadopulos et al.,
53 2015, Bachtrog, 2005, Bachtrog, 2013, Hallast et al., 2023). Deprived of recombination, these
54 genomic regions experience a reduced efficacy of selection (Felsenstein, 1974, Muller, 1932,
55 Hill & Robertson, 1966, Rice & Chippindale, 2001, Otto, 2009, Colegrave, 2002). As a
56 consequence, it has been theoretically suggested that non-recombining regions are bound to
57 degenerate (Smith & Haigh, 2009, Muller, 1932, Hill & Robertson, 1966, Fisher, 1930), i.e.,
58 to accumulate recessive deleterious mutations following recombination suppression. In
59 addition, theoretical models have shown that recessive deleterious alleles can be maintained
60 at the margin of self-incompatibility loci in linkage disequilibrium with it (Tezenas et al.,
61 2023, Antonovics & Abrams, 2004, Glémin et al., 2001, Uyenoyama, 2005). It has even been
62 suggested that the presence of recessive deleterious mutations around permanently
63 heterozygous alleles (e.g. a mating-type allele or a Y-like sex-determining allele) might be
64 the cause of the selection of recombination suppression, and not only a consequence (e.g., for
65 sheltering recessive deleterious mutations around a permanently heterozygous allele; (Branco
66 et al., 2017, Jay et al., 2021, Jay et al., 2024, Lenormand & Roze, 2022)). In both cases, one
67 expects recessive deleterious mutations to occur in non-recombining regions around sex-
68 determining or mating-type determining alleles. Such recessive deleterious mutations

69 associated with a permanently heterozygous allele constitute a sheltered load, as they are also
70 heterozygous and therefore not exposed to selection.

71

72 The presence of deleterious mutations in non-recombining mating-type and sex chromosomes
73 has been indirectly inferred through genomic analyses, that have for example revealed gene
74 disruptions, gene losses, non-synonymous substitutions, transposable element accumulation
75 and chromosomal rearrangements (Carpentier et al., 2022, Vittorelli et al., 2023, Duhamel et
76 al., 2023, Bachtrog, 2005, Bachtrog, 2013, Charlesworth & Charlesworth, 2000, Fontanillas
77 et al., 2015, Hough et al., 2014, Papadopulos et al., 2015, Whittle et al., 2011). However,
78 there has been little direct evidence for the existence of such sheltered load through
79 experimental fitness assays so far. To perform such experiments, one indeed needs to
80 compare fitness between a haploid phase or a homozygous state to the heterozygous diploid
81 state, i.e. between situations where mutations are exposed to selection to situations where
82 deleterious mutations are sheltered. Such situations can be found in autosomal supergenes,
83 i.e., non-recombining regions in autosomes governing adaptive and complex phenotypes
84 (Schwander et al., 2014). Using autosomal supergenes that can be homozygous, the existence
85 of sheltered load could be demonstrated in a few cases (Jay et al., 2021). A few balanced
86 haplo-lethal systems even exist, in which recessive deleterious mutations are lethal at the
87 homozygous state in non-recombining supergenes (Wielstra, 2020, Hill et al., 2023).
88 However, in the majority of organisms with non-recombining regions on sex-related
89 chromosomes, haploid or homozygous states do not occur in the life cycle and cannot be
90 generated in these non-recombining regions. The rare exceptions include self-incompatibility
91 loci in plants that can be rendered homozygous under certain conditions. Sheltered load was
92 found associated with the most dominant self-incompatibility allele in *Arabidopsis halleri*, at
93 several stages of the life cycle, using elevated CO₂ concentrations for disrupting self-

94 incompatibility (Stone, 2004, Llaurens et al., 2009). Sheltered load has also been
95 experimentally shown around the self-incompatibility locus in the plant *Solanum carolinense*
96 by using life stages where self-incompatibility is less strong to obtain homozygous plants at
97 the self-incompatibility locus (Mena-Alí et al., 2009). However, in the case of self-
98 incompatibility loci, regions without recombination are generally small, restricted to the self-
99 incompatibility locus (Goubet et al., 2012), and the existence of multiple alleles may render
100 unlikely the mechanism driving recombination suppression for sheltering pre-existing load
101 (Jay et al., 2022).

102

103 Fungi can be highly tractable models for investigating the existence of a sheltered load in
104 sex-related chromosomes. Indeed, there can be recombination suppression around their
105 mating-type loci in species with a long heterokaryotic (diploid-like) phase, i.e., with nuclei of
106 opposite mating types per cell, but unfused, which is functionally similar to a diploid stage
107 (Branco et al., 2017, Branco et al., 2018, Hartmann et al., 2021b, Fraser et al., 2004, Jay et
108 al., 2024, Grognet et al., 2014, Bakkeren & Kronstad, 1994, Menkis et al., 2008, Vittorelli et
109 al., 2023). In addition, many fungi display only two mating types (Billiard et al., 2011), and
110 recombination suppression around the mating-type locus has been detected only in those (Jay
111 et al., 2024). Genomic footprints of degeneration have been found in these non-recombining
112 regions, in the form of gene losses, transposable element accumulation, non-synonymous
113 substitutions and non-optimal codon usage and expression (Carpentier et al., 2022, Vittorelli
114 et al., 2023, Duhamel et al., 2023, Fontanillas et al., 2015, Whittle et al., 2011). In addition,
115 the life cycle of many fungi allows fitness comparison between haploid and heterokaryotic
116 stages (Grognet et al., 2014).

117

118 Mating types in fungi do not prevent selfing, as mating compatibility is controlled at the
119 haploid stage (Billiard et al., 2012, Giraud et al., 2008). As a matter of fact, the fungal
120 lineages with recombination suppression around their mating-type loci most often undergo
121 regular automixis i.e. intra-tetrad selfing (Billiard et al., 2012, Giraud et al., 2008, Hood &
122 Antonovics, 2000, Zakharov, 2005, Zakharov, 2023, Grognet et al., 2014, Jay et al., 2024,
123 Vittorelli et al., 2023), so that their genomes are highly homozygous in their extended
124 diploid-like phase, except in the non-recombining region around the mating type locus
125 (Branco et al., 2017, Branco et al., 2018, Hartmann et al., 2021a, Vittorelli et al., 2023). Such
126 life cycles in fungi are called pseudo-homothallic, with the production of self-fertile
127 heterokaryotic spores, encompassing two nuclei of opposite mating types originating from a
128 single tetrad after meiosis, thus germinating as a self-fertile heterokaryon and undergoing
129 mostly automixis. Self-sterile spores with a single mating type, germinating as homokaryotic
130 mycelia, can also generally be produced in laboratory conditions, although their importance
131 in nature is unknown. With such life cycles, experiments can be set up for comparing the
132 fitness between homokaryons (i.e. with a single type of nuclei per cell) and heterokaryons
133 (i.e. with nuclei of opposite mating types in each cell) in order to study the heterozygous
134 advantage in the region around the mating-type locus (Fig 1A). Indeed, in natural
135 heterokaryotic strains, that are typically highly homozygous genome-wide except around the
136 mating-type locus, because of high selfing rates, any variability in fitness between
137 homokaryotic and heterokaryotic stages should mainly be due to heterozygous recessive
138 deleterious mutations present in the non-recombining region around the mating-type locus
139 and possibly in its flanking region due to linkage disequilibrium (Branco et al., 2017, Tezenas
140 et al., 2023, Glémin et al., 2001). Some recessive lethal alleles have been revealed in anther-
141 smut fungi of the *Microbotryum* genus, by the lack of growth of haploids of one mating type
142 in some populations or species (Hood & Antonovics, 2000, Thomas et al., 2003, Oudemans

143 et al., 1998). Balanced lethals linked to the mating-type locus have also been shown in the
144 fungal-like oomycete *Phytophthora infestans* (Judelson et al., 1995).

145

146 We studied here five species of two phylogenetically distant fungal species complex within
147 the Sordariales order of Ascomycota: *Schizothecium tetrasporum* and *Schizothecium*
148 *tritetraperum*, belonging to the *Schizothecium tetrasporum* species complex (Vittorelli et al.,
149 2023), and *Podospora anserina*, *Podospora comata* and *Podospora pauciseta*, belonging to
150 the *Podospora anserina* species complex (Grognet et al., 2014, Hartmann et al., 2021a,
151 Boucher et al., 2017). These species are coprophilous, saprophyte or endophyte, and can be
152 found in herbivore faeces and soil (Silar, 2020). These fungi are excellent biological models
153 to investigate the presence of a sheltered load around their mating-type locus, displaying only
154 two mating-type alleles (*MATI-1* and *MATI-2*, also called *mat-* and *mat+*, respectively).
155 Indeed, these fungi display recombination suppression around their mating-type loci, as
156 shown by progeny segregation and nucleotide divergence analyses (Grognet et al., 2014,
157 Hartmann et al., 2021a, Vittorelli et al., 2023), and are pseudo-homothallic, i.e. with their
158 main life cycle phase being heterokaryotic (Grognet et al., 2014, Vittorelli et al., 2023), so
159 that sheltered load can be expected. Recombination suppression around the mating-type locus
160 spans approximately 1 Mb in the *S. tetrasporum* and *P. anserina* species complexes and has
161 been suggested to be quite recent, as indicated by the low divergence between mating types
162 and the lack of trans-specific polymorphism (Vittorelli et al., 2023, Grognet et al., 2014,
163 Hartmann et al., 2021a). Indirect evidence of sheltered load has been reported by genomic
164 analyses in their non-recombining regions, in particular a few gene losses or disruptions
165 (Vittorelli et al., 2023, Grognet et al., 2014). Furthermore, recombination suppression around
166 the mating-type locus represents convergent events between the *S. tetrasporum* and *P.*
167 *anserina* species complexes, that diverged over 150 million years ago, which provides

