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Fanny E. Hartmann, Alodie Snirc, Amandine Cornille, Cécile Godé, Pascal Touzet, Fabienne van Rossum, Elisabeth Fournier, Stéphanie Le Prieur, Jacqui A. Shykoff, Tatiana Giraud

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1 **Congruent population genetic structures and divergence histories in anther-**  
2 **smut fungi and their host plants *Silene italica* and the *S. nutans* species**  
3 **complex**

4

5 **Running title:**

6 Co-structure of anther-smuts and their hosts

7

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23

24 **Abstract**

25 The study of population genetic structure congruence between hosts and pathogens gives  
26 important insights into their shared phylogeographic and coevolutionary histories. We studied the  
27 population genetic structure of castrating anther-smut fungi (*Microbotryum* genus) and of their  
28 host plants, the *Silene nutans* species complex, and the morphologically and genetically close *S.*  
29 *italica*, which can be found in sympatry. Phylogeographic population genetic structure related to  
30 persistence in separate glacial refugia has been recently revealed in the *S. nutans* plant species  
31 complex across Western Europe, identifying several distinct lineages. We genotyped 171  
32 associated plant-pathogen pairs of anther-smut fungi and their host plant individuals using  
33 microsatellite markers and plant chloroplastic SNPs. We found clear differentiation between  
34 fungal populations parasitizing *S. nutans* and *S. italica* plants. The population genetic structure of  
35 fungal strains parasitizing the *S. nutans* plant species complex mirrored the host plant genetic  
36 structure, suggesting that the pathogen was isolated in glacial refugia together with its host and/or  
37 that it has specialized on the plant genetic lineages. Using random forest approximate Bayesian  
38 computation (ABC-RF), we found that the divergence history of the fungal lineages on *S. nutans*  
39 was congruent with the one previously inferred for the host plant and likely occurred with ancient  
40 but no recent gene flow. Genome sequences confirmed the genetic structure and the absence of  
41 recent gene flow between fungal genetic lineages. Our analyses of host-pathogen individual pairs  
42 contribute to a better understanding of co-evolutionary histories between hosts and pathogens in  
43 natural ecosystems, in which such studies are still scarce.

44  
45 **Key words:** population genetic structure, host-pathogen interaction, cryptic speciation, genetic  
46 divergence, approximate Bayesian computation, coevolution

## 47 **Introduction**

48 Host-pathogen interactions are pervasive in natural ecosystems, with many important ecological  
49 and evolutionary consequences (Poulin, 2005; Thompson, 2005). Pairs of tightly interacting hosts  
50 and pathogens may share common evolutionary histories due to co-evolution and/or shared  
51 geographic and climatic constraints, or may follow distinct evolutionary trajectories due to  
52 differences in reproductive systems, dispersal ranges, population sizes or contingent histories  
53 (Tellier, de Vienne, Giraud, Hood, & Refrégier, 2010; Thompson, 2005). The comparison of  
54 genetic divergence histories between host and pathogen populations gives important insights into  
55 their shared phylogeographic history and possible local adaptation or host specialization (Croll &  
56 Laine, 2016; Feurtey et al., 2016). Studies of phylogeny congruence of hosts and pathogens can  
57 for example allow identifying host shifts or co-speciation events (de Vienne et al., 2013; Hafner  
58 & Page, 1995; Wilson, Falush, & McVean, 2005). Several cases of host shift or co-speciation  
59 events between hosts and pathogens have led to damaging diseases in plants, animals and humans  
60 (Fisher, Gow, & Gurr, 2016; McDonald & Stukenbrock, 2016; Wolfe, Dunavan, & Diamond,  
61 2007). The study of population genetic structure congruence within species or species complexes  
62 can help identifying and understanding patterns of local adaptation (Gandon, Capowiez, Dubois,  
63 Michalakis, & Olivieri, 1996), which play an important role in the dynamics of pathogen and host  
64 communities (Gandon & Michalakis, 2002; Laine, 2005; Laine, 2008). Studies investigating the  
65 congruence of population genetic structure and divergence histories between hosts and pathogens  
66 are yet surprisingly still scarce despite their importance for understanding the evolutionary  
67 mechanisms and histories leading to host specialization and local adaptation (Barrett, Thrall,  
68 Burdon, & Linde, 2008; Croll & Laine, 2016), but see (Dybdahl & Lively, 1996; Feurtey et al.,  
69 2016; McCoy, Boulinier, & Tirard, 2005; Michalakis, Sheppard, Noel, & Olivieri, 1993; Tsai &

70 Manos, 2010).

71 Anther-smut fungi (*Microbotryum* genus) are generally highly specialized on their host plant  
72 species of the Caryophyllaceae family (Hartmann et al., 2019; Kemler, Göker, Oberwinkler, &  
73 Begerow, 2006; Le Gac, Hood, Fournier, & Giraud, 2007; Refrégier et al., 2008) and patterns of  
74 local adaptation have been reported in the system (Feurtey et al., 2016; Kaltz, Gandon,  
75 Michalakis, & Shykoff, 1999). Therefore, they constitute a highly suitable system to study the  
76 congruence of population genetic structure and divergence history between hosts and pathogens  
77 at different evolutionary scales. Comparisons of phylogenies at the genus level suggested a  
78 prevalence of host shifts at large evolutionary scales (Refrégier et al., 2008). The plant *Silene*  
79 *latifolia* and its anther-smut fungus *M. lychnidis-dioicae* display strong congruence of population  
80 genetic structures and plant local adaptation at regional and continental scales, suggesting the  
81 existence of co-evolution in the system (Delmotte, Bucheli, & Shykoff, 1999; Feurtey et al.,  
82 2016; Kaltz et al., 1999). Population genetic structures of both the host and the fungal pathogen  
83 likely resulted from past climatic events, showing hallmarks of recolonization from former  
84 glacial refugia in Europe (Badouin et al., 2017; Gladieux, Devier, Aguileta, Cruaud, & Giraud,  
85 2013; Gladieux et al., 2011; Taylor & Keller, 2007; Vercken et al., 2010). The congruence of  
86 host and pathogen population genetic structures has not been investigated in other anther-smut  
87 fungi-*Silene* pairs despite their importance as models of pathosystems in natural ecosystems  
88 (Bernasconi et al., 2009; Toh & Perlin, 2016) and the importance of assessing whether  
89 congruence in population genetic subdivision is a general pattern (Croll & Laine, 2016).  
90 Furthermore, the evolutionary history of population genetic divergence and of gene flow levels  
91 occurring between pathogens infecting closely related hosts with overlapping distribution ranges  
92 still remain poorly studied at the genome-wide-scale (but see Badouin et al., 2017).

93 *Silene nutans* is an assemblage of strongly differentiated cryptic genetic plant lineages,  
94 corresponding to at least seven “evolutionary significant units”, which can be grouped into two  
95 main phylogeographic (eastern and western) genetic clusters in Europe (Van Rossum et al.,  
96 2018). The *S. nutans* genetic lineages display several morphological and ecological differences,  
97 as well as nuclear and plastid genetic differentiation (Martin et al., 2016; Van Rossum et al.,  
98 2018), and strong postzygotic reproductive isolation has been shown between the eastern and  
99 western clusters in Western Europe (Martin et al., 2017). Geographic distribution patterns suggest  
100 that *S. nutans* lineages have diverged in allopatry during the Quaternary climate oscillations, and  
101 then recolonized northwards, without admixture (Martin et al., 2016; Martin et al., 2017; Van  
102 Rossum et al., 2018). The anther-smut fungal species *Microbotryum violaceum sensu stricto* is  
103 specialized on *S. nutans* (Kemler et al., 2006; Lutz et al., 2005). *Microbotryum* fungi are  
104 pollinator-borne pathogens castrating plants by replacing the pollen by their own spores and  
105 aborting ovaries, and they are usually highly specific on their *Silene* host plant species (Le Gac,  
106 Hood, & Giraud, 2007; Refrégier et al., 2008). The existence of cryptic lineages within *S. nutans*  
107 therefore raises the question of whether cryptic lineages also exist in the fungus and whether their  
108 genetic divergence history mirrors that of the host. In Southern Europe, *S. nutans* can be found in  
109 sympatry with *S. italica*, a closely related species, in particular in the Cévennes (Lafuma &  
110 Maurice, 2006). *Silene nutans* and *S. italica* can be difficult to distinguish, being very similar  
111 morphologically, mainly differing in petal shape, in the length of the gynophore and with rather  
112 nodding or erect flowers, respectively (Rameau, Mansion, & Dumé, 1989, 2008; Tison & de  
113 Foucault, 2014). Their flowering time, pollinator guilds and ecological niches overlap, although  
114 they slightly differ in their ecological requirements, with *S. italica* being strictly calcicolous,  
115 more xero-thermophilous and heliophilous than *S. nutans* (Rameau et al., 1989, 2008; Tutin et al.,  
116 2001). *Microbotryum* fungi have been found on the two plant species and appeared differentiated

117 based on a small sample and a few genetic markers (Bucheli, Gautschi, & Shykoff, 2000);  
118 however, spillover, i.e., non-sustainable cross-species disease transmission, has been shown to  
119 occur in *Microbotryum* fungi (Antonovics, Hood, & Partain, 2002; Gladioux et al., 2011). The  
120 study of the population genetic structure of *M. violaceum* s. s. on *S. nutans* therefore requires  
121 molecular typing to check plant and fungal species identities.

