

Co-occurrence among three divergent plant-castrating fungi in the same Silene host species

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CO-OCCURRENCE AMONG THREE DIVERGENT PLANT-CASTRATING FUNGI IN THE SAME SILENE HOST SPECIES

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1	CO-OCCURRENCE AMONG THREE DIVERGENT PLANT-CASTRATING FUNGI	
2	IN THE SAME <i>SILENE</i> HOST SPECIES	
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28 The competitive exclusion principle postulates that different species can only coexist in 29 sympatry if they occupy distinct ecological niches. The goal of this study was to understand 30 the geographical distribution of three species of *Microbotryum* anther-smut fungi that are 31 distantly related but infect the same host plants, the sister species Silene vulgaris and S. 32 uniflora, in western Europe. We used microsatellite markers to investigate pathogen 33 distribution in relation to host specialization and ecological factors. Microbotryum violaceo-34 irregulare was only found on S. vulgaris at high elevations in the Alps. Microbotryum 35 lagerheimii could be subdivided into two genetically differentiated clusters, one on S. uniflora 36 in the UK and the second on S. vulgaris in the Alps and Pyrenees. The most abundant 37 pathogen species, M. silenes-inflatae, could be subdivided into four genetic clusters, co-38 occurring in the Alps, the UK and the Pyrenees, and was found on both S. vulgaris and S. 39 uniflora. All three fungal species had high levels of homozygosity, in agreement with the 40 selfing mating system generally observed in anther-smut fungi. The three pathogen species 41 and genetic clusters had large range overlaps, but occurred at sites with different elevations, 42 temperatures and precipitation levels. The three *Microbotryum* species thus do not appear to 43 be maintained by host specialization or geographic allopatry, but instead may occupy 44 different ecological niches in terms of environmental conditions.

Key words: population structure, endemicity, speciation, hybrid zones, fungi, *Microbotryum violaceum, Silene maritima*, biogeography, altitude

48 Introduction

49	The competitive exclusion principle postulates that two species occupying the same	
50	niche in sympatry cannot coexist indefinitely (Gause 1934; Hardin 1960). In pathogens, this	Field Code Changed
51	means that species coexisting in sympatry in association with a common host species are	Field Code Changed
52	likely to differ in other components of their ecological niches. The coexistence of multiple	
53	pathogens on the same crop species is in fact often associated with separation by time,	
54	resource use, abiotic conditions or geographical area (Fitt et al. 2006; Giraud et al. 2017).	Field Code Changed
55	Closely related pathogens can also specialize on either the early or the late season part of a	Field Code Changed
56	given plant life cycle (Hamelin et al. 2016; Mailleret et al. 2012). For example, two eyespot	Field Code Changed
57	wheat pathogens, O. yallundae and O. acuformis, differ in temperature optima for growth and	Field Code Changed
58	fungicide resistance (Fitt et al. 2006). In oilseed rape, the pathogens Leptosphaeria maculans	Field Code Changed
59	and L. biglobosa differ in their location in stem and leaf tissues and in their maturation rates	
60	under low temperatures (Fitt et al. 2006). In malaria, variation in host resistance to different	Field Code Changed
61	Plasmodium species has been invoked to explain pathogen coexistence (Snounou & White	Field Code Changed
62	2004). Furthermore, some <i>Plasmodium</i> species are specialized to attack only the youngest or	
63	oldest red blood cells, leading to complex within-host dynamics during co-infection	
64	(McQueen & McKenzie 2004a, b). Pathogens with different transmission modes, e.g. sexual	Field Code Changed
65	versus non-sexual transmission, can also stably coexist (Thrall & Antonovics 1997).	Field Code Changed
66	The goal of this study was to delineate and understand the coexistence of different	
67	species of anther-smut fungi on two closely related host plants, Silene vulgaris and S.	
68	uniflora. Microbotryum is a species complex of basidiomycete fungi responsible for anther-	
69	smut disease in many plants in the Caryophyllaceae. Microbotryum anther-smut fungi are	
70	obligate pathogens that sterilize their hosts. Infected plants produce fungal teliospores in place	
71	of pollen, and female structures do not mature; in dioecious or gynodioecious species,	

72	infected female plants also develop spore-bearing anthers. Within Microbotryum, there are	
73	numerous phylogenetically divergent lineages many of which have been given species status	
74	(Denchev et al. 2009; Kemler et al. 2006; Le Gac et al. 2007a). These typically show inter-	F
75	sterility (de Vienne et al. 2009b; Le Gac et al. 2007b) and strong host specificity (de Vienne	Fi
76	et al. 2009a). There is usually only one endemic Microbotryum species per host plant species) Fi
77	(Le Gac et al. 2007a; Refrégier et al. 2008), although transient host shifts are not uncommon	Fi
78	(Antonovics et al. 2002; Gladieux et al. 2011; Hood et al. 2003).	F
79	A conspicuous exception to this pattern is the presence of three divergent	F
80	Microbotryum species on S. vulgaris and its closely related sister species S. uniflora (Figure	F
81	1). These are: 1) <i>M. lagerheimii</i> (Denchev 2007) (MvSv1 in Le Gac <i>et al.</i> 2007a), 2) <i>M.</i>	F
82	silenes-inflatae (MvSv2 in Le Gac et al. 2007a), and 3) M. violaceo-irregulare (Kemler et al.	Fi Fi
83	2006; Lutz et al. 2008). These three Microbotryum species are not particularly closely related	` - [Fi
84	to one-another (Figure 1), and show differences in spore morphology (color and	
85	ornamentation; Figure 1; Denchev 2007; Gold et al. 2009; Lutz et al. 2005; Lutz et al. 2008)).	F
86	Microbotryum lagerheimii has lighter spore color, while verrucose spore ornamentation is	F
87	unique to <i>M. violaceo-irregulare</i> (Figure 1; Lutz et al. 2005; Vánky 1994). All three species	Fi Fi
88	castrate host flowers in the same way, replacing the pollen by their spores and aborting	F
89	ovaries, and are sexually transmitted (the spores are dispersed by pollinators), suggesting that	
90	it is unlikely they can have distinct ecological niches within host individuals. None of the	
91	three species has ever been found to persist in a host species other than S. vulgaris or S.	
92	uniflora, indicating that they are maintained endemically on these host plants.	
93	One explanation for coexistence of these three fungal species could be that they are	
94	allopatric, although they have been found to co-occur in the same sites (Abbate & Antonovics	- F
95	2014; Chung et al. 2012; Le Gac et al. 2007a); however sampling has been limited thus far	- F