168 independent events of recombination suppression to study. Even within each of these two
169 species complexes, recombination suppression has likely evolved independently in the
170 various species, as suggested by the lack of trans-specific polymorphism and sizes of the non-
171 recombining regions (Hartmann et al., 2021a). In addition, natural strains are highly
172 homozygous genome-wide, except around the mating-type locus (Vittorelli et al., 2023,
173 Grognet et al., 2014, Hartmann et al., 2021a). Another asset of these models is that one can
174 cultivate *in vitro* homokaryotic and heterokaryotic mycelia (Figs 1A and 1B). In addition,
175 while most ascospores are heterokaryotic, homokaryotic spores are sometimes produced and
176 are easily distinguishable based on their smaller size (Vittorelli et al., 2023, Silar, 2020) (Fig
177 1C). In *S. tetrasporum* and *P. anserina*, previous experiments on a single strain did not detect
178 any heterokaryon advantage (Grognet et al., 2014, Vittorelli et al., 2023). However, a
179 difference in mycelium growth speed between *MATI-1* and *MATI-2* homokaryons has been
180 reported in *P. anserina*, but again using a single strain, when grown at stressful temperature
181 (37°C; (Contamine et al., 2004)). *Podospora anserina* indeed does not tolerate high
182 temperatures (Silar, 2020). Light is also an important regulator of diverse physiological
183 processes in *P. anserina* (Shen et al., 2022). Studying multiple strains is essential in
184 evolutionary biology, as the effects of deleterious mutations can depend on epistatic effects
185 and/or be present in only some individuals in populations, while still having important
186 population effects.

187

188 Here, we thus conducted assays using different fitness proxies on a dozen strains of each of *S.*
189 *tetrasporum*, *S. tritetrasporum* and *P. anserina*, by comparing heterokaryons and
190 homokaryons (Fig 1), in terms of mycelium growth speed under light or dark, at different
191 temperatures, and in terms of spore germination dynamics. We also ran exploratory fitness
192 experiments on small numbers of strains on *P. comata* and *P. pauciseta*. Our analyses reveal

193 a fitness advantage of heterokaryons over one of the homokaryons (either *MATI-1* or *MATI-*
194 2) in at least one tested condition for *S. tetrasporum*, *S. tritetrasporum* and *P. anserina*,
195 indicating the likely existence of a sheltered load in the non-recombining region present
196 around the mating-type locus and/or in its flanking region. In contrast, we found a faster
197 growth of one of the homokaryons compared to heterokaryons at 37°C in *P. pauciseta* and no
198 difference in *P. comata*, but small numbers of strains were available for these species.

199 Material and Methods

200 Strains

201 The information on the strains is given in Supplementary Table 1 (strain ID, species,
202 collection, year, substrate and geographic region of collection). We used 10 heterokaryotic
203 strains of *S. tetrasporum* and 13 heterokaryotic strains of *S. tritetrasporum* stored at -80°C.
204 We obtained homokaryotic mycelium of the *MATI-1* and *MATI-2* mating types for each
205 heterokaryotic strain as described previously (Vittorelli et al., 2023, Silar, 2020). Briefly, a
206 plug of heterokaryotic mycelium (5 mm³) was grown for each strain on a V8 medium Petri
207 dish for two to three weeks until perithecia formation. Then, agar Petri dishes were added on
208 the top, upside down, to collect projected spores (Silar, 2020). Homokaryotic spores were
209 then isolated, identified by their smaller size compared to heterokaryotic spores, and grown
210 on Petri dishes with G medium (Silar, 2020) (Fig 1C). These Petri dishes were subjected to a
211 heat shock (30 minutes at 65°C) to induce spore germination. After a few days of growth,
212 mycelia were transferred on M2 medium for growth. The mating-type allele of homokaryotic
213 mycelium grown from those spores was identified by PCR. For this goal, DNA was extracted
214 using Chelex and the mating-type allele (*MAT-1-1* or *MATI-2*) was determined using the
215 PCR conditions described previously (Vittorelli et al., 2023) and the following primer pairs:

216 *MAT-1-2* 5'‘GAGGGTGCAGTCGTGAT’3’ and 3'‘CCAGCCACACGAAAATCC’5’;
217 *MAT-1-1* 5'‘GATCGGATTCGCTCCAGA’3’ and 3'‘CGACCGTTGGAAATGACC’5’.

218 We also used 17 heterokaryotic strains of *Podospora anserina*, one heterokaryotic strain of *P.*
219 *comata* and three heterokaryotic strains of *P. pauciseta* (Supplementary Table 1), with
220 *MATI-1* and *MATI-2* homokaryotic mycelium stored at -80°C.

221

222 **Inoculum preparation**

223 Prior to each experiment, *MAT-1-1* and *MATI-2* homokaryotic mycelia of the studied
224 heterokaryotic strains were grown on M2 medium (Silar, 2020) for about 7 to 15 days at
225 27°C, 50% humidity and light and then stored at 4°C for a few days until the beginning of the
226 experiments. On the day of inoculation, fresh inoculum was prepared. Mycelium square plugs
227 of the two mating types for each strain were crushed separately (to form homokaryotic
228 mycelium solutions) and also together (to generate heterokaryotic mycelium solutions; (Silar,
229 2020, Vittorelli et al., 2023)), with 200 µL of sterile water for *S. tritetrasporum* and *S.*
230 *tetrasporum* and 150 µL of sterile water for the other species, using a TissueLyser at 30 Hz
231 for 20 to 30 min.

232

233 **Growth speed**

234 Spots of 20 µL of the obtained solutions for homokaryotic mycelium *MATI-1*, homokaryotic
235 mycelium *MATI-2* or heterokaryotic mycelium were deposited on Petri dishes with M2
236 medium (Silar, 2020), with three replicates per strain and condition. One central spot was
237 deposited per Petri dish, corresponding to a replicate. For *S. tritetrasporum*, two batches were
238 performed at different times. Plates were incubated at the conditions of temperature and light
239 exposure detailed in Table 1. Pictures of the plates were taken on two days (see details of the
240 days for each species in Table 1). As the mycelium limits of *P. anserina*, *P. comata* and *P.*

241 *pauciseta* were not always clearly distinguishable on pictures, the contour of the mycelial
242 colony was drawn with a marker pen under the Petri dish before taking the picture. Diameters
243 of mycelia were measured using ImageJ 1.54g (Schneider et al., 2012), and the growth speed
244 was computed as the difference in diameter between the two days divided by the number of
245 growth days. We measured one diameter per Petri dish, at the same location for the different
246 time points, using as a reference the label that had been put under the Petri dishes before
247 growth. Some Petri dishes were excluded from the analysis when contamination, dryness or
248 satellite colonies occurred (see Table S2 with raw data).

249

250 PCRs were performed on heterokaryotic mycelia 14 days post-inoculation for all *S.*
251 *tritetrasporum* strains in the two experiment batches to check whether they remained
252 heterokaryotic. Nine plugs (5 mm³) were sampled in each Petri dish from the inner to the
253 outer of the mycelium ring. DNA was extracted using Chelex and the mating-type allele
254 (*MAT-1-1*, *MAT-1-2* or both) was determined using the PCR conditions and the primer pairs
255 described above. When only a faint band appeared for one of the two mating types, the PCR
256 was re-run to check the presence of the second band. Some PCRs did not yield any band at
257 all, for neither mating type, and the corresponding plugs were excluded from the analysis on
258 heterokaryon persistence (see Table S2 with raw data).

259

260 **Germination dynamics**

261 For the 10 heterokaryotic strains of *S. tetrasporum*, three spots of 20 µL of heterokaryotic
262 mycelium solution were deposited on a single Petri dish with V8 medium (Vittorelli et al.,
263 2023) and incubated at 22°C until perithecia formation (about 15 days). PSN881A and
264 PSN779 strains did not produce any perithecia and were excluded from the analysis. Agar
265 Petri dishes were added on the top, upside down, to collect projected spores (Vittorelli et al.,

266 2023). We isolated 10 large spores and 10 small spores per heterokaryotic strain, which were
267 deposited on a Petri dish with G medium (Silar, 2020), allowing germination (two batches per
268 strain). Small spores are homokaryotic, while large spores are heterokaryotic at the mating-
269 type locus with a 90% probability, as estimated in (Vittorelli et al., 2023) based on
270 genotyping of the germinated mycelium, which is a minimum estimate, as the mycelium
271 could also have lost a nucleus during growth before genotyping. These Petri dishes were
272 foiled with aluminium paper, subjected to a heat shock of 30 min at 65°C and incubated at
273 27°C to induce germination (Silar, 2020). Germinated spores were counted on days 2, 3 and 7
274 to analyse the germination dynamics of small and large spores, i.e. the rates of germination
275 over time (Table 1). A few spores were excluded from the analysis when there was
276 contamination or two spores were observed germinating instead of one (see Table S3 with
277 raw data).