122 In this study, we analyzed associated plant-pathogen samples of *S. nutans* and *S. italica*  
123 populations from Europe in order to address the following questions: 1) Is there genetic  
124 differentiation between anther-smut fungi parasitizing the closely related plant species *S. nutans*  
125 and *S. italica*? Are there hybrids and/or spill-overs in anther-smut fungi? 2) Is the population  
126 genetic structure of the anther-smut fungi parasitizing *S. nutans* and *S. italica* congruent with  
127 those of their hosts? 3) What is the divergence history of these anther-smut fungi on their hosts?  
128 Did genetic divergence occur with gene flow? Does the divergence history of the fungi mirror  
129 that of their hosts? In order to address these questions, we used genetic markers (nuclear  
130 microsatellite markers and/or chloroplastic SNPs) in both plants and anther-smut fungi to analyse  
131 diseased material (171 plant-pathogen pairs) collected across Western Europe and infer  
132 population genetic structures. We used approximate Bayesian computation (ABC) to compare  
133 genetic divergence scenarios in anther-smut fungi in order to assess whether the genetic  
134 divergence history in anther-smut fungi mirrored that inferred previously for their host plants. We  
135 also sequenced 53 genomes of *Microbotryum* fungi and analysed them together with 46 available  
136 *Microbotryum* genomes to assess whether the inferences on population genetic structure and gene  
137 flow based on microsatellite markers hold at the genome-wide levels and whether there were  
138 spill-overs or gene flow from other sympatric *Microbotryum* species than those analysed with  
139 microsatellite markers.

140

## 141 **Materials and Methods**

### 142 **Fungal and plant materials**

143 We analyzed 171 anther-smut fungi collected from diseased plant individuals of *S. nutans* and *S.*  
144 *italica* that were sampled in 55 distinct geographic sites across Europe, including the United  
145 Kingdom, France, Belgium, Switzerland, Italy, Germany and Norway (Table S1, Fig. 1A).  
146 Diseased plant individuals were stored in individual paper envelopes kept in plastic bags filled  
147 with silica gel, in dark conditions at 8°C. Small pieces of leaves were used as raw material for  
148 DNA extraction of host plant individuals. Diploid spores of *Microbotryum* anther-smut fungi  
149 were collected in buds of infected flowers and grown as yeasts on potato dextrose agar (PDA)  
150 medium, and then stored at -20°C. Spores did not grow for a few samples older than two years.  
151 For DNA extraction of these strains, we used the dried spores directly collected from anthers of  
152 diseased flowers. In most cases, spores from anthers of a single flower were used and therefore  
153 corresponded to a single *Microbotryum* genotype (López-Villavicencio et al., 2007). When spore  
154 material was limited, we used multiple anthers from several flowers of the same plant. We never  
155 observed more than two alleles per strain and found low levels of heterozygosity per strain,  
156 indicating that we unlikely genotyped more than one fungal genotype.

157

### 158 **Genotyping and identification of species and genetic lineages of the *Silene* plants based on** 159 **nuclear microsatellite markers and chloroplastic SNPs**

160 We extracted plant DNA of at least one diseased plant individual per site using the NucleoSpin®  
161 96 Plant II kit (Macherey-Nagel, Germany). We obtained DNA for 134 out of the 171 diseased



162 plant individuals. At least one host individual was genotyped per site, except for six sites due to  
163 the lack of plant material (Table S1). In addition, we genotyped three plant individuals for which  
164 no anther-smut fungus was isolated but strains were isolated at the same sites (sample # 874\_4,  
165 874\_6, 512\_3). Using morphological criteria of the host plant species, sample collectors initially  
166 identified 153 *S. nutans* and 18 *S. italica* diseased individuals. However, as the morphologies of  
167 *S. nutans* and *S. italica* are very similar, misidentification could occur. Therefore, we sequenced  
168 four chloroplast fragments (*psbA*, *LF*, *MATK*, *GS*; Lahiani et al., 2013) to check species identity  
169 of the diseased plants collected in southeastern France and in Italy, where both *S. nutans* and *S.*  
170 *italica* can be found. We thus reassigned one individual to *S. nutans* and seven individuals to *S.*  
171 *italica*, while one host individual could not be assigned to either *S. nutans* or *S. italica* species  
172 (from the pair # 1436). We excluded this host-pathogen individual pair from our analyses. We  
173 thus had 145 diseased *S. nutans* plants and 25 diseased *S. italica* plant (Table S1).

174  
175 To further genotype the plant individuals for studying the population genetic structure, we used a  
176 combination of plastid (chloroplast) SNPs and nuclear microsatellite markers as previously  
177 described (Godé et al., 2014; Martin et al., 2016; Martin et al., 2017). For plastid markers, using  
178 the KASPAR® protocol we genotyped six SNPs, named Cp42, Cp397, Cp540, Cp656, Cp730  
179 and Cp804, and polymorphic for [T/G], [A/C], [C/T], [G/T], [C/T], and [T/G], respectively.  
180 Individual haplotypes were defined as combinations of allelic states for all six SNPs. For nuclear  
181 markers, we used six multiplexes genotyping 24 microsatellite markers (Table S2). We followed  
182 the previously published protocols (Godé et al., 2014; Martin et al., 2016; Martin et al., 2017),  
183 except that we used different dye colors, bought from Eurofins Genomics. We used the Multiplex  
184 PCR Kit (Qiagen) following manufacturer instructions for PCR reactions, performed separately  
185 for each multiplex in 15 µL volume containing 3 µL of DNA, 3.4 µL of H<sub>2</sub>O, 7.1 µL of multiplex

186 PCR Kit (Buffer 2X, Qiagen, USA), and 1.4  $\mu$ L of the primer mix. The primer mix included 2  
187  $\mu$ M of unlabelled forward and reverse primers and 0.5  $\mu$ M or 0.75  $\mu$ M of the labelled forward  
188 primer depending on the dye label. We used the same PCR cycling program as described in  
189 (Godé et al., 2014) with some modifications of the final elongation for multiplex 1 (Table S3A-  
190 C). We checked successful PCR amplifications on 2% (w/v) agarose gel electrophoresis. We  
191 outsourced genotyping at the Gentyane Genotyping Platform (INRA, Clermont, France) and  
192 scored alleles with GENEMAPPER v.4.0 (Applied Biosystems). We excluded three markers  
193 (SIL18, SIL26, SIL42) for which we had less than 50% of the individuals successfully  
194 genotyped. Given the material available, we could genotype 136 host plant individuals using both  
195 plastid SNPs and nuclear microsatellite markers. All 136 genotyped host individuals had a  
196 determined plastid haplotype and a determined genotype for at least 50% of the 21 remaining  
197 nuclear microsatellite markers.

198

199 **Genotyping and species identification of the *Microbotryum* fungal strains: microsatellite**  
200 **markers and ITS sequences**

201 We extracted fungal DNA using the Chelex protocol (Biorad, USA) following (Giraud, 2004).  
202 We extracted DNA from the 170 strains of anther-smut fungi, either from mixes, stored at -20°C,  
203 of haploid sporidia resulting from clonal growth after meiosis or from diploid teliospores directly  
204 collected in anthers of diseased flowers and stored at 10°C. Diploid genomes were therefore  
205 genotyped. DNA was diluted half-fold for PCR amplification. We used the internal transcribed  
206 spacer (ITS) to check that the genus of the fungal strains belonged to the *Microbotryum* genus.  
207 To genotype the fungal strains, we used 22 microsatellite markers arranged into multiplex (Table  
208 S4 ; (Fortuna et al., 2016; Giraud et al., 2008)). We used the Multiplex PCR Kit (Qiagen)  
209 following manufacturer instructions for PCR reactions which were performed separately for each

210 multiplex as described above. We used the PCR cycling programs as in previous studies (Fortuna  
211 et al., 2016; Giraud et al., 2008; Table S3C-D). We checked the success of PCR amplifications on  
212 2 % agarose gel electrophoresis. For genotyping, we pooled multiplexes 7 and 8 as a single  
213 multiplex. We outsourced genotyping at the Gentyane Genotyping Platform (INRA, Clermont,  
214 France) and scored alleles with GENEMAPPER v.4.0 (Applied Biosystems). We could  
215 determine the genotypes for all strains for at least 50% of the 22 markers. We identified 21 fungal  
216 strains that were likely siblings of other fungal strains based on null genetic distances and we  
217 excluded them from the STRUCTURE software analyses (Table S1; see below).