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96	and it remains possible that contact zones of allopatric distributions were sampled. An	
97	alternative hypothesis is that the species display differential specialization towards S. vulgaris	
98	versus S. uniflora. Previous studies further suggest that this hypothesis is unlikely, given that	
99	both host species have been found to be parasitized in nature by at least two of the	
100	Microbotryum species also parasitizing S. vulgaris: M. lagerheimii and M. silenes-inflatae	
101	(Chung <i>et al.</i> 2012; Smith <i>et al.</i> 2017).	Field Code Changed
102	A more plausible explanation for the species coexistence could be adaptation to	
103	different abiotic conditions. All three species affecting S. vulgaris were found almost	
104	exclusively in host populations at higher elevations in the French Alps, where overall	
105	temperatures were lower and precipitation more consistently higher than conditions	
106	experienced by lower-elevation host populations (Abbate & Antonovics 2014). Here we	Field Code Changed
107	investigated the generality of this pattern with a larger and broader sampling in order to	
108	further understand their coexistence. Differentiation in disjunct refugia during the last	
109	glaciation in Europe may also have shaped differential adaptation to abiotic conditions and/or	
110	generated allopatry. Strong population differentiation has been found in other Microbotryum	
111	species that reflects footprints of persistence in glaciation refugia, e.g. in M. lychnidis-dioicae	
112	parasitizing S. latifolia (Badouin et al. 2017; Feurtey et al. 2016; Gladieux et al. 2011;	Field Code Changed
113	Vercken et al. 2010) and M. silenes-acaulis parasitizing S. acaulis (Bueker et al. 2016). High	Field Code Changed Field Code Changed
114	selfing rates were inferred for previously studied Microbotryum species (Bueker et al. 2016;	Field Code Changed
115	Giraud 2004; Gladieux et al. 2011; Hood & Antonovics 2000; Hood & Antonovics 2004),	Field Code Changed
116	and such a closed mating system may contribute to generate and maintain strong population	Field Code Changed
117	structure and genetic isolation between species (Gibson et al. 2012; Vercken et al. 2010).	Field Code Changed
110	To assess the wider geographical and elevational distributions of the three different	Field Code Changed
110	To assess the wider geographical and elevational distributions of the three different	Field Code Changed
119	Microbotryum species occurring on S vulgaris and S uniflora we collected anther-smut	

fungi from both host populations in different regions in western Europe, and genotyped them with microsatellite markers. We then examined whether the three *Microbotryum* species 1) had contrasting geographical or elevational distributions, 2) showed different frequencies on the two sibling host species *S. vulgaris* and *S. uniflora*, 3) co-occurred in the same sampling sites, and if so, whether hybrids could be detected, 4) exhibited within-species geographical population subdivision, and 5) displayed high selfing rates like other *Microbotryum* species.

126

127 Materials and Methods

128 Teliospore collection

129 The Microbotryum samples analyzed in this study were collected across western Europe 130 (Supplementary Figure S1, Supplementary Table S1) from 51 sites with S. vulgaris (n = 407), 131 and 12 sites with S. uniflora (n = 59). A sampling site was defined as a location in which 132 samples were collected a few meters apart. Sampling was performed by numerous 133 investigators throughout Europe and was carried out as part of general disease surveys, other 134 field trips, or as part of more formal investigations (Chung et al. 2012; Le Gac et al. 2007a, 135 and unpublished studies). Within sampling sites, a single diseased flower was collected per 136 individual plant, stored in silica gel, and DNA from the diploid teliospores was extracted for 137 genetic analyses. Multiple infections by different genotypes are not uncommon in Silene-138 Microbotryum systems (Bueker et al. 2016; Buono et al. 2014; Chung et al. 2012; Hood 139 2003; López-Villavicencio et al. 2011), but teliospores within a single flower originate from a 140 single diploid genotype (Gold et al. 2009; Lopez-Villavicencio et al. 2007). Teliospores 141 sampled from a given flower were therefore assumed to be from a particular pathogen 142 individual. Because of the difficulty of identifying the different Microbotryum species in the

field, sampling was carried out without knowledge of fungal species identity, so our sample corresponds to an unbiased representation of the *Microbotryum* species frequencies in the sampled regions. We obtained permits for collections in national parks and all samples were collected before 2014, so not falling under the Nagoya protocol. Dried spores from each sample are freely available upon request.