278

279 **Graphical summaries and statistical analyses**

280 Statistical analyses and graphic displays were performed using R software v 4.3.2 (R Core
281 Team, 2023). For the growth experiment analyses, a linear mixed-effect model was used for
282 all tested conditions for *S. tritetrasporum*, *S. tetrasporum*, *P. anserina* and *P. pauciseta*,
283 explaining growth speed using the nuclear status (i.e. heterokaryotic, *MATI-1* or *MATI-2*
284 homokaryotic) as a fixed effect and the strain ID as a random effect. In *S. tritetrasporum*, a
285 random effect for the batch was added. For *P. comata*, a single strain was used; a linear
286 model was therefore used with growth speed as the outcome and nuclear status (i.e.
287 heterokaryotic, *MATI-1* or *MATI-2* homokaryotic) as a predictor. For the germination
288 experiment analysis in *S. tetrasporum*, a generalised linear mixed-effect model with a
289 binomial distribution was used, modelling germination rate as a linear combination of day (as
290 a continuous predictor) and spore size class (large or small) treated as fixed effects, and strain

291 ID and batch as random effects. A statistical significance level of 5% was considered for all
292 statistical tests. For linear and linear mixed-effect models, predictions with standard
293 deviations are represented and p-values from Student t-tests are reported (with Satterthwaite
294 correction for the linear mixed-effect models), with default contrast coding (i.e, for a three-
295 level categorical predictor, only two regression coefficients are tested, excluding the
296 reference category, here set to be the heterokaryotic nuclear status, which is subsumed in the
297 intercept term). Omnibus tests for the overall significance of statistical models were based on
298 F-tests, with Satterthwaite's approximation for the degrees of freedom (DoF) in the case of
299 mixed-effects models, where the first and second DoFs correspond to the effect and the
300 residuals of the model, respectively. When considering a statistical model with a single
301 categorical predictor with k levels, this test can be seen as a joint test of all k levels (instead
302 of individual t-tests for all k-1 levels, excluding the reference level). In the case of the
303 generalised linear mixed-effects model, predictions with standard deviation obtained with the
304 Laplace approximation are represented and p-values from χ^2 -tests are reported. The linearity
305 of the response and predictor relationship and the Gaussian distribution of the residuals in all
306 models (including random effects intercept and slope in the case of random-effects models)
307 were assessed visually using R built-in facilities (fitted vs. residual values plot and QQ-plot,
308 respectively).

309

310 Results

311 To investigate the presence of sheltered load in the non-recombining regions around the
312 mating-type locus, or at their margin, in *S. tetrasporum*, *S. tritetrasporum* and *P. anserina*,
313 experiments were performed in each species to compare the fitness of heterokaryons to that

314 of homokaryons using strains as biological replicates (10, 13 and 17 strains, respectively;
315 Table S1; Fig 1). Exploratory fitness analyses were run on smaller numbers of strains in *P.*
316 *comata* and *P. pauciseta* (one and three strains, respectively). For *S. tetrasporum* and *S.*
317 *tritetrasporum*, we used as fitness proxies the mycelium growth speed under light at 22°C
318 between day 6 and day 14. In addition, we used as fitness proxies for *S. tetrasporum* the
319 mycelium growth speed under dark at 22°C and assessed the spore germination dynamics
320 between day 2 and day 7. For *P. anserina*, *P. comata* and *P. pauciseta*, we measured
321 mycelium growth speed between day 2 and day 6 under light at 18°C and 37°C. These
322 conditions were chosen for each species based on preliminary experiments or previous
323 knowledge on the different species to use more or less stressful conditions. Table S2 gives the
324 raw measures for the growth experiment for which 5% of plates have been excluded on
325 average per analysis due to the presence of satellite colonies, contaminants or dryness. Table
326 S3 gives the raw measures for the germination experiment, for which two out of the 10
327 strains of *S. tetrasporum* were excluded due to lack of perithecia and less than 1% of the
328 spores were excluded, due to contamination or the germination of two spores, meaning that
329 two spores had been deposited instead of one.

330 In *S. tritetrasporum*, heterokaryons grew significantly faster than homokaryons *MATI-1*
331 under light at 22°C by 5.6% (Figs 2 and S1; $F(2, 192.3)=7.9683$, $p\text{-value}=0.0005$). In *S.*
332 *tetrasporum*, heterokaryons also grew significantly faster than homokaryons *MATI-1* under
333 light at 22°C by 5.5% (Figs 3A and S2A; $F(2, 70.2)=4.5497$, $p\text{-value}=0.0139$), while
334 heterokaryons grew significantly faster than homokaryons *MATI-2* in the dark at 22°C by
335 4.0% (Fig 3B and S2B; $F(2, 67.2)=5.9887$, $p\text{-value}=0.0040$).

336 Given that some level of nuclear loss of one mating type has been reported to occur during
337 heterokaryotic mycelium growth in the *S. tetrasporum* species complex (Vittorelli et al.,

338 2023), we used PCR to test whether and to what extent such a phenomenon occurred in our *S.*
339 *tritetraperum* heterokaryotic mycelia used for growth experiments, by genotyping the
340 mating-type locus for nine plugs per Petri dish. We found that mycelia had remained mostly
341 heterokaryotic (Figs S1 and S3).

342

343 In *P. anserina* under light conditions, heterokaryons grew significantly faster than
344 homokaryons *MATI-2* at 18°C, by 8.1% (Figs 4A and S4A; $F(2, 128.4)=5.7881$, p -
345 value=0.0039). The tendency was similar at 37°C but the difference was not significant (Figs
346 4B and S4B; $F(2, 133.0)=1.4409$, p -value=0.2404).

347

348 We found a similar tendency in *P. pauciseta* at 18°C, but not significant (Figs 5A and S5A;
349 $F(2, 22)=1.4184$, p -value=0.2634). In contrast, we found a significantly slower growth of
350 heterokaryons compared to *MATI-1* homokaryons at 37°C in *P. pauciseta*, by 27.6% (Fig 5B
351 and S5B; $F(2, 21)=4.0481$, p -value=0.0326). In *P. comata*, both homokaryons also seemed to
352 grow faster than heterokaryons at 18°C (Figs 5C and S5D; $F(2, 6)=2.348$, p -value=0.1765)
353 and 37°C (Figs 5D and S5D; $F(2, 6)=1.1963$, p -value=0.3654), although it was not
354 significant. However, we had only three and one strains for these species, respectively, so
355 little conclusion can be inferred from these species at the population scale. Note that *P.*
356 *comata* grew very little at 37°C, indicating that it represents a too stressful condition for this
357 species.

358

359 The comparison of the germination dynamics of small spores (i.e. homokaryotic spores)
360 versus large spores (i.e. mainly heterokaryotic spores; (Vittorelli et al., 2023)) in *S.*
361 *tetrasporum* did not reveal any evidence of heterozygous advantage (Figs 6 and S6).

362

363 Discussion

364

365 **Likely presence of recessive deleterious mutations around the mating-type locus**

366 Our fitness assay showed that heterokaryons were fitter than at least one homokaryon under
367 light at 18°C in *P. anserina* and at 22°C in *S. tetrasporum* and *S. tritetrasporum*, and also at
368 22°C in the dark in *S. tetrasporum*, suggesting heterozygote advantage. Such results are most
369 likely due to the presence of recessive deleterious mutations in the non-recombining region
370 around the mating-type locus, being associated to a single mating-type allele, and/or in
371 linkage disequilibrium with the mating-type locus in the margin of the non-recombining
372 region, these mutations being expressed at the homokaryotic state but sheltered at the
373 heterokaryotic state. Our findings thus suggest the existence of a sheltered load near the
374 mating-type locus in the studied fungi with a sample size of at least 10 strains.

375

376 Our study is one of the very rare experimental studies showing the existence of a sheltered
377 load for sex-related chromosomes. Only a few studies could investigate a sheltered load so
378 far, around self-incompatibility loci in plants (Stone, 2004, Llaurens et al., 2009) as this is
379 one of the rare systems where mating-type loci can be rendered homozygous. To investigate
380 the presence of a sheltered load, we leveraged on another unique biological system, pseudo-
381 homothallic fungi, whose life cycle allows fitness comparison between individuals in which
382 mutations around the mating-type locus are sheltered versus exposed to selection, while the
383 rest of the genome is exposed to selection, being highly homozygous due to high selfing rates
384 in nature. Our study thus provides unique experimental evidence of a sheltered load around
385 mating-type loci, i.e. of heterozygous recessive deleterious mutations in the non-recombining
386 region, in three fungal species with independent events of recombination suppression.

387 Previous evidence in fungi include haplo-lethal alleles linked to mating types in anther-smut
388 fungi and oomycetes, in which one or the two mating types fail to grow at the haploid stage
389 or cause a segregation bias in progenies (Hood & Antonovics, 2000, Thomas et al., 2003,
390 Oudemans et al., 1998, Judelson et al., 1995), but the non-recombining regions were much
391 older in these cases. Here, we reveal the existence of a sheltered load in very young non-
392 recombining regions (Hartmann et al., 2021a, Vittorelli et al., 2023, Grognet et al., 2014).
393 Recessive deleterious mutations have also been experimentally shown to occur in a few
394 autosomal supergenes (Jay et al., 2021, Wielstra, 2020, Hill et al., 2023). Altogether, such
395 direct experimental evidence of sheltered load is important for understanding the evolution of
396 non-recombining regions.