218

### 219 **Population genetic structure based on microsatellite markers**

220 To analyze and compare host and pathogen population genetic structures, we used a combination  
221 of three complementary approaches using microsatellite nuclear markers on both the *Silene* host  
222 plants and anther-smut fungal datasets. First, we used the model-based Bayesian clustering  
223 approach implemented in the software STRUCTURE version 2.3.4 (Pritchard, Stephens, &  
224 Donnelly, 2000). The program performs partitions of multilocus genotypes into genetic clusters  
225 and assigns individuals to genetic clusters, minimizing the departure from expected frequencies  
226 and linkage equilibrium among loci. We tested an admixture model with correlated frequencies  
227 and no prior information for  $K = 2$  to  $K = 10$  clusters. A total of 10 repetitions were run for each  
228  $K$  value. We used 50,000 samples as a burn-in period and 100,000 samples per run for the Monte  
229 Carlo Markov Chain (MCMC) replicates. Cluster assignment probabilities were computed using  
230 the CLUMPP program (Jakobsson & Rosenberg, 2007) implemented in the R package  
231 {Pophelper}. We used the R package {Pophelper} (<https://github.com/royfrancis/pophelper>) to  
232 build the barplots. We choose as the biologically most relevant  $K$  value the finer population  
233 structure, as the highest  $K$  value for which a new cluster could be identified with individuals

234 highly assigned to it, the new cluster at  $\{K+1\}$  having only admixed individuals (i.e. mean  
235 membership coefficient  $<0.80$  to the given cluster). For the anther-smut fungus dataset, genetic  
236 data were haploidized as individuals were highly homozygous. High homozygosity levels might  
237 bias inferences in the software STRUCTURE as these are based on Hardy-Weinberg expectations  
238 in a diploid setting (Pritchard et al., 2000). Furthermore, 21 strains were removed, appearing as  
239 siblings of other strains, with identical genotypes. *Microbotryum* fungi have one obligatory  
240 sexual event before plant infection, so that clonemates cannot be found in different plants, but  
241 high selfing rates may allow the same genotype to be found in neighbor plants if they are  
242 parasitized by the same diploid spores or offspring (Giraud, 2004). For the host plant dataset,  
243 genetic data were kept as diploid and five individuals were removed as they had identical  
244 genotypes as other neighbor plants and may be clonemates. We identified clonemates and  
245 siblings in both datasets using the `dist(X)` function in the R package `{Ape}` (Paradis, Claude, &  
246 Strimmer, 2004) and considered two individuals to be siblings if their distance was equal to 0.  
247 We then used two methods for assessing population genetic structure that do not assume  
248 outcrossing or a lack of linkage disequilibrium. We performed a discriminant analysis of  
249 principal components (DAPC) using the R package `{ADEGENET}` (Jombart, 2008; Jombart &  
250 Ahmed, 2011) and used a principal component analysis (PCA) with the `dudi.pca` function using  
251 the R package `{ade4}` (Dray & Dufour, 2007) on the entire set of individuals in both datasets.  
252 Maps showing genotypes per locality were drawn using the R package `{maps}` (Becker, Wilks,  
253 Brownrigg, Minka, & Deckmyn, 2017) and `{mapplots}` (Gerritsen, 2013). Scatter plots were  
254 performed using the R package `{ggplot2}` (Wickham, 2009).

255

## 256 **Population statistics of genetic diversity and structure based on microsatellite markers**

257 We computed, using the R package `{diveRsity}` (Keenan, McGinnity, Cross, Crozier, & Prodöhl,

2016), the following estimates of genetic diversity per locus, site, genetic cluster identified in the STRUCTURE analysis and/or species for the host plants and for the anther-smut fungi datasets: the number of alleles, allelic richness ( $A_R$ ), observed heterozygosity ( $H_O$ ), expected heterozygosity ( $H_E$ ), the fixation indexes ( $F_{ST}$ ,  $F_{IS}$  and  $F_{IT}$ ), and the Jost's D statistics corresponding to the fraction of allelic variation found among genetic clusters. We tested whether genotype frequencies fitted the Hardy-Weinberg expectations using a standard  $\chi^2$  goodness of fit method and assessed the significance of  $F_{ST}$ ,  $F_{IS}$  and  $F_{IT}$  values using a bootstrap procedure with 1000 iterations and calculating 95% confidence intervals. Sites with less than three sampled individuals were pooled with other sites of the same genetic lineage when they were closer than 1/10th of latitude or longitude or were otherwise excluded from the analyses. We thus excluded nine sites for the plants (site # 1014, 303, 1532, 719, 1546, 1547, 1437, 429, 1548, 1438) and 13 sites for the fungal pathogens (site # 1068, 1014, 303, 1532, 719, 1546, 940, 1547, 1437, 333, 1249, 6809, 1548) as they contained too few samples for computing relevant population genetics statistics and were too far from other populations to be pooled with them. In total, we considered 20 groups of sites for the host and 19 groups of sites for the pathogen. We included sibling individuals but excluded individuals with admixed membership between genetic clusters inferred from the STRUCTURE analysis (i.e. individuals with mean membership coefficient  $<0.80$  to the given cluster), which may be due to low assignment power or admixture. To take into account differences of sample size between sites, we also estimated allelic richness using ADZE (Szpiech, Jakobsson, & Rosenberg, 2008) which corrects for sample size difference. Calculations were performed using a standardized sample size of  $N=3$ , corresponding to the smallest number of observations per site. We tested for mean differences in diversity statistics between species and genetic clusters using a Wilcoxon rank sum test and a Kruskal-Wallis rank sum test in the R software v3.5.3, respectively, considering values across groups of sites. To study isolation-by-

282 distance patterns in the *S. nutans* and fungi parasitizing *S. nutans* datasets, we computed  
283 correlations between matrices of genetic and geographic distances of plant and fungal  
284 populations using a Mantel test. Genetic distances between populations at each group of sites  
285 were calculated as the Nei's distance (Nei, 1972) using the `dist.genpop()` function of the R  
286 package {ADEGENET} (Jombart, 2008; Jombart & Ahmed, 2011). Geographic distances were  
287 calculated using the `dism()` function of the R package {geosphere}. We computed Mantel tests  
288 using the `mantel.rtest()` function of the R package {ade4} with 1,000,000 resamples for the null  
289 distribution. We also studied the correlations between matrices of genetic distances of plant and  
290 fungal populations using a Mantel test as above. To remove the effect of the correlation between  
291 genetic and geographic distances in the correlation between matrices of genetic distances, we also  
292 performed a partial Mantel test using the `partial.mantel.test()` function of the R package {ncf}  
293 with 1,000,000 resamples for the null distribution (Feurtey et al., 2016). We considered 16 group  
294 of sites, the same both for the host and the pathogen, corresponding to *S. nutans* host plants and  
295 fungal strains belonging to the genetic cluster parasitizing *S. nutans*. We excluded sites of *S.*  
296 *italica* host plants and fungal strains belonging to the genetic cluster parasitizing *S. italica*, and  
297 one site studied in the host but not studied in the pathogen (site # 333). We investigated the  
298 occurrence of recent events of effective population size reduction (i.e. bottlenecks) within the  
299 identified fungal genetic clusters using the program BOTTLENECK version 1.2.02 (Piry,  
300 Luikart, & Cornuet, 1999).

301

### 302 **Inference of anther-smut fungi divergence history based on microsatellite markers**

303 We studied the divergence history of the anther-smut fungi sampled on the *S. nutans* lineage  
304 complex using an approximate Bayesian computation-random forest (ABC-RF) procedure that

305 performs ABC inferences based on the machine learning tool named “random forest” (Breiman,  
306 2001; Pudlo et al., 2016; Raynal et al., 2019). The divergence history of the host had already been  
307 previously inferred (Martin et al., 2016; Martin et al., 2017; Van Rossum et al., 2018). To  
308 increase our power to disentangle between different evolutionary scenarios, we performed model  
309 choice and parameter estimation by comparing scenarios or groups of scenarios in sequential  
310 rounds, each round testing a particular type of evolutionary event, either divergence time, order  
311 of divergence or presence of gene flow (Estoup, Raynal, Verdu, & Marin, 2018; Liu et al., 2019);  
312 Table 1; Table S5). We built scenarios based on the genetic clusters obtained using microsatellite  
313 markers and on previous analyses of divergence of anther-smut fungi and their host species  
314 (Badouin et al., 2017; Gladieux et al., 2013; Martin et al., 2016; Martin et al., 2017; Van Rossum  
315 et al., 2018). The tested scenarios varied regarding the time of divergence, the relative order of  
316 divergence of fungal genetic clusters and the occurrence of gene flow among genetic fungal  
317 clusters (Table S5). We did not include scenarios with variation in effective population sizes as  
318 the BOTTLENECK analysis did not identify any signature of effective population size reduction  
319 (see ‘Results’ section). To test if the order of sequential rounds had an effect on the outcome of  
320 the analysis, we tested the time of divergence and the relative order of divergence as either the  
321 first or the second round (Note S1). We used as populations the fungal genetic clusters identified  
322 through population structure analyses and removed fungal strains that were likely siblings of  
323 other fungal strains in each genetic cluster.