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149 Microsatellite, ITS genotyping and spore morphology

- 150 DNA was extracted and genotyped using 11 microsatellite loci as described previously
- 151 (SVG1, SVG2, SVG5, SVG6, SVG8, SVG15, SN2, SN5, SN11, DC2, and DC5; Giraud
- 152 2004; Giraud et al. 2008). Microsatellite scoring was performed as described previously
- 153 (Vercken et al. 2010). Analyses using GENCLONE (Arnaud-Haond & Belkhir 2007) showed
- 154 that the eleven markers were sufficient to discriminate multilocus genotypes in the dataset as
- 155 a plateau was reached in terms of number of genotypes detected (Supplementary Figure S2).
- 156 In order to assign the genetic clusters from microsatellite analyses to the three
- 157 Microbotryum species, we sequenced the ribosomal internal transcribed spacer (ITS),
- spanning ITS1, 5.8S and ITS2 fragments, in 27 individuals using the fungus-specific primers
- 159 ITS1 and ITS4 (White *et al.* 1990) and matched these to published ITS sequences from *M*.
- 160 silenes-inflatae, M. lagerheimii, M. violaceo-irregulare (Kemler et al. 2006; Lutz et al. 2008).
- 161 Additionally, we verified in 15 individuals spore color (light versus dark purple) under the
- 162 binocular and spore ornamentation (i.e. verrucose versus reticulate) under light microscopy.
- 163 The samples chosen for ITS sequencing and for spore morphology assessment were picked at
- 164 random within each cluster, and there was complete agreement in the species assignments
- 165 between ITS, spore morphology and genetic clusters.



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167	Data analyses	
168	ITS tree. A maximum likelihood phylogeny of ITS region sequenced was inferred using	
169	PhyML 3.0 (Guindon et al. 2010).	Field Code Changed
170		
171	Map. The sample map (Supplementary Figure 2) was generated using the GGMAP package in	
172	R environment (Kahle & Wickham 2013).	Field Code Changed
173		
174	Descriptive statistics. Microsatellite variability was quantified by the unbiased gene diversity	
175	(H _d), allelic richness (A_r), and the fixation index (F_{IS}) using FSTAT 2.9.3.2 (Goudet 2001).	Field Code Changed
176	Observed and unbiased expected heterozygosities (H_O and H_E) were computed using a custom	
177	python script. Analyses of variance (ANOVA) and Kruskall-Wallis tests were performed	
178	using JMP v7.0 (SAS Institute).	
179		
180	Population subdivision. We assessed population subdivision using Bayesian clustering	
181	implemented in STRUCTURE 2.3.4 (Falush et al. 2003; Pritchard et al. 2000). The method	Field Code Changed
182	partitions multilocus genotypes into clusters while minimizing departure from expected	
183	frequencies and linkage equilibrium among loci. For this analysis, we used a haploid setting	
184	because Microbotryum individuals were highly homozygous (Table 1), which could bias	
185	structure inferences based on Hardy-Weinberg expectations in a diploid setting. We	
186	conducted independent runs with different number of clusters ($K = 1$ to 15). Each run of	
187	400,000 iterations and 100,000 burn-in iterations was repeated ten times, using a model	
188	allowing for admixture and correlated allele frequencies. We used CLUMPP 1.1.1 (Jakobsson	Field Code Changed
	9	

190 replicate runs for each K ('Greedy' algorithm; 100 random input sequences; G' statistic), and 191 to average individual assignment probabilities (q) over replicated runs with identical 192 clustering solution. 193 Because M. lagerheimii and M. violaceo-irregulare were minority components of the 194 data set, they could not be differentiated in the overall analysis. We therefore also ran 195 STRUCTURE on a sub-dataset encompassing only genotypes assigned to these species based on 196 ITS sequencing. We retained for this sub-dataset the genotypes with total memberships $q \ge 0.8$ 197 in the cluster corresponding to both *M. lagerheimii* and *M. violaceo-irregulare* in the analysis 198 on the whole dataset (blue cluster at K=5, see results). The 0.8 threshold is arbitrary but is 199 typically used for assigning individuals, and the genotypes with main assignments below 0.8

& Rosenberg 2007) to identify potential distinct solutions among the results of independent

200 had assignment probabilities balanced among the different clusters, indicating they likely

201 corresponded to individuals with low assignment power (see results).

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203 Hybrid detection

204 To assess whether individuals with intermediate assignment probability to clusters 205 were high-confidence inter-cluster hybrids or may be simply genotypes with low power of 206 assignment, possibly carrying alleles frequent in all species, we used a two-step procedure. In 207 the first step, we used the membership proportions of individual genomes in different clusters 208 (q values) inferred using the admixture model of STRUCTURE to identify 'pure' representatives 209 of the different species or of clusters within species. Based on the STRUCTURE outputs of the 210 full dataset at K=5 (identified as the most relevant subdivision, see results), pure 211 representatives of a given species were identified as genotypes for which the sum of q values