397

398 The lack of significant heterozygote advantage detected in certain conditions might be due to
399 the genes with putative deleterious alleles having little role under these conditions, the weak
400 effect of the mutations, and/or the low number of mutations. In all three fungal species with a
401 sample size of at least 10 strains, the heterokaryons indeed grew faster than at least one
402 homokaryon in at least one condition, and showed similar tendencies across all conditions.
403 The growth advantage was around 5%, which may constitute a non-negligible advantage to
404 colonise short-lined habitats such as faeces. The spore germination dynamics in contrast did
405 not seem different between small and large spores. Heterozygous advantage need not be
406 expressed in all stages of the life cycle or in all strains to have a significant fitness effect in
407 natural populations: even if the heterozygous advantage only holds at some temperature and
408 during growth (and not during germination), it may incur non-negligible fitness advantage if
409 these conditions are frequent enough in natural populations.

410

411 No significant heterozygote advantage was detected in *P. comata* and we even found a
412 heterozygote disadvantage in *P. pauciseta* at 37°C, but we only used one or three strains for
413 these species as preliminary experiments. Such a heterozygote disadvantage could be due to
414 negative epistasis or outbreeding depression; indeed, although these species are highly
415 selfing, some rare events of outcrossing do occur (Hartmann et al., 2021a). However, further
416 natural strains would need to be analysed to draw conclusive inferences. In *S. tetrasporum*
417 and *P. anserina* for example, experiments on a single strain did not detect any heterokaryon
418 advantage in previous studies (Grognet et al., 2014, Vittorelli et al., 2023), while it was
419 revealed here with a dozen strains. For *P. pauciseta* at 18°C, the heterokaryon seemed to
420 grow faster than homokaryons, and this condition may be worth exploring using additional
421 strains and conditions. Indeed, in previous studies in which a single strain of *P. anserina* had
422 been studied (Grognet et al., 2014), a significant difference had been found at 37°C between
423 the two homokaryons but not at 22°C (Grognet et al., 2014, Contamine et al., 2004). Here, *P.*
424 *comata* did not grow much at 37°C, and it may be worth exploring lower but still stressful
425 temperatures.

426

427 Few other studies have compared fitness proxies between homokaryons and heterokaryons in
428 fungi. In three lineages of *Neurospora tetrasperma*, another Sordariales fungus with
429 recombination suppression around its mating-type locus, fitness estimates have been
430 computed comparing heterokaryons with different nuclei ratios and also compared to
431 homokaryons, using as a proxy the quantity of asexual haploid spores (conidia) produced by
432 mycelia. In each lineage, one mating type produced more spores than the other mating types,
433 and heterokaryons appeared intermediate (Meunier et al., 2018). This suggests codominance
434 for the alleles controlling spore quantity, and no sheltered load affecting this trait. Few strains
435 have however been analysed in this study (Meunier et al., 2018).

436

437 **Sheltered load originating post- or pre-recombination suppression and models of sex**
438 **chromosome evolution**

439 Our results on the three species with at least ten strains, i.e., *S. tetrasporum*, *S. tritetrasporum*
440 and *P. anserina*, support the prediction that recessive deleterious mutations occur in the non-
441 recombining regions around mating-type loci and/or at its margin. However, our
442 experimental setting does not allow distinguishing whether the load occurred before or after
443 recombination suppression. Indeed, recessive deleterious mutations might have accumulated
444 as a consequence of recombination suppression (Smith & Haigh, 2009, Muller, 1932, Hill &
445 Robertson, 1966, Fisher, 1930) and/or before recombination suppression (Tezenas et al.,
446 2023, Antonovics & Abrams, 2004, Glémin et al., 2001, Uyenoyama, 2005), and then even
447 possibly driving the evolution of recombination suppression (Jay et al., 2024, Jay et al., 2022,
448 Branco et al., 2017). The young age of the recombination suppression in these species gives
449 plausibility to an origin of the deleterious mutations before recombination suppression.

450

451 We found significant differences in terms of growth speed, in the species with at least ten
452 strains, i.e., *S. tetrasporum*, *S. tritetrasporum* and *P. anserina*, between heterokaryons and a
453 single one of homokaryons. In *S. tetrasporum*, the homokaryon displaying a disadvantage
454 was not the same in the different conditions, suggesting the existence of deleterious mutations
455 in both haplotypes, but different ones. This altogether may be because of stochastic
456 accumulation of recessive deleterious mutations following recombination suppression in the
457 non-recombining region or at its margin, with specific mutations affecting by chance one
458 mating type and not the alternative one. It may also be because recombination suppression
459 was actually due to selection for a non-recombining fragment with fewer deleterious
460 mutations than average in the population (Jay et al., 2021, Jay et al., 2024); in this case, the

461 corresponding homokaryon would be expected to initially carry a particularly light load
462 compared to the alternative mating type.

463

464 We found relatively weak differences in fitness (ca. 5% differences in mycelium growth
465 speed) and the heterozygous advantage was not detected in all traits, which may be explained
466 by the young age of the recombination suppression and/or by a non-negligible haploid phase
467 that would purge to some extent recessive mutations with large detrimental effect. Indeed,
468 although rare, some homokaryotic spores are produced in these fungi: in *S. tetrasporum*, 2%
469 to 10% of asci with small (i.e. homokaryotic) spores have been reported (Raju & Perkins,
470 1994, Vittorelli et al., 2023). In *P. anserina*, 5% of spores are homokaryotic (Ament-
471 Velásquez & Vogan, 2022). The loss of nuclei of one mating type during mycelium growth in
472 *Schizothecium* fungi may also expose recessive deleterious mutations to selection. Therefore,
473 because of the young age of recombination suppression and the existence of a non-negligible
474 haploid phase, even if limited, we do not expect a high load in the non-recombining region.
475 Nevertheless, we documented the existence of a sheltered load, which may have pre-existed
476 and/or accumulated following recombination suppression.

477

478 **Alternative hypotheses to sheltered load**

479 There may however be alternative hypotheses explaining the higher fitness of heterokaryons.
480 Although the species used mainly reproduce by selfing and most strains display genome-wide
481 homozygosity (Grognet et al., 2014, Hartmann et al., 2021a, Vittorelli et al., 2023), some rare
482 events of outcrossing can generate short tracks of heterozygosity in genomic regions far from
483 the mating-type locus, which could also have provided a heterozygote advantage in *S.*
484 *tetrasporum*, *S. tritetrasporum* and/or *P. anserina*. It could also be due to a heterozygous
485 advantage not related to deleterious mutations, although such advantages are rare in nature

486 and mostly related to host-parasite interactions (Gemmell & Slate, 2006), and therefore
487 unlikely in our experimental setting.

488

489 The heterokaryon advantage detected may also be due, in principle, to a ploidy advantage, for
490 example, related to gene expression dosage, which may be expected to be twice as high in
491 heterokaryons as in homokaryons. It is not clear, however, whether heterokaryons have twice
492 as many nuclei as homokaryons, as there are multiple nuclei per cell in both types of mycelia
493 (Sylvain Brun, pers. comm.) and the nuclei number differences per cell between
494 homokaryons and heterokaryons have not been investigated.

495

496 **Possible confounding effects**

497 Nuclear losses have been shown to occur within a heterokaryotic mycelium in the *S.*
498 *tetrasporum* species complex (Vittorelli et al., 2023). However, such a phenomenon, if any
499 effect, would only have attenuated differences between homokaryons and heterokaryons, so
500 that our experiments are conservative regarding this possibility. Furthermore, we found that
501 most heterokaryotic plugs tested had kept nuclei of the two mating types. Even for the
502 mycelia in which we detected a single mating type, it may be that the two mating types are in
503 unbalanced proportions, with the minor one being in frequency beyond the PCR sensitivity
504 threshold (Meunier et al., 2018, Meunier et al., 2022). In addition, large spores have been
505 shown to be sometimes homokaryotic in *S. tetrasporum* (less than 13% of large spores
506 germinated as homokaryons, which may also be due to nucleus loss during growth (Vittorelli
507 et al., 2023)), which might explain the non-significant effect of spore size on germination
508 dynamics.

509

510 **Conclusions**

511 In conclusion, our findings suggest the presence of a sheltered load, i.e. recessive deleterious
512 mutations at the heterozygous state around the mating-type locus, in three species of
513 Sordariales fungi with young regions of recombination suppression. Leveraging on the
514 experimental assets of fungi, allowing cultivating separately haploid-like and diploid-like life
515 stages, in a system with two mating types permanently heterozygous and genome-wide
516 homozygosity except around the mating-type locus, our experiments provided one of the rare
517 direct experimental evidence of sheltered load around mating-compatibility loci, which is
518 crucial for our understanding of sex-related chromosome evolution.