324 We ran the ABC procedure modified from (Liu et al., 2019). Briefly, we simulated datasets with  
325 22 fungal microsatellite markers using the ABCtoolbox program (Wegmann, Leuenberger,  
326 Neuenschwander, & Excoffier, 2010) and used fastsimcoal 2.5 for coalescent-based simulations  
327 (Excoffier & Foll, 2011). We simulated 10,000 genetic datasets per scenario using coalescent

328 simulations with model parameters drawn from prior distributions (Table S6A). We set prior  
329 distributions based on previous analyses of divergence of anther-smut fungi and of their host  
330 species (Badouin et al., 2017; Branco et al., 2018; Gladieux et al., 2013; Martin et al., 2016;  
331 Martin et al., 2017; Van Rossum et al., 2018). We set a generation time of one year for the  
332 pathogen based on its life cycle (Thrall, Biere, & Antonovics, 1993). We estimated the following  
333 parameters: effective size of each genetic cluster ( $N$ ), divergence time and migration rate per  
334 generation between two genetic clusters  $x$  and  $y$  ( $T_{xy}$  and  $m_{xy}$ , respectively). We computed 16  
335 summary statistics for the observed and simulated datasets with the program arlsumstats (Table  
336 S6B; (Excoffier & Lischer, 2010). We assumed a generalized stepwise model of microsatellite  
337 evolution (Estoup, Jarne, & Cornuet, 2002) and allowed the mutation rate to vary across  
338 microsatellite markers. We drew locus-specific mutation rates from a gamma distribution ( $\alpha, \alpha/\mu$ )  
339 in which  $\mu$  is the mutation rate per generation and  $\alpha$  is a shape parameter (Cornille et al., 2012;  
340 Liu et al., 2019). We used the R package {abcrf} v1.7.0 (Pudlo et al., 2016) to compute a  
341 classification vote through ABC-RF, representing the number of times a scenario was selected  
342 among classification  $n$  trees of the constructed random forest. We chose  $n=500$  trees. For each  
343 round, we selected the scenario or group of scenarios with the highest number of classification  
344 votes. We computed the posterior probability and the prior error rates over 10 replicated analyses.  
345 We performed a linear discriminant analysis (LDA) in the R package {abcrf} v1.7.0 (Pudlo et al.,  
346 2016) on the simulated and observed datasets to visually check the fit of the model to the  
347 observed data. Finally, we performed parameter inferences using the group of models eventually  
348 selected.

349

350 **Sequencing data and genome assemblies**



351 We performed whole-genome sequencing for 23 anther-smut fungal strains parasitizing *S. italica*  
352 and 30 strains parasitizing *S. nutans*. Out of these 53 strains, 38 strains belonged to the 170 host-  
353 pathogen pairs that were genotyped with microsatellite markers (Table S7). Due to limited fungal  
354 material, we sequenced six additional strains that were collected at our studied sites but not  
355 genotyped for microsatellite markers and nine additional strains collected at three new sites  
356 (Table S7). We extracted DNA from fresh spores stored at -20°C using the Nucleospin Soil Kit  
357 (Macherey-Nagel, Germany). Genomes were sequenced using Illumina 150 bp paired-end  
358 sequencing technology at 46X coverage on average. We also included outgroups using available  
359 whole-genome sequences of 56 anther-smut fungal strains parasitizing closely-related host  
360 species of *S. nutans* and *S. italica* occurring in the studied geographic range (Le Gac, Hood,  
361 Fournier, et al., 2007; Refrégier et al., 2008): 33 *M. lychnidis-dioicae* strains parasitizing *S.*  
362 *latifolia* (Badouin et al., 2017), 19 *M. silenes-dioicae* strains parasitizing *S. dioica* (Badouin et al.,  
363 2017) and four *M. violaceum* var *paradoxa* strains parasitizing *S. paradoxa* (Branco et al., 2018).  
364 We downloaded raw data publicly available from the NCBI Sequence Read Archive (SRA) under  
365 the BioProject IDs PRJNA295022 and PRJEB16741 (Table S7). We thus analysed a total of 109  
366 *Microbotryum* genomes. For read mapping, we used as reference genome the high-quality  
367 haploid genome assembly of the MvSn-1249 *M. violaceum* s. str. strain corresponding to the a<sub>2</sub>  
368 mating type (collected on *S. nutans*) previously obtained with P6/C4 Pacific Biosciences SMRT  
369 technology and annotated for gene models (Branco et al., 2017). The MvSn-1249-A2 assembly  
370 was accessed from GenBank BioProject accession number PRJEB12080 (BioSample ID:  
371 SAMEA3706514, assembly: GCA\_900014965).

372

### 373 **Genome read mapping and variant calling procedure**

374 We performed read mapping and SNP calling of the 109 analysed genomes against the MvSn-

375 1249-A2 genome as previously described (Branco et al., 2018; Hartmann, Rodríguez de la Vega,  
376 Brandenburg, Carpentier, & Giraud, 2018). First, we trimmed Illumina raw reads for sequence  
377 quality and removed adapter sequences using the software Cutadapt v1.8.3 (Martin, 2011) with  
378 the options: `-q 10, 10; --minimum-length 50; -a`  
379 `AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC; -`  
380 `AAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATT.`  
381 We aligned trimmed reads using the short read aligner bowtie2 v2.1.0 (Langmead, Trapnell, Pop,  
382 & Salzberg, 2009) with the three following software options: `--very-sensitive-local; --phred33; -X`  
383 `1000`. We removed PCR duplicates using the MarkDuplicates tool of Picard tools version 2.14.1  
384 (<http://broadinstitute.github.io/picard>) and performed local realignment of mapped reads using  
385 the tools RealignerTargetCreator and IndelRealigner of the Genome Analysis Toolkit (GATK)  
386 version 3.8 (McKenna et al., 2010) to improve alignment accuracy in indel regions. Mean  
387 alignment rates to the reference genome ranged from 53 to 96% (Table S7). For SNP calling, we  
388 used GATK version 3.7 (McKenna et al., 2010) and ran HaplotypeCaller on each strain  
389 individually using a diploid mode. Then, we used GenotypeGVCFs on a merged gvcf variant file  
390 to perform joint variant calls. We filtered SNP calls for quality using VariantFiltration and  
391 following the GATK good practices for hard-filtering of variants ( $QUAL < 250$ ;  $QD < 2$ ;  $MQ <$   
392  $30.0$ ;  $-10.5 > MQRankSum > 10.5$ ;  $-5 > ReadPosRankSum > 5$ ;  $FS > 60$ ;  $SOR > 3$ ) and masked  
393 SNPs for repeats. We found 41 SNPs in the strain MvSn-1249-A2 (the very same fungal strain as  
394 the reference genome also sequenced with Illumina), indicating low rates of false positives in our  
395 SNP calling. We only kept bi-allelic SNPs with a high genotyping rate ( $> 90\%$ ) on autosomes  
396 (i.e., excluding SNPs on the mating-type contigs MvSn-1249-A2-R1\_MC15 and MvSn-1249-A2-  
397 R1\_MC16). We excluded SNPs on the mating-type chromosomes for population genomics  
398 analyses as they exhibit suppressed recombination on 90% of their lengths (Branco et al., 2018).

399 Removing the strain MvSn-1249-A2, we kept 108 fungal strains and 1,305,369 SNPs for  
400 population genomic analyses.

401

## 402 **Population genomics analyses**

403 To analyze the population genetic structures of anther-smut fungi parasitizing several *Silene*  
404 species using the genome-wide SNP dataset called on autosomes, we used the model-based  
405 Bayesian clustering approach implemented in the software STRUCTURE version 2.3.4  
406 (Pritchard et al., 2000), from K=2 to K=10 clusters, and performed a PCA, similarly as for the  
407 microsatellite datasets. For the STRUCTURE analysis, we selected a set of 233 unlinked SNPs  
408 randomly distributed at intervals of 100 kb along the autosomes, linkage disequilibrium decaying  
409 over 50-100 kb in *Microbotryum* populations (Badouin et al., 2017). We used the R package  
410 {Pophelper} (<https://github.com/royfrancis/pophelper>) to perform barplots. We performed the  
411 PCA on all SNPs (but excluding missing data and heterozygote genotypes) using the --pca  
412 command of the Plink v1.9 software (Chang et al., 2015; Purcell et al., 2007). To study footprints  
413 of gene flow between fungal strains, we performed a phylogenetic network analysis implemented  
414 in splitsTree (Huson, 1998; Huson & Bryant, 2006) with the neighborNet method.

415

## 416 **Results**

### 417 **Population genetic structure and diversity of *Silene nutans* and *S. italica* host plants**

418 After checking plant species identity with molecular markers, we retained 145 diseased *S. nutans*  
419 plants and 25 diseased *S. italica* plants (Table S1; Fig 1A). The 21 plant nuclear microsatellite  
420 markers distinguished well *S. nutans* and *S. italica* both in STRUCTURE analyses from K=2 and

421 in the principal component analysis (PCA; Fig 1B-C; Fig S1).