212	in the different clusters assigned to this species was above 0.8; genotypes not meeting this	
213	requirement were classified as putative hybrids. Pure representatives of the various clusters	
214	within a given species were defined as genotypes displaying q values above 0.8 within one of	
215	the clusters identified within this species; genotypes not meeting this requirement were	
216	considered as putative admixed genotypes among clusters.	
217	In the second step, we used pure representatives of the species or clusters as 'learning	
218	samples', allowing estimation of the membership proportions for the putative hybrid samples	
219	in 'learning clusters', using STRUCTURE, options USEPOPINFO and	
220	UPDATEPFROMPOPFLAGONLY. In assignment tests of putative hybrids between M.	
221	silenes-inflatae and either M. lagerheimii or M. violaceo-irregulare, genotypes were	
222	considered as hybrids if they had q values above 0.2 and below 0.8 in learning clusters. For	
223	M. lagerheimii and M. violaceo-irregulare, genotypes were considered as hybrids if they had	
224	a membership proportion q above 0.2 and below 0.8 in the two learning clusters	
225	corresponding to <i>M. lagerheimii</i> and <i>M. violaceo-irregulare</i> for differentiating these two	
226	species. Because clustering with STRUCTURE can be strongly influenced by unbalanced	
227	sample sizes (Kalinowski 2011), and because sample size for <i>M. silenes-inflatae</i> was one	Field Code Changed
228	order of magnitude higher than for the other species, datasets of pure representatives were	
229	randomly resampled ten times to the size of the smallest species sample (n=7, M. violaceo-	
230	irregulare), and STRUCTURE analyses were run on each resampled dataset.	
231	In order to check the validity of the genetic subdivision retrieved from the STRUCTURE	
232	analysis, using a method not assuming outcrossing or lack of genetic disequilibrium, we also	
233	carried out a principal component analysis (PCA) on the microsatellite data using the	
234	'dudi.pca' function in the R-package ADE4 (Dray & Dufour 2007). We then used a	Field Code Changed
235	discriminant analysis of the principal components (DAPC), as implemented in the R-package	

ADEGENET (Jombart 2008; Jombart *et al.* 2010). The 30 first principal components were
retained, representing 90% of total genetic variation, as well as the six first discriminant
functions. The membership probabilities of genotypes were computed using DAPC for
numbers of clusters *K* ranging from 1 to 25.

240

241 Host specificity, geographical and climatic distributions

242 To assess host specificity, we examined the differential occurrence of the pathogen species and clusters on S. vulgaris and S. uniflora. We also plotted the geographical 243 244 distribution of the Microbotryum species and their genetic clusters to assess their degree of 245 allopatry. Because there was large variance in sample size at each site, with sites often having 246 only one individual sample, we used analyses based on presence and absence of a species or 247 cluster at a site. Analyses based on raw numbers or relative frequencies often violated model 248 assumptions. The associations between host species and pathogen species or genetic clusters 249 were tested using the 'CATMOD' procedure in the SAS Statistical Computing Environment 250 (SAS Institute Inc. 2013). Associations between species or genetic clusters and geographic 251 regions were tested using Fisher's exact tests with the SAS procedure 'FREQ' due to small 252 sample size for some categories.

To assess environmental effects, we compared the elevational distributions of the *Microbotryum* species, and of the genetic clusters within *M. silenes-inflatae* and *M. lagerheimii.* In addition to elevation, we incorporated in our analysis a suite of bioclimatic variables for each of the sampling locations in order to assess whether components of climatic variation could help explain the observed distributions. Nineteen bioclimatic variables (Supplementary Table S2) were obtained from the WorldClim database (means across years Field Code Changed Field Code Changed

259	1950 – 2000) recorded	from a network of weather sta	ations and interpolated over a 30-arc
	/		*

- second resolution grid (<u>www.worldclim.org</u>) (Hijmans et al. 2005). For each sampling site,
- 261 values of the 19 variables were extracted using nearest-neighbor interpolation with the Spatial
- 262 Analyst Tool in ArcMap 9.2 software (ESRI (Environmental Systems Resource Institute)
- 263 2009). We used a principal components analysis to analyze and reduce the variation to three 264 major axes using the SAS procedure PRINCOMP (based on the correlation matrix). We then 265 carried out a logistic regression to examine the relationship between species presence and the 266 first three components generated by the PCA, using the 'glm' function in the R *stats* package 267 with a quasi-binomial link.

268

269 **Results**

270 Species identification, species distributions and population subdivision

271 The STRUCTURE analysis on the whole dataset, modeling K=2 to 15 clusters, revealed 272 several levels of strong genetic differentiation, with well-defined *Microbotryum* clusters 273 appearing up to K=5 (i.e., clusters encompassing multiple genotypes with membership 274 coefficients near the maximum; Figure 2 and Supplementary Figure S3). The genetic clusters 275 were overall broadly distributed among sampled regions, although a few clusters appeared 276 geographically more restricted (Figure 2 and Supplementary Figure S3). Some genetic 277 differentiation was observed among samples collected on S. uniflora and S. vulgaris, with 278 some genetic clusters being specific to either host species, but also with other genetic clusters 279 occurring on both host species.