519

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537

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722 chromosomes in fungi. *Trends in Genetics and Evolution* **6**: doi:
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728 **Table 1:** Number of strains from *Schizothecium tetrasporum*, *Schizothecium tritetrasporum*,
 729 *Podospora anserina*, *Podospora comata* and *Podospora pauciseta* used for experiments,
 730 summary of the conditions (light and temperature) and measure time points for which fitness
 731 proxies (growth speed or germination dynamics) were assessed according to species and
 732 strains. Information on strains can be found in Table S1.

Growth speed				
Species	Number of strains	Measure time points (days)	Temperature	Light exposure
<i>S. tetrasporum</i>	10	6 / 14	22°C	Yes
<i>S. tetrasporum</i>	10	6 / 14	22°C	No
<i>S. tritetrasporum</i>	13	6 / 14	22°C	Yes
<i>P. anserina</i>	17	2 / 6	18°C	Yes
<i>P. anserina</i>	17	2 / 6	37°C	Yes
<i>P. comata</i>	1	2 / 6	18°C	Yes
<i>P. comata</i>	1	2 / 6	37°C	Yes
<i>P. pauciseta</i>	3	2 / 6	18°C	Yes
<i>P. pauciseta</i>	3	2 / 6	37°C	Yes

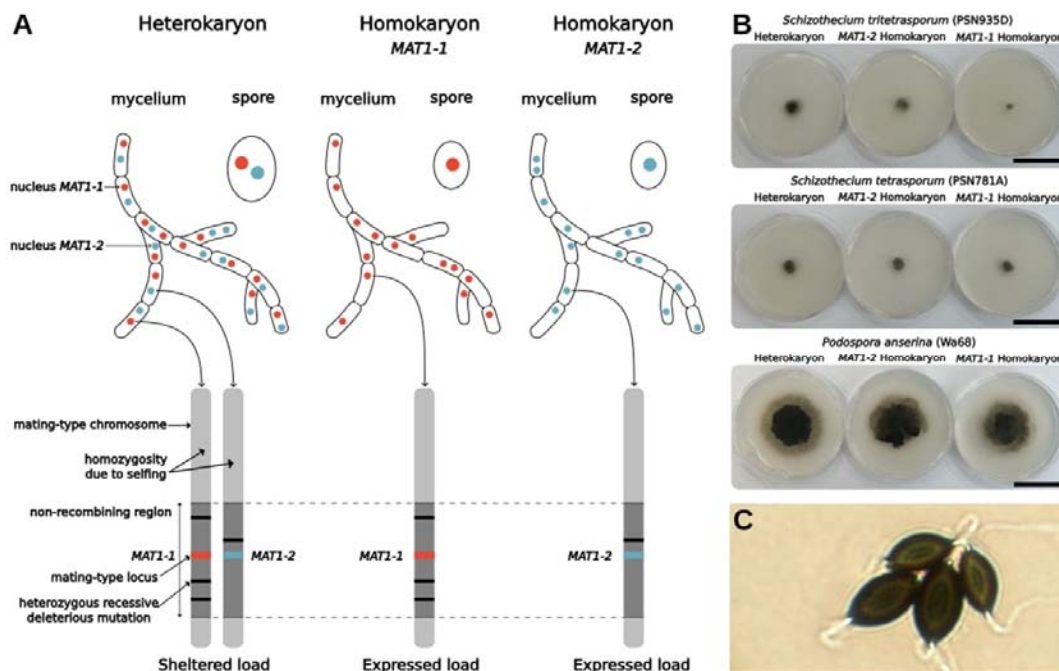
Germination dynamics		
Species	Number of strains	Measurement time points (days)
<i>S. tetrasporum</i>	8	2 / 3 / 7

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735 **Figures**

736 **Figure 1: Fitness experiments to test for the presence of a sheltered load in pseudo-**
737 **homothallic ascomycete fungi, i.e. producing heterokaryotic spores (with nuclei of the**
738 **two mating types), and with therefore a main phase of the life cycle being**
739 **heterokaryotic. A) Schema illustrating how the life cycle of pseudo-homothallic fungi can**
740 **be used to test for the existence of sheltered load (i.e the presence of heterozygous recessive**
741 **deleterious mutations) in the non-recombining region around mating-type loci. B) Pictures of**
742 **mycelium after six days of growth on Petri dishes under light conditions at 22°C, with**
743 **heterokaryons at left, *MAT1-2* homokaryons in the middle and *MAT1-1* homokaryons at right,**
744 **for *Schizothecium tritetrasporum* (strain PSN935D), *Schizothecium tetrasporum* (strain**
745 **PSN781A) and *Podospora anserina* (strain Wa68), from top to bottom. Scale bar: 4 cm. C)**
746 **Picture of *Schizothecium tetrasporum* spores (strain CBS815.71), with two small on *the* top**
747 **right and two large on *the* bottom left. The large spores have length and width of ca. 25 x 12**
748 **µm.**

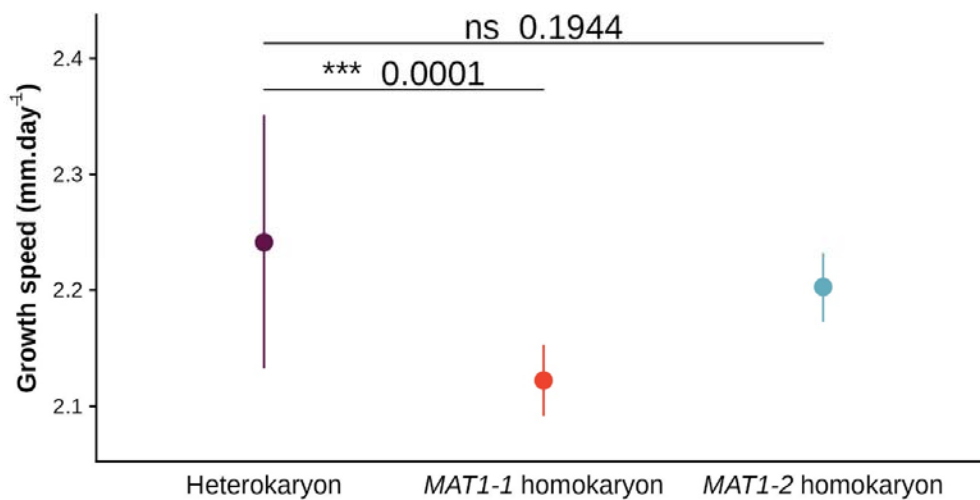


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751 **Figure 2: Mycelium growth speed of heterokaryons versus homokaryons in**

752 *Schizothecium tritetrasporum*. Mycelium growth speed ($\text{mm}\cdot\text{day}^{-1}$) of heterokaryons (in
753 purple), *MAT1-1* homokaryons (in red) and *MAT1-2* homokaryons (in blue), between day 6
754 and day 14 estimated from two batches of three replicates using 12 strains grown under light
755 conditions at 22°C. Dots and bars represent estimated means and standard deviations from a
756 linear mixed-effect model explaining growth speed using the nuclear status (*MAT1-1*
757 homokaryon, *MAT1-2* homokaryon or heterokaryon) as fixed effect and strain ID and batch
758 as random effects. P-values correspond to t-tests with Satterthwaite correction (ns: non
759 significant; *** = $p < 0.001$).



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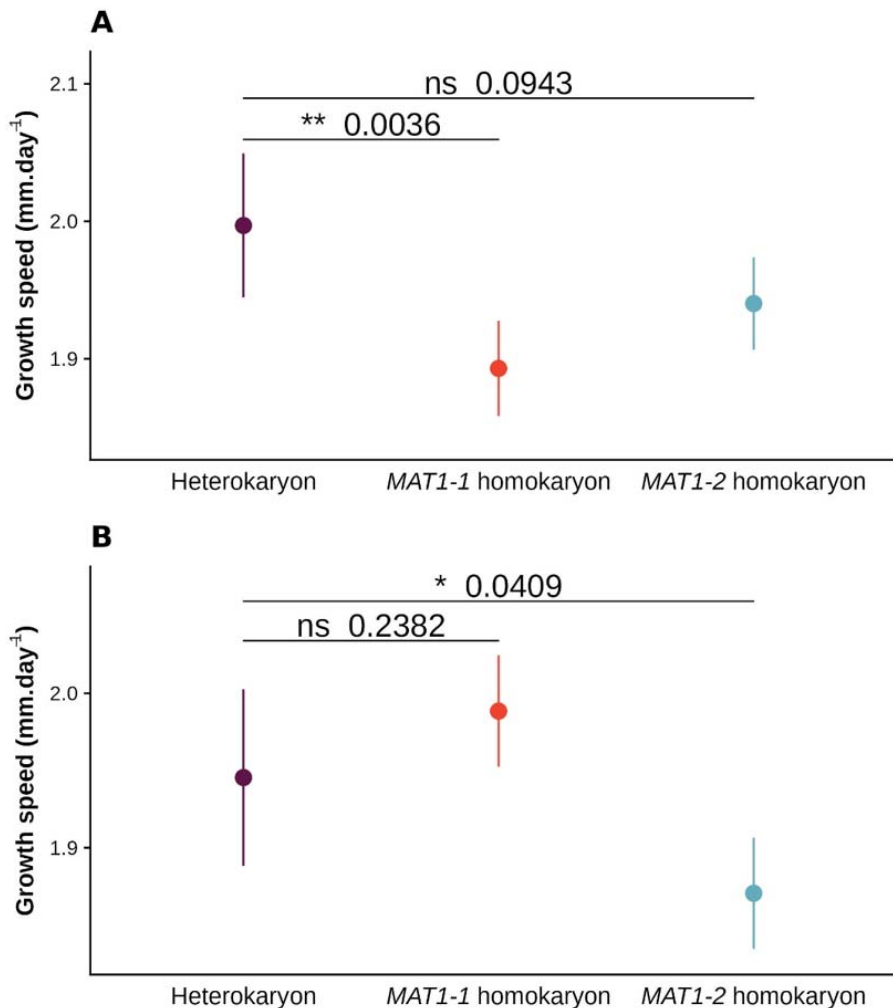
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768 **Figure 3: Mycelium growth speed of heterokaryons versus homokaryons in**
769 *Schizothecium tetrasporum*, under light and dark conditions. Mycelium growth speed
770 ($\text{mm}\cdot\text{day}^{-1}$) of heterokaryons (in purple), *MAT1-1* homokaryons (in red) and *MAT1-2*
771 homokaryons (in blue), between day 6 and day 14 estimated from three replicates using 10
772 strains grown under **A)** light or **B)** dark conditions, both at 22°C. Dots and bars represent
773 estimated means and standard deviations from a linear mixed-effect model explaining growth
774 speed using the nuclear status (*MAT1-1* homokaryon, *MAT1-2* homokaryon or heterokaryon)
775 as a fixed effect and strain ID as a random effect. P-values correspond to t-tests with
776 Satterthwaite correction (ns: non significant; * = $p < 0.05$).