422 Within *S. nutans*, we identified four distinct haplotypes based on the six plastid SNPs, retrieving  
423 in our sample the four major genetic lineages of *S. nutans* previously identified in Western  
424 Europe (Fig 1A; (Martin et al., 2016; Martin et al., 2017; Van Rossum et al., 2018). The blue,  
425 yellow, orange, and red haplotypes were found, as in previous studies, in the northeastern,  
426 southeastern, western and southwestern parts of Europe, respectively. All *S. italica* samples had a  
427 blue plastid haplotype (Fig 1A). Within *S. nutans*, the PCA based on the 21 plant nuclear  
428 microsatellite markers mainly differentiated the orange genetic lineage from the other ones (Fig  
429 1B; Fig S1B) and the STRUCTURE analysis detected four genetic clusters, corresponding to the  
430 different plastid haplotypes, except for the red haplotype for which we had too few samples (Fig  
431 1C; Fig S1A). The STRUCTURE analysis further identified two well-separated genetic clusters  
432 within the *S. nutans* orange plastid haplotype (Fig. 1C). These two genetic clusters also appeared  
433 separated on the second axis of the PCA and corresponded to plant individuals collected in the  
434 Northern part of France *versus* Belgium and Western part of France (Fig S1), in agreement with  
435 previous findings (Martin et al., 2016). From the STRUCTURE analyses, we identified five host  
436 individuals with admixed cluster memberships (mean membership coefficient <0.80 to the given  
437 cluster), which may be due to low assignment power or admixture between genetic clusters. We  
438 found no further clear subdivision within *S. nutans* at higher K values and no clear population  
439 subdivision within *S. italica* (Fig S1A).

440 We found no significant differences in allelic richness neither between *S. nutans* and *S. italica*  
441 (Wilcoxon rank sum (WRS) test on 20 groups of sites,  $W = 12$ , p-value = 0.169; Table S8A) nor  
442 between the four *S. nutans* genetic clusters (Kruskal-Wallis rank sum (KWRS) test on 16 groups  
443 of sites,  $\text{Chi}^2 = 4.92$ , degree of freedom (df) = 3, p-value = 0.178; Table S8A). In *S. nutans*, levels

444 of observed heterozygosity were significantly lower than those of expected heterozygosity under  
445 Hardy-Weinberg equilibrium for most loci, the mean  $F_{IS}$  value per marker being 0.33 (Table S2).  
446 In *S. italica*, levels of observed and expected heterozygosities were not significantly different for  
447 most markers (78%; Table S2), the mean  $F_{IS}$  value per locus being 0.37 (Table S2). As expected,  
448 pairwise  $F_{ST}$  and Jost's D indicated higher divergence between *S. italica* and *S. nutans* (mean  
449  $F_{ST}$ =0.32; mean Jost's D=0.57; Table S9A) than between genetic clusters of *S. nutans* (mean  
450  $F_{ST}$ =0.20; mean Jost's D=0.35; Table S9A; KWRS test for both statistics,  $W = 0$ , p-value =  
451 0.010). For *S. nutans*, we found on average lower pairwise  $F_{ST}$  and Jost's D values between the  
452 yellow Western cluster and the two orange Western and Northwestern genetic clusters (mean  
453  $F_{ST}$ =0.17; mean Jost's D=0.28; Table S9A) than between the blue Eastern genetic cluster and the  
454 Western genetic clusters (mean  $F_{ST}$ =0.24; mean Jost's D=0.42; Table S9A), although the  
455 difference was not significant (KWRS test for  $F_{ST}$ ,  $W = 8$ , p-value = 0.200; KWRS test for Jost's  
456 D,  $W = 9$ , p-value = 0.100). The correlation between the matrices of pairwise genetic and  
457 geographic distances for *S. nutans* (tested on 16 sites) was significantly positive (Mantel test:  $r =$   
458 0.54, p-value < 0.001), indicating an isolation-by-distance pattern.

459

#### 460 **Differentiation between anther-smut fungi parasitizing *Silene nutans* and *S. italica***

461 Population genetic structure analyses separated *Microbotryum* strains into two main genetic  
462 clusters, corresponding to their host plant of sampling, *S. italica* and *S. nutans*. The host of  
463 collection separated strains according to the first axis of the PCA (Fig 2B). The STRUCTURE  
464 analysis and the discriminant analysis of principal component (DAPC) also delimited two clear  
465 fungal genetic clusters according to the host of sampling at  $K=2$  (Fig 2C; Fig S2). In the PCA

466 (Fig. 2B), four *Microbotryum* strains sampled on *S. nutans* clustered with strains parasitizing *S.*  
467 *italica* and one *Microbotryum* strain sampled on *S. italica* clustered with strains parasitizing *S.*  
468 *nutans*. These strains may correspond to spill-overs, i.e. cross-species disease transmissions,  
469 between the two studied *Silene* species or from other *Silene* species. We excluded for further  
470 analyses the 11 strains that had their main cluster memberships < 80% in the STRUCTURE  
471 analysis at K=2 (Fig 2C), which can be due to low assignment power or admixture.

472 Anther-smut fungi parasitizing *S. italica* had on average higher levels of allelic richness (WRS  
473 test on 19 groups of sites,  $W = 48$ , p-value = 0.004) and observed heterozygosity (WRS test on  
474 19 groups of sites,  $W = 30$ , p-value = 0.014) than anther-smut fungi parasitizing *S. nutans* (Table  
475 S8B). Levels of observed heterozygosity were significantly lower than heterozygosity expected  
476 under Hardy-Weinberg equilibrium for eight markers for *Microbotryum* fungi parasitizing *S.*  
477 *nutans* and six markers for *Microbotryum* fungi parasitizing *S. italica* (Table S4).

478

479 **Similar genetic structure in anther-smut fungi as in their *Silene nutans* and *S. italica* host**  
480 **plants**

481 The existence of additional levels of population genetic structure within fungal strains was  
482 indicated by the STRUCTURE barplots (Fig S2), the second and third principal components of  
483 the PCA that explained 27.9 % of the total variance between strains (Fig 2B). To investigate the  
484 population structure within each of the two main genetic clusters of anther-smut fungi,  
485 corresponding mainly to populations parasitizing *S. nutans* and *S. italica*, respectively, we  
486 analyzed them separately, which confirmed the existence of a strong population structure within  
487 anther-smut fungi parasitizing *S. nutans* (Fig 3A). The first two PCA axes separated fungal

488 strains into three distinct genetic clusters, corresponding to the three most frequent plastid  
489 haplotypes identified in the host plant (Fig 3A), which was also supported by the DAPC and the  
490 STRUCTURE analyses (Fig 3B; Fig S3). We named these three fungal genetic clusters the blue  
491 Eastern, yellow Western and orange Western *Microbotryum* clusters, in reference to their *S.*  
492 *nutans* host plastid haplotypes. In contrast to the host plant *S. nutans*, we found no clear  
493 subdivision in the orange Western anther-smut fungal cluster (Fig S3). We found no genetic  
494 clusters specific to strains sampled from a host with a red haplotype, perhaps due to the low strain  
495 number. We excluded for further analyses the two admixed fungal strains (cluster memberships  
496 <0.80 in a given cluster) and the three fungal strains sampled on a host with a red haplotype. We  
497 found no further population structure in the set of 28 anther-smut strains parasitizing *S. italica*  
498 (Fig S4), as for the host plant.

499 All three fungal genetic clusters parasitizing *S. nutans* had similar levels of allelic richness  
500 (KWRS test on 16 groups of sites,  $\text{Chi}^2 = 1.33$ ,  $\text{df} = 2$ ,  $\text{p-value} = 0.514$ ; Table S8B) and observed  
501 heterozygosity (KWRS test,  $\text{Chi}^2 = 0.67$ ,  $\text{df} = 2$ ,  $\text{p-value} = 0.716$ ; Table S8B). The  $F_{IS}$  varied  
502 between genetic clusters from -0.11 to 0.82 (Table S8B). We found no significant differences in  
503 differentiation between the three fungal genetic clusters (KWRS tests for  $F_{ST}$  and Jost's D,  $\text{Chi}^2 =$   
504  $2$ ,  $\text{df} = 2$ ,  $\text{p-value} = 0.368$ ; Table S9B). The significant correlation between the matrices of  
505 genetic and geographic distances between pairs of fungal populations parasitizing *S. nutans* (16  
506 sites) indicated an IBD pattern (Mantel test:  $r = 0.62$ ,  $\text{p-value} < 0.001$ ). There was a significantly  
507 positive correlation between genetic distance matrices for the fungal and the *S. nutans*  
508 populations (16 sites; Mantel test:  $r = 0.80$ ,  $\text{p-value} < 0.001$ ), and it remained significant when  
509 controlling for the IBD effect (partial Mantel test:  $r = 0.70$ ,  $\text{p-value} < 0.001$ ). Altogether, our  
510 findings indicate strong congruence between the population genetic structures of the anther-smut

511 fungi parasitizing *S. nutans* and its host plant *S. nutans* (Fig 3C). We found no signatures of  
512 recent reduction in effective population size in either of the three fungal genetic clusters  
513 parasitizing *S. nutans* nor the fungal genetic cluster parasitizing *S. italica* using the  
514 BOTTLENECK software (Piry et al., 1999) under either stepwise or two-phase models of  
515 microsatellite evolution (Table S10; one-tailed Wilcoxon signed rank test, all P-values>0.36).