In order to assign the genetic clusters to the three previously described *Microbotryum* species, we sequenced ITS in 27 randomly chosen strains and built a maximum likelihood Field Code Changed

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282	tree, also including published ITS sequences from <i>M. silenes-inflatae</i> , <i>M. lagerheimii</i> , <i>M.</i>			
283	violaceo-irregulare and other Microbotryum species (Supplementary Figure S4: Genbank			
284	accession numbers MH491551-MH491577). Out of the 20 sequences in the fully supported			
285	clade containing the published M. silenes-inflatae ITS sequences, 19 were genotypes assigned			
286	to the largest STRUCTURE cluster in analyses assuming $K=2$ clusters (pink cluster;			
287	Supplementary Figure S4), indicating that this cluster corresponds to <i>M. silenes-inflatae</i> .			
288	Analyses at K>2 only further subdivided M. silenes-inflatae into several clusters (Figure 2).			
289	The ITS sequences falling in the fully supported clades containing the published M.			
290	<i>lagerheimii</i> ($n=5$) and <i>M. violaceo-irregulare</i> ($n=2$) sequences all belonged to genotypes in			
291	the second (blue) cluster of the $K=2$ STRUCTURE model (Supplementary Figure S4).			
292	The log-likelihood plateaued at $K>5$ with only a single clustering solution			
293	(Supplementary Figure S5A); the additional clusters appearing at K values above 5 mainly			
294	introduced additional subdivision within M. silenes-inflatae, produced many genotypes with			
295	intermediate assignment probability and never clearly separated the genotypes assigned to M .			
296	lagerheimii and M. violaceo-irregulare using ITS into distinct clusters. K=5 was therefore			
297	considered as the most relevant K value, with the blue cluster corresponding to both M .			
298	lagerheimii and M. violaceo-irregulare, and the four other clusters (green, orange, pink and			
299	yellow) to different lineages within <i>M. silenes-inflatae</i> .			
300	The species M. lagerheimii and M. violaceo-irregulare could not be separated into			
301	distinct clusters at any K values using the full dataset, despite their large phylogenetic			
302	distance (Figure 1). The lack of ability to distinguish these species is likely due to their small			
303	sample sizes compared to that collected for <i>M. silenes-inflatae</i> . Such difficulties in			
304	recovering species differentiation in cases of unbalanced sample sizes have been shown by			
305	simulations (Neophytou 2014). We therefore ran STRUCTURE on a sub-dataset with the	Fie		

306	genotypes from <i>M. lagerheimii</i> and <i>M. violaceo-irregulare</i> only. At K=3 in this sub-dataset
307	(Figure 3; Supplementary Figures S5B and S6), one cluster (brown) corresponded to M.
308	violaceo-irregulare genotypes while the other two clusters (dark and light blue) represented
309	M. lagerheimii genotypes, mainly separating isolates collected on S. uniflora in the UK and
310	Netherlands (light blue) versus genotypes collected on S. vulgaris in the Pyrenees and Alps
311	(dark blue). Spore morphology (color and ornamentation) of 15 individuals, chosen at random
312	within each species, provided further evidence for cluster assignment to species: all five
313	strains assigned to <i>M. silenes-inflatae</i> had dark purple spores with reticulate ornamentation,
314	all five strains assigned to M. lagerheimii had light purple spores with reticulate
315	ornamentation, and all strains assigned to M. violaceo-irregulare had dark purple spores with
316	verrucose ornamentation (Supplementary Table S3).
317	The principal component analysis (PCA) on the full dataset separated the three
318	species, with M. lagerheimii and M. silenes-inflatae in separate corners of the parameter space
319	and <i>M. violaceo-irregulare</i> appearing intermediate between them (Figure 4A). The ITS
320	sequence variants particular to M. silenes-inflatae were not confined to any of its four genetic
321	clusters (Supplementary Figure S3; Figures 4A and 4B), providing evidence that they
322	constitute within-species genetic subdivision. The M. lagerheimii and M. violaceo-irregulare
323	species and two M. silenes-inflatae clusters were found on both S. vulgaris and S. uniflora,
324	while the two other <i>M. silenes-inflatae</i> clusters occurred only on <i>S. vulgaris</i> (Figure 2). A
325	PCA run on the sub-dataset of the two less frequent Microbotryum species also discriminated
326	them well (Figures 4C and 4D).
327	Clustering using DAPC supported the clustering patterns found in STRUCTURE
328	analyses (Supplementary Figure S7 and Figure 2). The DAPC Bayesian information criterion

329 reached a minimum value at *K*=28 (Supplementary Figure S8). However, visual inspection of

membership probability patterns across K values indicated that DAPC and STRUCTURE
identified the same five main clusters (Supplementary Figure S7 and Figure 2). The DAPC on
the full dataset could not distinguish *M. lagerheimii* and *M. violaceo-irregulare* into different
clusters either, while the DAPC on the genotypes from these two species separately produced
a pattern of membership proportions largely similar to the one obtained with STRUCTURE
(Supplementary Figure S9).

- 336
- 337 *Hybridization and admixture checking*

338 We performed assignment tests using the option in STRUCTURE that uses pure 339 reference genotypes as learning samples to determine whether individuals with intermediate 340 assignment probabilities could simply be genotypes with low assignment power instead of 341 hybrids or admixed genotypes. Within M. silenes-inflatae, we identified 64, 40, 75 and 79 342 genotypes representatives of the yellow, orange, pink and green clusters, respectively, that we 343 used as learning samples. In assignment tests for within-species admixture, all individuals 344 with intermediate assignment probabilities had comparable q values in multiple learning 345 samples (q values ranging from 0.193 to 0.349, i.e. approximately one divided by the number 346 of clusters), suggesting that all genotypes with intermediate assignment probabilities could be 347 genotypes with low assignment power instead of genuine admixed individuals 348 (Supplementary Table S4). Within *M. lagerheimii*, we identified 24 and 12 pure 349 representatives of the dark and light blue clusters, respectively; the only individual with 350 intermediate assignment probabilities in the *M. lagerheimii* full dataset (q=0.465 in the blue 351 cluster; q=0.535 in the light blue cluster) had high membership in the blue cluster (q=0.731) 352 in the *M. violaceo-irregulare/M. lagerheimii* subset analysis.