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779 **Figure 4: Mycelium growth speed of heterokaryons versus homokaryons in *Podospora***

780 *anserina*, under two different temperatures. Mycelium growth speed ($\text{mm}\cdot\text{day}^{-1}$) of

781 heterokaryons (in purple), *MAT1-1* homokaryons (in red) and *MAT1-2* homokaryons (in

782 blue), between day 2 and day 6 estimated from three replicates using 17 strains grown at **A)**

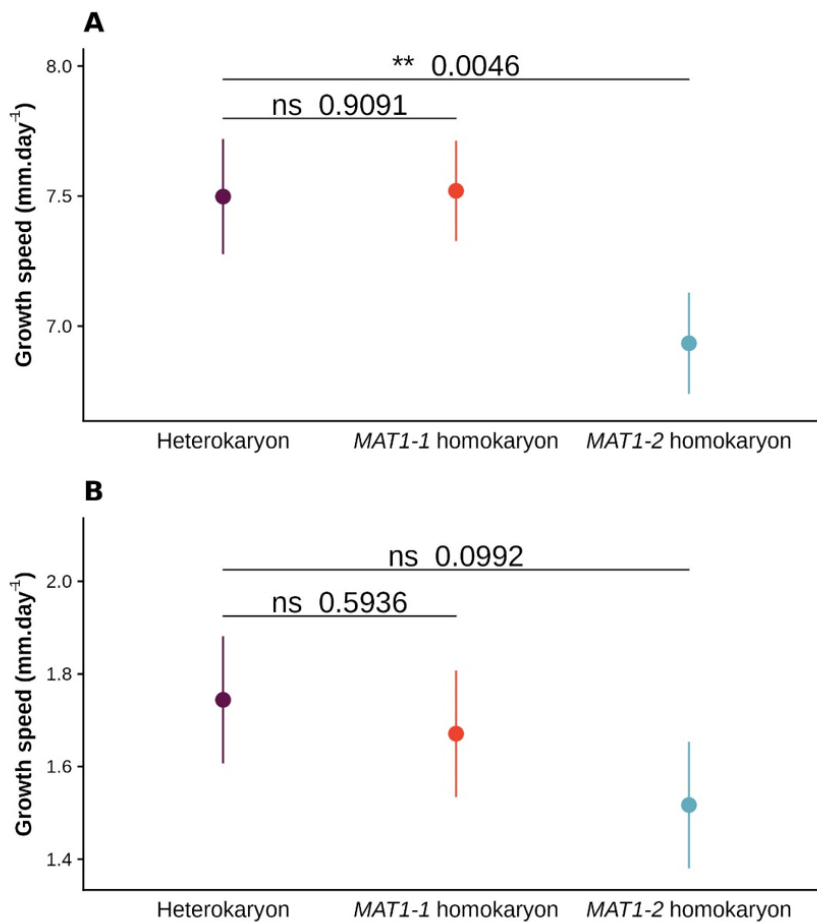
783 18°C or **B)** 37°C , both under light conditions. Dots and bars represent estimated means and

784 standard deviations using a linear mixed-effect model explaining growth speed using the

785 nuclear status (*MAT1-1* homokaryon, *MAT1-2* homokaryon or heterokaryon) as a fixed effect

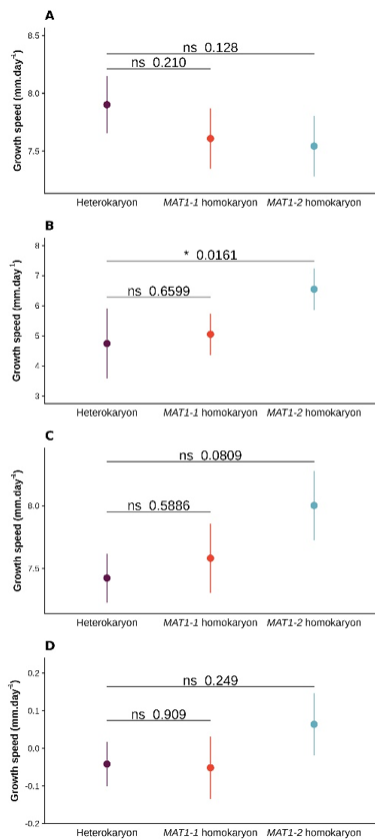
786 and strain ID as a random effect. P-values correspond to t-tests with Satterthwaite correction

787 (ns: non significant; ** = $p < 0.01$).



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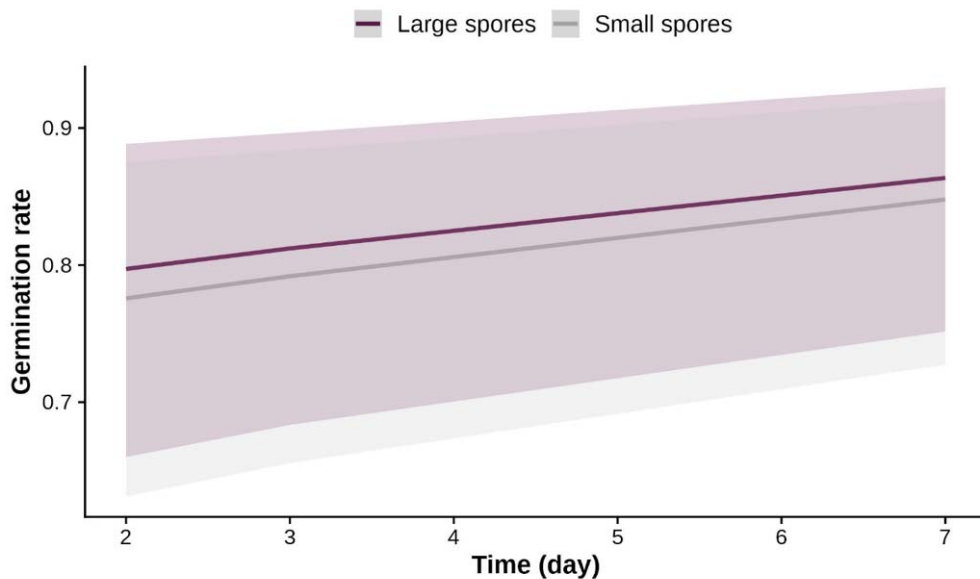
789 **Figure 5: Mycelium growth speed of heterokaryons versus homokaryons in *Podospora***
790 ***pauciseta* and *Podospora comata*, under two different temperatures.** Mycelium growth
791 speed (mm.day⁻¹) of heterokaryons (in purple), *MAT1-1* homokaryons (in red) and *MAT1-2*
792 homokaryons (in blue), between day 2 and day 6 estimated from 3 replicates using three
793 strains for *P. pauciseta* (A and B) and a single strain for *P. comata* (C and D) grown at 18°C
794 (A and C) or 37°C (B and D) under light conditions. Dots and bars represent estimated means
795 and standard deviations from a linear mixed-effect model for *P. pauciseta* or a linear model
796 for *P. comata* explaining the growth speed using the nuclear status (*MAT1-1* homokaryon,
797 *MAT1-2* homokaryon or heterokaryon) as a fixed effect and strain ID as a random effect for
798 *P. pauciseta*. P-values correspond to t-tests (with Satterthwaite correction for the mixed-
799 effect models) (ns: not significant; * = p<0.05).