516

### 517 **Inferred divergence history of anther-smut fungi on *Silene nutans* congruent with of their** 518 **host plant**

519 To study if the anther-smut fungi on *S. nutans* shared the same history of divergence into cryptic  
520 lineages as the one previously inferred for their host plants, the *S. nutans* cryptic species complex  
521 (Martin et al., 2016; Martin et al., 2017; Van Rossum et al., 2018), we used an approximate  
522 Bayesian computation random forest (ABC-RF) procedure. We compared various divergence  
523 scenarios for the three identified *Microbotryum* genetic clusters parasitizing *S. nutans*. We  
524 removed the 21 fungal strains that were likely siblings of other fungal strains, keeping 110  
525 anther-smut fungal strains belonging to the blue Eastern, yellow Western and orange Western  
526 *Microbotryum* genetic clusters parasitizing *S. nutans* and we used as outgroup the 25 genotyped  
527 *Microbotryum* strains parasitizing *S. italica*.

528 We performed three rounds of scenario comparison, each testing a particular evolutionary event,  
529 including divergence times, divergence order and the occurrence of gene flow (Table 1; Table S5;  
530 Fig S5). At each round, we retained the inferred most likely evolutionary scenario to be used as  
531 backbone for the subsequent rounds. To assess our power to discriminate between scenarios, we  
532 checked posterior probability (Table 1) and assessed visually whether the observed data fell



533 within the clouds of simulated data of the compared scenarios (Fig S6).

534 In the first round ("time of divergence"; Table 1, "round 1"), we tested four different time periods  
535 of divergence for various divergence orders (A, B or C; Fig S5A). The group of scenarios with  
536 strongest support included a first divergence occurring between 100,000-1,500,000 years ago,  
537 and a second divergence occurring between 0-20,000 years ago (posterior probability = 0.54 +/-  
538 0.02, prior error rate = 34%; Table 1, "round 1"; Fig S6A). In the second round ("divergence  
539 order"; Table 1, "round 2"), we found with a high posterior probability for the scenario assuming  
540 a first divergence of the blue Eastern *Microbotryum* genetic cluster, followed by the divergence  
541 between the yellow and orange Western *Microbotryum* genetic clusters (posterior probability =  
542 0.79 +/-0.02, prior error rate = 2%; Table 1, "round 2"; Fig S6B). When testing the order of  
543 divergence between clusters as first round and the time of divergence as second round, we  
544 selected the same scenarios with a high posterior probability (Note S1; Table S11).

545 In the third round ("occurrence of gene flow"; Table 1, "round 3, step 1"), we compared in a first  
546 step three groups of scenarios differing in the clusters affected by gene flow. The most supported  
547 group of scenarios assumed gene flow among *Microbotryum* genetic clusters parasitizing *S.*  
548 *nutans* but not with *S. italica* (posterior probability = 0.60 +/-0.02, prior error rate = 5%; Table 1,  
549 "round 3, step 1"; Fig S6C). In the following steps (Table 1, "round 3, steps 2 to 4"), we  
550 compared groups of scenarios of divergence with different timing of gene flow. Scenarios of  
551 divergence with ancient gene flow were the most supported, which suggested that episodes of  
552 ancient gene flow occurred during divergence or just following divergence of the three  
553 *Microbotryum* genetic clusters parasitizing *S. nutans* and then completely stopped (posterior  
554 probability = 0.76 +/-0.02, prior error rate = 35%; Table 1, "round 3, step 2"; Fig S6D-F).  
555 However, we did not have enough power to differentiate between scenarios of ancient gene flow

556 that involved different genetic clusters (either all three genetic clusters or only the two Western  
557 genetic clusters) or different times of past gene flow (either over a 0-20000 year period or a  
558 50,000-500,000 year period; Table S5; posterior probability = 0.49  $\pm$  0.03, prior error rate = 60%;  
559 Table 1, “round 3, step 5”). We performed parameter inferences for the group of four models  
560 with ancient migration (“round 3, step 2”, group 3 see Table 1; Table S12). The inferred dates  
561 and order of divergence between fungal genetic clusters parasitizing *S. nutans* were consistent  
562 with the history of divergence previously inferred for the host plant *S. nutans* (Fig 4; Martin et  
563 al., 2016; Martin et al., 2017; Van Rossum et al., 2018).

564

565 **No genome-wide signatures of gene flow between anther-smut fungi on distinct *Silene***  
566 **species**

567 To check whether genome-wide data support the patterns inferred above on population genetic  
568 structure in the anther-smut fungi and lack of recent gene flow, we sequenced the genomes of 53  
569 *Microbotryum* strains parasitizing *S. nutans* or *S. italica* (Table S7). We also used previously  
570 sequenced genomes of anther-smut fungi parasitizing species with overlapping geographical  
571 ranges in order to check if some of the strains that appeared as admixed or did not cluster  
572 according to their host species may actually correspond to other *Microbotryum* species. After  
573 filtration, we obtained 1,305,369 SNPs for 108 *Microbotryum* strains. We performed PCA  
574 analyses on the genome-wide SNPs and STRUCTURE analyses and confirmed strong population  
575 differentiation between fungal strains parasitizing *S. nutans* and *S. italica* (Fig 5A-B; Fig S7A).  
576 Only one strain parasitizing *S. nutans* clustered with strains parasitizing *S. italica*, similarly as  
577 found from microsatellite data (strain # 1438; Fig 2C), therefore likely being a genuine spill-over

578 between *S. nutans* and *S. italica*. Two other strains parasitizing *S. nutans* clustered with strains  
579 parasitizing *S. latifolia*. These strains had admixed cluster membership (i.e. mean membership  
580 coefficient <0.80 to the given cluster) or clustered with strains parasitizing *S. italica* from  
581 microsatellite data (Fig 2C). These strains likely represent spill-overs between *S. nutans* and *S.*  
582 *latifolia*.

583 Genome sequences retrieved the same genetic structure as microsatellites for anther-smut fungi  
584 parasitizing *S. nutans*, with a strong differentiation between *Microbotryum* strains sampled on the  
585 *S. nutans* blue plastid haplotype on the one hand and those sampled on yellow, red or orange  
586 plastid haplotypes on the other hand (Fig S7B), and confirmed the absence of strong population  
587 structure within anther-smut fungi parasitizing *S. italica* (Fig S7C). We also found strong  
588 population differentiation between fungal strains parasitizing the three closely related host  
589 species of *S. nutans* and *S. italica* (Fig 5A-B). STRUCTURE analyses showed no signatures of  
590 recent admixture between strains parasitizing different hosts (Fig 5B).

591 The phylogenetic network analysis implemented in SplitsTree also supported the absence of  
592 recent gene flow at the genome-wide levels between the *Microbotryum* species parasitizing  
593 different hosts and the close genetic similarity of the three genetic clusters of anther-smut fungi  
594 parasitizing the *S. nutans* complex (Fig 5C). Within *Microbotryum* strains parasitizing *S. nutans*,  
595 we found no footprint of recombination between strains of the two main genetic clusters (Fig 5C)  
596 which was consistent with a scenario of divergence with ancient migration and no contemporary  
597 gene flow inferred from ABC demographic inferences.

598

599 **Discussion**

600 We found that genetic structure and divergence history of anther-smut fungi parasitizing the *S.*  
601 *nutans* plant species complex closely mirrored those of their host plant genetic lineages. Such a  
602 congruence in population structure and divergence history of the host and the pathogen in the *S.*  
603 *nutans* complex, the distribution area of the genetic clusters in Western Europe, as well as the  
604 inferred dates of divergence suggest that the host and the pathogen differentiated in parallel  
605 following isolation in shared glacial refugia. To confirm the co-divergence events between *S.*  
606 *nutans* lineages and anther-smut fungi genetic clusters, we need to more accurately date the node  
607 of lineage divergence events in the host and the pathogen. Such molecular dating is essential to  
608 support co-divergence events as preferential shifts of the pathogen to closely related host species  
609 can also produce congruent phylogenies (de Vienne et al., 2013), but is currently difficult due  
610 lack of appropriate calibration points.