353	We identified 7, 38 and 400 pure genotype representatives of <i>M. violaceo-irregulare</i> ,
354	M. lagerheimii and M. silenes-inflatae, respectively, that we used as learning samples. In
355	assignment tests for inter-specific hybrid identification, all genotypes had substantial
356	membership in all learning samples considered. Therefore, no genotype could be identified as
357	a high-confidence hybrid, neither between species nor between lineages within M. silenes-
358	inflatae (Supplementary Table S5), and they may instead represent genotypes with low
359	assignment power. This inference was supported by the heterozygosity values of the
360	genotypes with intermediate assignment probabilities, whose mean ($H_0=0.18$) was not higher
361	than that in other samples (Table 1), in contrast to what would be expected for hybrids.
362	
262	Hast anoith it. account is all and alimetic distributions

363 *Host specificity, geographical and climatic distributions*

364 Pathogen species and several clusters within M. silenes-inflatae and M. lagerheimii were 365 differentially distributed among host species. Because pathogens were sampled without prior 366 knowledge of their genotype, their differential distribution was not due to sampling bias. The 367 rarest species, M. violaceo-irregulare, was found only on S. vulgaris in the western and 368 central Alps (Figure 5H and Supplementary Figure S10). The two other species, M. 369 lagerheimii and M. silenes-inflatae, were both found in the Alps, the Pyrenees and the UK 370 Figure 5H and Supplementary Figure S10). However, the two genetic clusters within M. 371 lagerheimii were well-separated both geographically and with regard to host species, with the 372 light blue cluster occurring only on S. uniflora in the UK and the dark blue cluster only on S. 373 vulgaris in the Alps and the Pyrenees (Figure 5, F and G). While all Microbotryum species 374 were found on S. vulgaris, M. silenes-inflatae was found in 90% of S. vulgaris populations 375 but just 50% of S. uniflora populations, the difference in frequency being significant (Fisher's 376 exact test, p = 0.004). Clusters within *M. silenes-inflatae* had largely overlapping

distributions on S. vulgaris, except the pink cluster which occurred only in the Alps and the
Pyrenees (Figure 5 A-E). The green cluster of <i>M. silenes-inflatae</i> was found commonly on <i>S.</i>
uniflora in the UK (Fig 5 B), while only single records of the yellow and orange clusters were
identified there. This bias towards occurrence in S. vulgaris populations was statistically
significant only for the yellow cluster (Fisher's exact test, $p = 0.025$). In the single site where
host plants were identified as S. vulgaris var. prostrata (Site 22), the high elevation form of
the host, all strains were assigned to M. violaceo-irregulare.
Different species and clusters within species often occurred in the same sampling
site. Among the 45 sites with more than one assigned sample, nine displayed sympatry of
Microbotryum species (M. silenes-inflatae and either M. lagerheimii or M.violaceo-
irregulare). Within M. silenes-inflatae, 11 sampling sites out of 32 displayed sympatry among
at least two genetic clusters (Supplementary Figure S10). Genotypes with intermediate
assignment probabilities in STRUCTURE were not more frequently found in sites with co-
occurring clusters ($\chi^2 = 0.36$, df = 1, $p = 0.54$), supporting the interpretation that they
represent genotypes with low assignment power rather than hybrid individuals. Moreover, the
genotypes with intermediate assignment probabilities in different species were present as
frequently in sites where only a single species was found as in sites where two or more
species were found ($\chi^2 = 1.17$, df = 1, $p = 0.26$). Genotypes with intermediate assignment
probabilities in different <i>M. silenes-inflatae</i> clusters were in 33 out of 40 (82%) sites with a
single cluster, and the genotypes with intermediate assignment probabilities in different M.
lagerheimii clusters were in 13 out of 17 (76%) sites with a single cluster (grey genotypes in
Supplementary Figure S10).

Where our sampling was most extensive (Pyrenees, western Alps and central Alps),we investigated whether the distributions of the species (51 sites) and of clusters within *M*.