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802 **Figure 6: Spore germination dynamics in *Schizothecium tetrasporum*, depending on**
803 **their heterokaryotic versus homokaryotic nuclear status.** Germination rate dynamics for
804 small spores (in purple) and large spores (in grey) estimated from eight strains from
805 germination rates on day 2, day 3 and day 7 from two batches of 10 spores per strain and per
806 spore size class. The fit of the germination dynamics was done by maximum likelihood with
807 Laplace approximation using a generalised linear mixed-effect model with a Binomial
808 distribution modelling germination rate as a linear combination of day (as a continuous
809 predictor) and spore size class (large or small) treated as fixed effects and strain ID and batch
810 as random effects. P-values correspond to χ^2 -tests (spore size effect: $p=0.4459$; day effect: $p=$
811 0.0178).



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818 **Supplementary Figures**

819 **Supplementary Figure S1: Mycelium growth speed of heterokaryons versus**

820 **homokaryons for 13 strains of *Schizothecium tritetrasporum*.** Mycelium growth speed

821 (mm.day⁻¹) of heterokaryons (in purple), *MAT1-1* homokaryons (in red) and *MAT1-2*

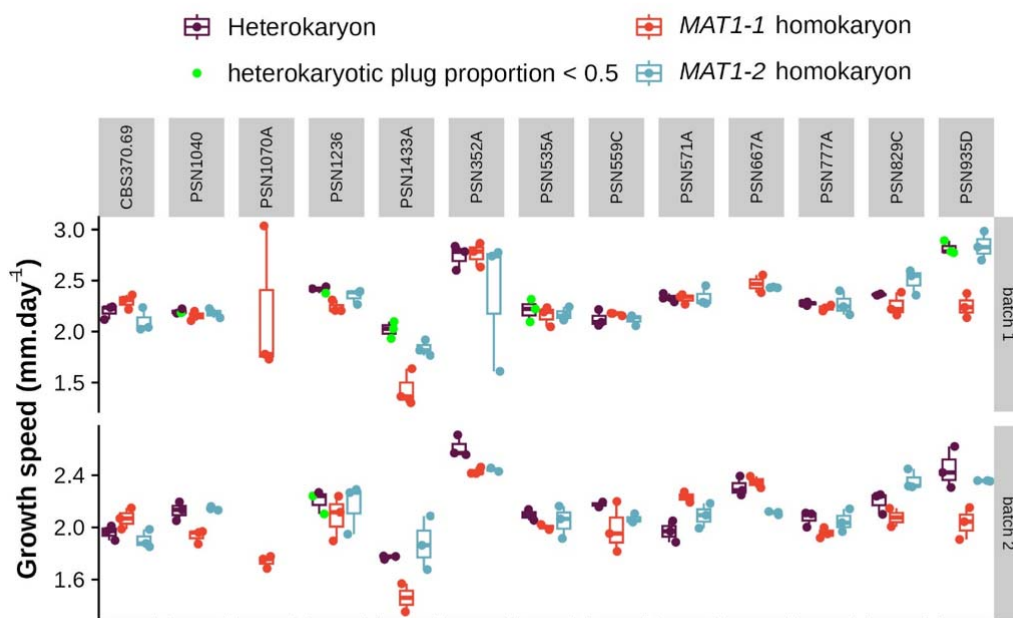
822 homokaryons (in blue), of the three replicates for each of two batches, for the 13 strains of *S.*

823 *tritetrasporum*, between day 6 and day 14 grown under light conditions at 22°C. Green dots

824 highlight the heterokaryon replicates for which less than 50% of the nine mycelium plugs

825 from the Petri dish were genotyped heterokaryotic. Boxplot elements: central line: median,

826 box limits: 25th and 75th percentiles, whiskers: 1.5× interquartile range.



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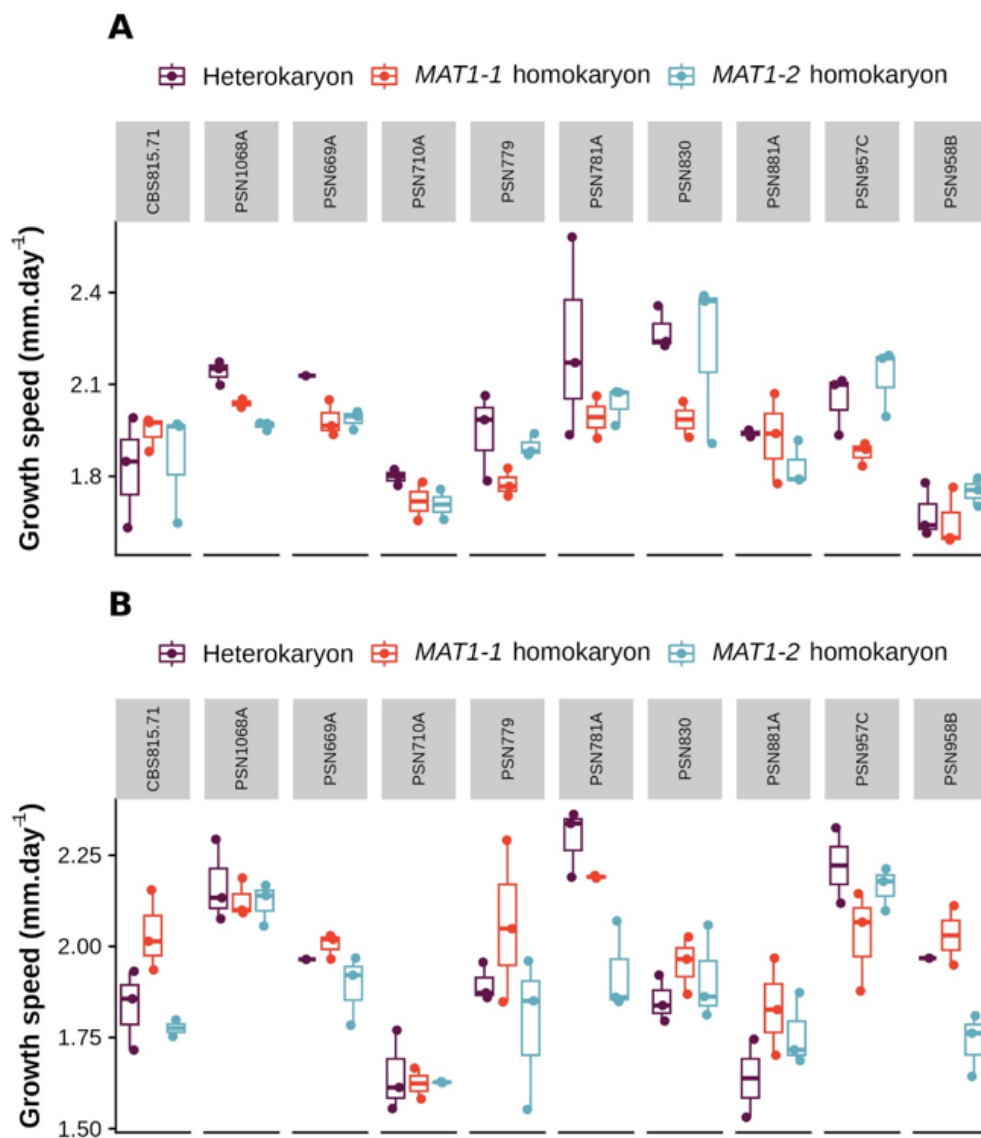
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833 **Supplementary Figure S2: Mycelium growth speed of heterokaryons versus**
834 **homokaryons for 10 strains of *Schizothecium tetrasporum*.** Mycelium growth speed
835 (mm.day⁻¹) of heterokaryons (in purple), *MAT1-1* homokaryons (in red) and *MAT1-2*
836 homokaryons (in blue), of the three replicates of the 10 strains of *S. tetrasporum* between day
837 6 and day 14 grown under **A) light** or **B) dark** conditions at 22°C. Boxplot elements: central
838 line: median, box limits: 25th and 75th percentiles, whiskers: 1.5× interquartile range.
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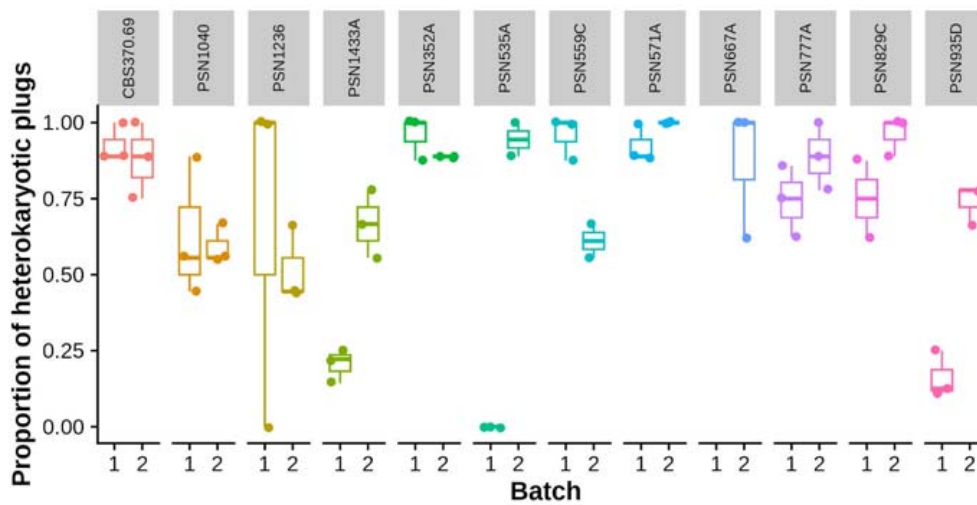
843 **Supplementary Figure S3: Estimates of nuclear loss in *Schizothecium tritetrasporum***

844 **mycelium.** Proportion of heterokaryotic plugs (i.e. with both *MATI-1* and *MATI-2* alleles

845 detected by PCR) in *S. tritetrasporum* mycelium after 14 days of growth. Proportions were

846 calculated out of nine plugs per plate in three replicates per strain in two batches that were

847 genotyped by PCR.