611 Nevertheless, our results rule out the hypothesis that anther-smut fungi on some of the *S. nutans*  
612 genetic lineages may result from specialization by host shifts from distant anther-smut fungi  
613 parasitizing other *Silene* species in sympatry or parapatry. This contrasts with the prevalence of  
614 host shifts between distant lineages observed in the Caryophyllaceae-*Microbotryum* system at  
615 larger evolutionary scales (Refrégier et al., 2008), but is consistent with the strong congruence  
616 reported between the population genetic structures of *S. latifolia* and its anther-smut fungi, also  
617 corresponding to glacial refugia footprints (Feurtey et al., 2016). Our study thus contributes  
618 towards gaining general insights into the processes of divergence in host-pathogen systems.  
619 While at large evolutionary scales, host shifts seem to be the rule (de Vienne, Hood, & Giraud,  
620 2009; Thines, 2019), finer and more recent population subdivisions may more often result from  
621 codivergence due to shared geographic and climatic constraints, such as glacial refugia. The  
622 discrepancy between the two evolutionary scales may be due to recurrent pathogen lineage

623 extinctions followed by recolonizations through host shifts: codivergence may occur frequently,  
624 but pathogen lineages would regularly go extinct over longer evolutionary scales and plant  
625 lineages would be recolonized by host shifts. This novel hypothesis can be tested by studying  
626 further closely related pairs of natural host-pathogen associations, which unfortunately still  
627 remain scarce.

628 The significant IBD pattern in both the *S. nutans* plant and anther-smut fungi and the correlation  
629 between the genetic distances between host and pathogen pairs when controlling for IBD suggest  
630 that the anther-smut fungi followed similar recolonization routes as the plant and/or became  
631 specialized on the host genetic lineages. However, contrary to the system *S. latifolia* - *M.*  
632 *lychnidis-dioicae* (Feurtey et al., 2016) and other host-pathogen systems (Barrett et al., 2008;  
633 Criscione, Poulin, & Blouin, 2005; Nieberding et al., 2008; Nieberding & Olivieri, 2007), we  
634 found higher levels of subdivision in *S. nutans* than in its associated anther-smut fungi. The  
635 weaker genetic structure in anther smut fungi compared to its host suggests again extinction of  
636 the pathogen lineage in a plant lineage, followed by recolonization from another fungal lineage,  
637 or the ability of an anther-smut lineage to remain generalist on two closely related plant lineages.

638 Future studies using cross-inoculation experiments between strains and host plants of different  
639 genetic clusters within the *S. nutans* complex could assess whether the congruence of host-  
640 pathogen genetic structure is associated with a pattern of local adaptation of the pathogen or of  
641 the host, as in the system *S. latifolia* - *M. lychnidis-dioicae* (Delmotte et al., 1999; Feurtey et al.,  
642 2016; Kaltz et al., 1999). We could not obtain enough fresh material for this study to be able to  
643 test local adaptation. Furthermore, strong asymmetric postzygotic reproductive isolation was  
644 found between the Eastern and Western *S. nutans* plant lineages (Martin et al., 2017) and it would  
645 be interesting in future studies to explore if a similar reproductive isolation pattern is present

646 between the Eastern and Western fungal lineages on *S. nutans*, and whether reproductive  
647 isolation can be found among anther-smut fungi.

648 We found clear differentiation between fungal populations parasitizing the two closely related *S.*  
649 *nutans* and *S. italica* species, supporting previous findings of differentiation based on a few  
650 strains and loci (Bucheli et al., 2000). No strong population structure was found within *S. italica*  
651 or within its associated anther-smut fungi. However, future studies with larger sampling  
652 distribution in the *S. italica* range in the Mediterranean Basin (Naciri, Pasquier, Lundberg,  
653 Jeanmonod, & Oxelman, 2017) may identify population differentiation. Cross-species disease  
654 transmissions, i.e. spill-overs of fungal strains, were more frequent on *S. nutans* than on *S. italica*,  
655 which may be due to unequal sampling size between the two *Silene* species or to biological  
656 differences. Some Caryophyllaceae plants indeed seem more susceptible to cross-species  
657 transmissions (Antonovics et al., 2002; de Vienne, Hood, et al., 2009; Hood et al., 2019). Putative  
658 spill-overs may actually correspond to other *Microbotryum* species, that were too rare to be  
659 identified in our population analyses using microsatellite markers, as revealed with the whole  
660 genome sequence data. In fact, two strains collected on *S. nutans* were actually assigned, based  
661 on whole genome sequences, to *M. lychnidis-dioicae*, specialized on *S. latifolia*.

662 The statistical comparison of demographic models and the whole-genome SNP analyses indicated  
663 the absence of recent gene flow between *Microbotryum* species parasitizing closely related *Silene*  
664 species, as well as between the Eastern and Western fungal lineages parasitizing *S. nutans*,  
665 although some spill-over cases were detected. Noteworthy, we also found no evidence of hybrid  
666 individuals between the two host plants species *S. nutans* and *S. italica* based on SNP data. The  
667 admixed barplots observed in the microsatellite STRUCTURE analyses must therefore have been  
668 due to low power of assignment rather than recent hybridization. This result was consistent with

669 previous studies on several other anther-smut fungi, in which no hybrids were detected in natural  
670 populations, even between very closely related species that can hybridize in the laboratory  
671 (Abbate et al., 2018; Badouin et al., 2017; Gladieux et al., 2013; Petit et al., 2017). The only case  
672 where hybrids were detected was among closely related anther-smut fungi with overlapping host  
673 and geographic ranges, on *Dianthus* species (Petit et al., 2017). Ecological factors, such as  
674 different habitats, pollinator guilds or flowering time of the host species (Jürgens, Witt, &  
675 Gottsberger, 1996; Kephart, Reynolds, Rutter, Fenster, & Dudash, 2006), may constitute pre-  
676 zygotic factors favoring reproductive isolation of host-specialized anther-smut fungi in natural  
677 populations. Both extrinsic and intrinsic post-zygotic barriers can be strong in anther-smut fungi  
678 (Giraud & Gourbière, 2012; Le Gac, Hood, & Giraud, 2007). Strong host specialization may  
679 indeed play a role in reproductive isolation, through migrant inviability and hybrid maladaptation  
680 on parental hosts, especially given the life cycle of *Microbotryum* fungi, with many spores falling  
681 on a plant and competing for systemic infection, and selfing being frequent, exposing hybrids to  
682 systematic competition with non-hybrids (Gibson, Hood, & Giraud, 2012). In addition,  
683 comparative genomics of anther-smut fungi showed presence of large genomic rearrangements  
684 and gene content variation between species (Branco et al., 2018; Hartmann et al., 2018), and  
685 experimental crosses suggested high frequency of hybrid sterility and abnormal genomic contents  
686 in hybrids (de Vienne, Refrégier, et al., 2009).

687 The low levels of gene flow among anther-smut fungi parasitizing different hosts found in  
688 *Microbotryum* fungi stand in high contrast with frequent reports of signatures of introgression in  
689 other fungal pathogens, such as crop pathogens or human disease-associated pathogens (Feurtey  
690 & Stukenbrock, 2018). Few studies have focused on fungal pathogens diversification in natural  
691 host communities while several evolutionary processes, such as time scale of divergence, host

692 density and heterogeneity, are likely very different than those occurring on human-modified  
693 environment pathogens (Laine, 2005; Laine, Barrès, Numminen, & Siren, 2019; Stukenbrock &  
694 McDonald, 2008). To understand how biodiversity arises and what the mechanisms of host-  
695 pathogen evolution are over large evolutionary scales, we need more studies on co-evolutionary  
696 histories of parasites and their hosts in natural ecosystems. Studies of population genetic structure  
697 and divergence in plant fungal pathogens indeed remain so far mostly focused on crop pathogens  
698 (Barrès et al., 2008; Enjalbert, Duan, Leconte, Hovmøller, & De Vallavieille-Pope, 2005;  
699 Fournier & Giraud, 2008; Linde, Zhan, & McDonald, 2002; Saleh, Milazzo, Adreit, Fournier, &  
700 Tharreau, 2014; Stukenbrock, Banke, & McDonald, 2006; Zaffarano, McDonald, & Linde,  
701 2008), in which patterns are heavily impacted by host genetic homogeneity and high abundance,  
702 as well as by human-mediated plant and pathogen dispersal. We found here strong population  
703 structure congruence between hosts and pathogens, in agreement with a previous study on other  
704 anther-smut lineages (Feurtey et al., 2016). Further studies on other natural systems are needed to  
705 assess whether this represents a general pattern in natural pathogen-host communities.

706

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721

## 722 **Datasets**

723 dataset 1: Hartmann F.E., Snirc A., Cornille A., Godé C., Touzet P., Van Rossum F., Fournier E.,  
724 Le Prieur S., Shykoff J., Giraud T. 2020. Genotype data from : Congruent population genetic  
725 structures and divergence histories in anther-smut fungi and their host plants *Silene italica* and  
726 the *S. nutans* species complex, Dryad, Dataset, <https://doi.org/10.5061/dryad.m63xsj3z3>

727 dataset 2: Hartmann F.E., Snirc A., Cornille A., Godé C., Touzet P., Van Rossum F., Fournier E.,  
728 Le Prieur S., Shykoff J., Giraud T. 2020. Whole genome sequencing raw data of *Microbotryum*  
729 fungi infecting *Silene nutans* and *S. italica*. NCBI Sequence Read Archive (SRA). BioProject ID  
730 PRJNA589725.