401	silenes-inflatae (37 sites with unambiguous assignments) were associated with local	
402	geography, elevation and/or climate. The occurrence of M. silenes-inflatae did not differ	
403	between the Pyrenees, western Alps or central Alps (Fisher's exact test, $p = 0.168$). The	
404	occurrence of <i>M. violaceo-irregulare</i> and <i>M. lagerheimii</i> differed significantly among regions	
405	(Fisher's exact tests, $p = 0.031$ and $p = 0.001$ respectively), with the former being absent in	
406	the Pyrenees and the latter absent in the Western Alps (Figure 5 and Supplementary Figure	
407	10). Within M. silenes-inflatae, the green cluster showed significant differences in occurrence	
408	among regions (Fisher's exact test, $p = 0.025$), being more common in the western Alps. The	
409	analysis approached significance for the orange cluster (Fisher's exact test, $p = 0.052$) which	
410	was more common in the Pyrenees (Figure 5 and Supplementary Figure 10). The other two	
411	clusters showed no significant differences in occurrence among regions (Fisher's exact tests,	
412	p = 0.634 for pink and $p = 0.408$ for yellow).	
413	The three species occurred at significantly different elevations (Figure 6; ANOVA,	
413 414	The three species occurred at significantly different elevations (Figure 6; ANOVA, $df = 2$, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was	
413414415	The three species occurred at significantly different elevations (Figure 6; ANOVA, $df = 2$, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman 1974) and a	Field Code Changed
413414415416	The three species occurred at significantly different elevations (Figure 6; ANOVA, $df = 2$, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman 1974) and a non-parametric test also indicated significant differences in elevation among species	Field Code Changed
 413 414 415 416 417 	The three species occurred at significantly different elevations (Figure 6; ANOVA, df = 2, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman 1974) and a non-parametric test also indicated significant differences in elevation among species (Kruskall-Wallis non-parametric test, df = 2, χ^2 = 16.7, <i>p</i> = 0.002). The species <i>M. violaceo</i> -	Field Code Changed
 413 414 415 416 417 418 	The three species occurred at significantly different elevations (Figure 6; ANOVA, df = 2, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman 1974) and a non-parametric test also indicated significant differences in elevation among species (Kruskall-Wallis non-parametric test, df = 2, χ^2 = 16.7, <i>p</i> = 0.002). The species <i>M. violaceo-</i> <i>irregulare</i> was found only at high elevations, <i>M. lagerheimii</i> at the lowest elevations, while	Field Code Changed
 413 414 415 416 417 418 419 	The three species occurred at significantly different elevations (Figure 6; ANOVA, df = 2, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman 1974) and a non-parametric test also indicated significant differences in elevation among species (Kruskall-Wallis non-parametric test, df = 2, χ^2 = 16.7, <i>p</i> = 0.002). The species <i>M. violaceo-</i> <i>irregulare</i> was found only at high elevations, <i>M. lagerheimii</i> at the lowest elevations, while <i>M. silenes-inflatae</i> was found across a greater range of elevations (Figure 6). The two clusters	Field Code Changed
 413 414 415 416 417 418 419 420 	The three species occurred at significantly different elevations (Figure 6; ANOVA, df = 2, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman 1974) and a non-parametric test also indicated significant differences in elevation among species (Kruskall-Wallis non-parametric test, df = 2, χ^2 = 16.7, <i>p</i> = 0.002). The species <i>M. violaceo-</i> <i>irregulare</i> was found only at high elevations, <i>M. lagerheimii</i> at the lowest elevations, while <i>M. silenes-inflatae</i> was found across a greater range of elevations (Figure 6). The two clusters within <i>M. lagerheimii</i> were also found at significantly different elevations (Figure 6;	Field Code Changed
 413 414 415 416 417 418 419 420 421 	The three species occurred at significantly different elevations (Figure 6; ANOVA, df = 2, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman 1974) and a non-parametric test also indicated significant differences in elevation among species (Kruskall-Wallis non-parametric test, df = 2, χ^2 = 16.7, <i>p</i> = 0.002). The species <i>M. violaceo-</i> <i>irregulare</i> was found only at high elevations, <i>M. lagerheimii</i> at the lowest elevations, while <i>M. silenes-inflatae</i> was found across a greater range of elevations (Figure 6). The two clusters within <i>M. lagerheimii</i> were also found at significantly different elevations (Figure 6; ANOVA, df = 1, Sum of squares = 21620792, <i>F</i> -ratio = 167.9, <i>p</i> < 0.0001; Kruskall-Wallis	Field Code Changed
 413 414 415 416 417 418 419 420 421 422 	The three species occurred at significantly different elevations (Figure 6; ANOVA, df = 2, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman 1974) and a non-parametric test also indicated significant differences in elevation among species (Kruskall-Wallis non-parametric test, df = 2, χ^2 = 16.7, <i>p</i> = 0.002). The species <i>M. violaceo-</i> <i>irregulare</i> was found only at high elevations, <i>M. lagerheimii</i> at the lowest elevations, while <i>M. silenes-inflatae</i> was found across a greater range of elevations (Figure 6). The two clusters within <i>M. lagerheimii</i> were also found at significantly different elevations (Figure 6; ANOVA, df = 1, Sum of squares = 21620792, <i>F</i> -ratio = 167.9, <i>p</i> < 0.0001; Kruskall-Wallis test, df = 1, χ^2 = 24.1, <i>p</i> < 0.0001), as well as the four clusters within <i>M. silenes-inflatae</i>	Field Code Changed
 413 414 415 416 417 418 419 420 421 422 423 	The three species occurred at significantly different elevations (Figure 6; ANOVA, df = 2, Sum of squares = 4486478, <i>F</i> -ratio = 8.7, <i>p</i> = 0.0002). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman 1974) and a non-parametric test also indicated significant differences in elevation among species (Kruskall-Wallis non-parametric test, df = 2, χ^2 = 16.7, <i>p</i> = 0.002). The species <i>M</i> , <i>violaceo-</i> <i>irregulare</i> was found only at high elevations, <i>M</i> . <i>lagerheimii</i> at the lowest elevations, while <i>M</i> . <i>silenes-inflatae</i> was found across a greater range of elevations (Figure 6). The two clusters within <i>M</i> . <i>lagerheimii</i> were also found at significantly different elevations (Figure 6; ANOVA, df = 1, Sum of squares = 21620792, <i>F</i> -ratio = 167.9, <i>p</i> < 0.0001; Kruskall-Wallis test, df = 1, χ^2 = 24.1, <i>p</i> < 0.0001), as well as the four clusters within <i>M</i> . <i>silenes-inflatae</i> (Figure 6; ANOVA, df = 3, Sum of squares = 10011015, <i>F</i> -ratio = 24.3, <i>p</i> < 0.0001; Kruskall-	Field Code Changed

425	its two clusters was associated with the differences in host species, as described above, i.e.
426	coastal S. uniflora in the UK versus broad elevational distribution of S. vulgaris elsewhere.
427	The Figure 2 also illustrates the differences in elevation between species and clusters within
428	each geographical region: clusters remain well separated when genotypes are sorted by
429	elevation, in particular at K=5.