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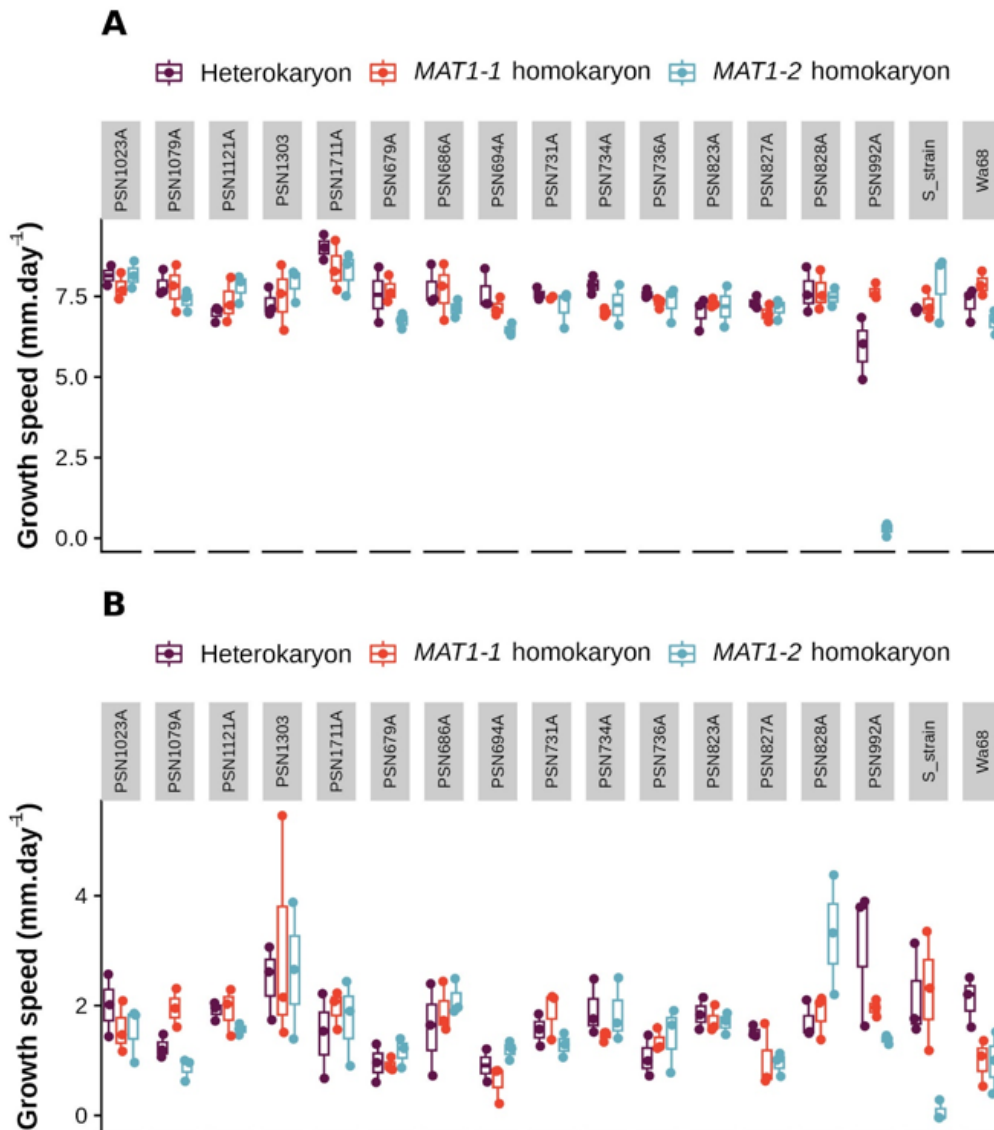
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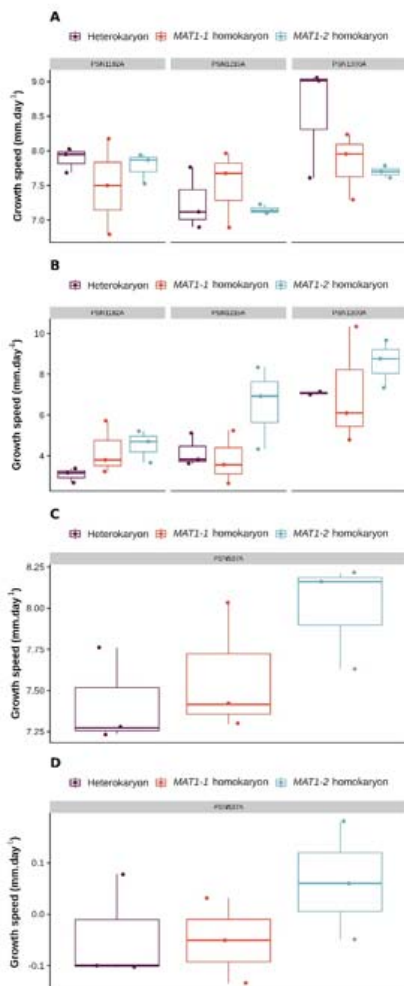
857 **Supplementary Figure S4: Mycelium growth speed of heterokaryons versus**
858 **homokaryons for 17 strains of *Podospora anserina*.** Mycelium growth speed ($\text{mm}\cdot\text{day}^{-1}$)
859 of heterokaryons (in purple), *MAT1-1* homokaryons (in red) and *MAT1-2* homokaryons (in
860 blue), of the three replicates of the 17 strains of *P. anserina* between day 2 and day 6 grown
861 at **A) 18°C** or **B) 37°C** under light conditions. Boxplot elements: central line: median, box
862 limits: 25th and 75th percentiles, whiskers: $1.5\times$ interquartile range.



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865 **Supplementary Figure S5: Mycelium growth speed of heterokaryons versus**
866 **homokaryons for one strain of *Podospora comata* and three strains of *Podospora***
867 ***pauciseta*. Mycelium growth speed ($\text{mm}\cdot\text{day}^{-1}$) of heterokaryons (in purple), *MAT1-1***
868 **homokaryons (in red) and *MAT1-2* homokaryons (in blue), of the three replicates of the three**
869 **strains for *P. pauciseta* (A and B) and the single strain for *P. comata* (C and D) between day**
870 **2 and day 6 grown at 18°C (A and C) or 37°C (B and D) under light conditions. Boxplot**
871 **elements: central line: median, box limits: 25th and 75th percentiles, whiskers: 1.5×**
872 **interquartile range.**



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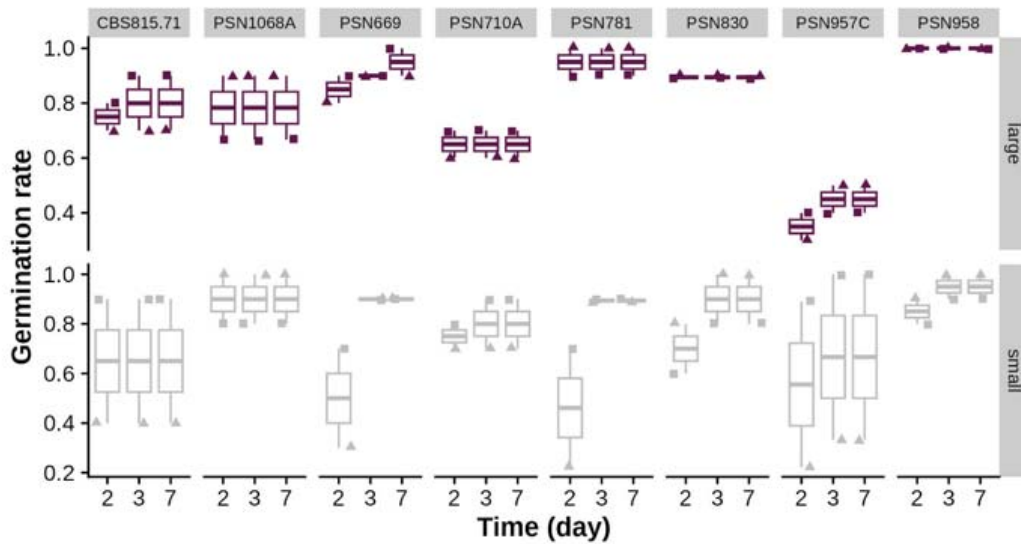
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875 **Supplementary Figure S6: Spore germination rate for eight strains of *Schizothecium***
876 ***tritetrasporum*, depending on their heterokaryotic versus homokaryotic nuclear status.**

877 Germination rate on days 2, 3 and 7 for small spores and large spores of the 10 spores of the
878 two batches of the eight strains of *S. tetrasporum*. Symbol shapes correspond to batches.

879 Boxplot elements: central line: median, box limits: 25th and 75th percentiles, whiskers: 1.5×
880 interquartile range.

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