731

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## 1067 **Data accessibility**

1068 The plant microsatellite and chloroplastic genotypes and the fungal microsatellite genotypes are

1069 available at Dryad, Dataset, <https://doi.org/10.5061/dryad.m63xsj3z3>. We deposited the genome



1070 raw data at NCBI Sequence Read Archive (SRA) under the BioProject ID PRJNA589725 (see  
1071 Table S7 for BioSample accession numbers).

1072

1073 **Author contributions**

1074 TG and FH conceived and designed the project with the help of FVR. TG, PT, FVR, JS and EF  
1075 collected samples. AS, CG, PT, SLP and FH genotyped the samples. FH and AC performed the  
1076 analyses. FH, TG and FVR wrote the manuscript. All authors read and approved the final version  
1077 of the manuscript and declare no conflict of interest.

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1080 **Supporting information**

1081 Additional Supporting Information may be found in the online version of this article.

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1084 **Tables**

1085 **Table 1: Comparisons of scenarios and group of scenarios used for approximate Bayesian**  
1086 **computation (ABC) to reconstruct the fungi divergence history.** Scenarios are described in  
1087 Table S5 and Fig S5. Results of ABC random forest procedure are shown, with the percentage of  
1088 vote for each scenario/group of scenarios, the posterior probability and the prior error rate. The  
1089 best scenario is indicated with bold and three stars in the percentage of vote.

1090

1091

## 1092 **Figures**

1093 **Figure 1: Population genetic structure of *Silene nutans* and *S. italica* host plants based on**  
1094 **six plastid (chloroplast) SNPs and 21 microsatellite markers.** A. Plastid haplotypes according  
1095 to their geographic distribution. On the right, a zoom of the map in the northeastern region of  
1096 France and Belgium is shown. The size of the symbols is proportional to the number of  
1097 individuals sampled by site (1-9 plants). B. Principal component analysis (PCA), with  
1098 information of plastid haplotypes. The first and second principal component axes are shown and  
1099 the percentage of variance explained by each axis is indicated into brackets. C. Results of  
1100 STRUCTURE on both host plant species for K=5 clusters. On panels A and B, symbol shape  
1101 indicates species and color indicates plastid haplotypes. The two genetic clusters identified within  
1102 the orange plastid haplotype based on the STRUCTURE analysis (panel C) are shown with light  
1103 and dark orange colors, respectively.

1104

1105 **Figure 2: Population genetic structure of anther-smut fungi (*Microbotryum*) parasitizing**  
1106 ***Silene nutans* and *S. italica* based on 22 microsatellite markers.** A. Anther-smut fungi  
1107 parasitizing *S. italica* (top; photo credit M.E. Hood) and *S. nutans* (bottom; photo credit M.  
1108 Strack van Schijndel). B. Principal component analysis (PCA). The first and second principal  
1109 component axes are shown and the percentage of variance explained by each axis is indicated  
1110 into brackets. The symbol shape indicates the sampling host species. The dark and light blue  
1111 colors indicate assignment probability >0.80 to the two corresponding clusters identified in the  
1112 STRUCTURE analysis for K=2 (see panel C). Pink color indicates strains for which no cluster  
1113 could be assigned with a probability >0.80. Genetic variance explained by the principal  
1114 component axes is shown in the right bottom corner. C. STRUCTURE barplot for K=2. Strains  
1115 are ordered according to their sampling host species.

1116

1117 **Figure 3: Congruence of population genetic structure between *Silene nutans* host plants and**  
1118 **their anther-smut fungal (*Microbotryum*) strains based on microsatellite markers.** A.  
1119 Principal component analysis (PCA) on the fungal strains with information of the population  
1120 genetic structure of their hosts. The first and second principal component axes are shown.  
1121 Symbol shape indicates the sampling host species and color indicates the plastid haplotypes of  
1122 the sampling host. B. STRUCTURE barplots for (1) K=3 in 112 *Microbotryum* strains and (2)  
1123 K=4 in 110 *S. nutans* plant individuals. Fungal strains are ordered according to their sampling  
1124 host plastid haplotypes. Host plant individuals are ordered according to their plastid haplotypes.  
1125 C. Map of mean cluster membership proportions per site for (1) the 112 fungal strains in the  
1126 STRUCTURE analysis at K=3 and (2) the 110 *S. nutans* plant individuals in the STRUCTURE  
1127 analysis at K=4. The pie diameter reflects the sample size in the corresponding site (1 - 14  
1128 individuals).

1129

1130 **Figure 4: Most likely scenario for the divergence between the fungal genetic clusters**  
1131 **inferred with approximate Bayesian computation (ABC).**

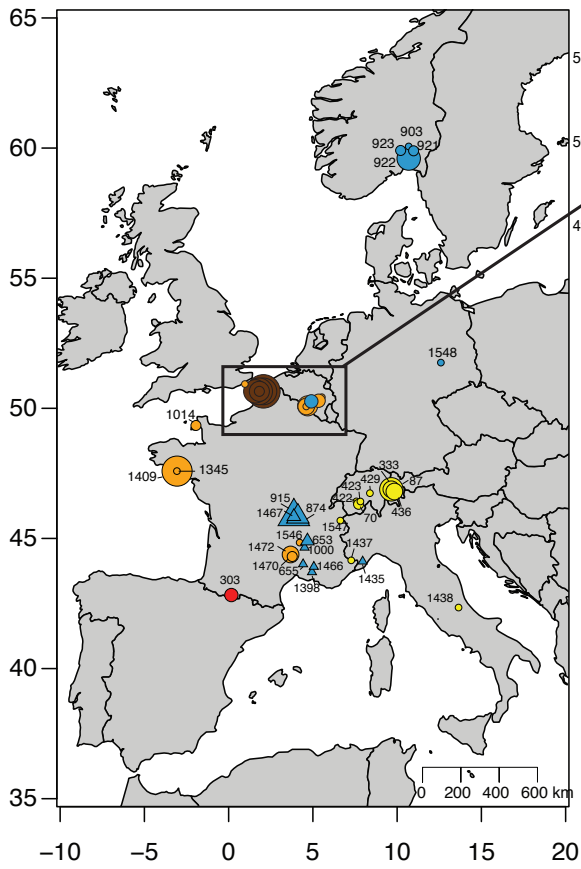
1132

1133 **Figure 5: Absence of genome-wide signature of recent gene flow among anther-smut fungal**  
1134 **strains parasitizing closely related *Silene* species.** A. Principal component analysis (PCA)  
1135 based on 1,305,369 genome-wide SNPs. B. STRUCTURE analyses based on 233 unlinked SNPs  
1136 for K=5. The Y axis indicates the estimated membership proportions in the K clusters for each  
1137 fungal strain (X axis). C. Neighbornet tree from a SplitsTree analysis based on 595,002 genome-  
1138 wide SNPs with no missing data and heterozygote genotypes. The inner plot shows a zoom of the  
1139 tip of the tree for strains parasitizing *S. nutans*. Information on the species or plastid haplotype of  
1140 the host of sampling of the strains is given.

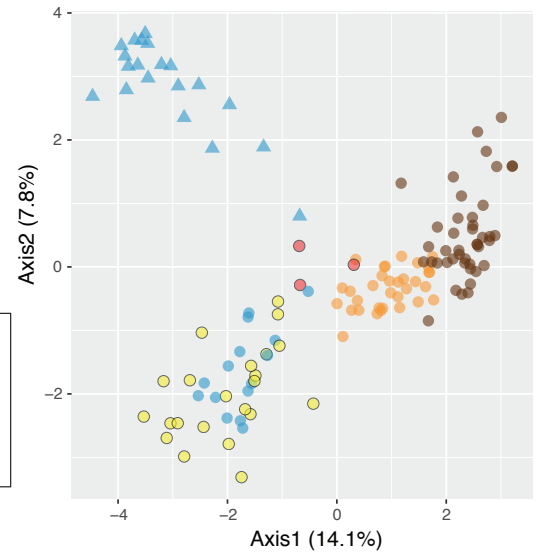
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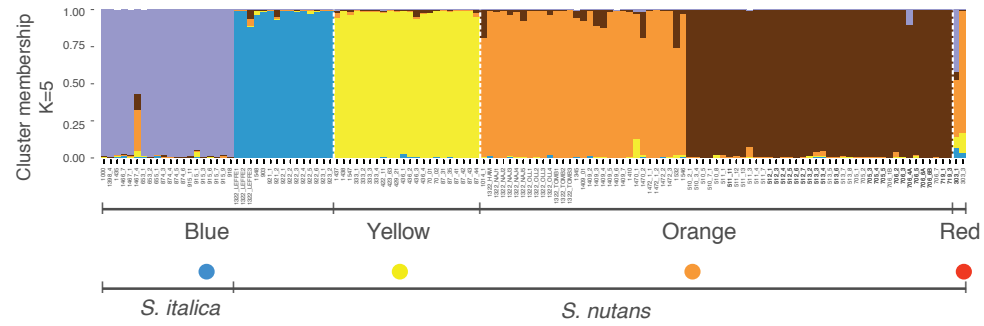
### A Host plant individuals



### B Host plant individuals



### C



Host plant individuals, plastid haplotypes and species