430 Differences in Microbotryum distributions among both elevations and geographic 431 regions could indicate impacts of climate. To test whether species or clusters were associated 432 with different elevations and/or climates among the most heavily sampled regions (S. vulgaris 433 populations in the Pyrenees, western Alps, and central Alps), we carried out a two-step 434 analysis to first reduce climatic variables to uncorrelated principal components (PCs), and 435 then test for the relative ability of climate, elevation and geographical location to explain 436 species and cluster distributions. For both species and cluster datasets, the 19 bioclimatic 437 variables (Supplementary Table S2) were reduced to three axes using PCA. All PC axis 438 loadings were very similar for both species and cluster analyses (Supplementary Tables S2 439 and S6). Axis 1 (explaining 63 and 66% of the variation for species and cluster, respectively) 440 represented decreasing temperature and increasing precipitation; axis 2 (19.3 and 19.4% of 441 the variation) was weighted heavily on increased seasonality of precipitation and more 442 moderate interactions between temperature and rainfall; axis 3 (8.3 and 7.6% of the variation) 443 was weighted positively on more mild winter temperatures and lower temperature seasonality 444 (Supplementary Tables S2 and S6). 445 Due to the high degree of collinearity among variables, effects of climate, elevation

and geographic location were then analyzed in a second PCA that incorporated latitude,

447 longitude, elevation, as well as scores for the first three principal components of the

448 bioclimatic analysis. Three major axes summarized the variation, similarly for species and

449	cluster analyses (Supplementary Table S7). The first axis (accounting for 66.3 and 48.1% of
450	the variation in the species and cluster analyses, respectively) was weighted heavily on
451	geographical position (latitude and longitude) and precipitation seasonality (PC axis 2 in the
452	bioclimate analysis). The second axis (33.0 and 32.6% of the variation) was weighted heavily
453	on elevation and the three climatic PC axes (especially axis 1 for temperature and rainfall)
454	and was almost completely independent of location. Axis 3 (17.0 and 17.1%) was relatively
455	independent of both location and elevation, but weighted strongly on climatic axes
456	representing fewer extremes of cold and dry periods.
457	The logistic regression analysis on species (Supplementary Table S8) showed a
458	significant negative association between the occurrence of <i>M. lagerheimii</i> and location and
459	seasonality in precipitation (PC axis 1), suggesting that either climate, location, or both
460	contributed to explain this species' distribution. For the logistic regression analysis on M.
461	silenes-inflatae genetic clusters (Supplementary Table S8), occurrence of the orange cluster
462	was significantly associated with PC axis 2 (reflecting increased elevation and its
463	characteristic colder and wetter climate), and the yellow cluster approached significant
464	negative association with PC3 (indicating greater chance of occurrence at sites with more
465	extreme cold and dry periods, independent of geography or elevation) (Supplementary Table
466	S8).

467

468 Levels of genetic variation and mating system

Summary statistics were computed for clusters identified at K=5 in analyses of the full dataset
for *M. silenes-inflatae*, and at K=3 in analyses of the *M. lagerheimii-M. violaceo-irregulare*dataset (Table 1). The genotypes with intermediate assignment probabilities in STRUCTURE
outputs were excluded. Low heterozygosity levels were found in all three species, leading to

high levels of inferred selfing rates (mean of 0.67, with range of 0.33-0.92, Table 1). Higher
selfing rates were estimated in *M. violaceo-irregulare* (0.92) and *M. lagerheimii* (0.80-0.86)
than in *M. silenes-inflatae* (0.33-0.73).

- 476
- 477
- 478 Discussion

479 This study aimed to understand the coexistence of three distant species of anther-smut fungi, 480 all castrating pathogens of the sister host species, Silene vulgaris and S. uniflora. The three 481 recognized *Microbotryum* species that are specific to these hosts were found in our sampling, 482 although with contrasting frequencies: M. silenes-inflatae was by far the most prevalent, 483 followed by *M. lagerheimii* and then *M. violaceo-irregulare*. These three phylogenetically 484 distant species, previously distinguished based on morphological characters, were 485 unambiguously identified based on microsatellite markers complemented with ITS sequences 486 and spore morphology. We had to analyze a restricted microsatellite dataset to discriminate 487 between M. lagerheimii and M. violaceo-irregulare, likely because of their low abundances 488 compared to *M. silenes-inflatae* (Neophytou 2014). Despite co-occurring in the same host 489 species and sites, no high-confidence inter-specific hybrids were identified between the 490 pathogen lineages, in agreement with the strong post-zygotic isolation generally found among 491 distant Microbotryum species (de Vienne et al. 2009b; Le Gac et al. 2007b; Petit et al. 2017). 492 However, no crossing studies have experimentally investigated post-zygotic isolation among 493 these particular *Microbotryum* species, and we cannot exclude the possibility that our 494 microsatellite markers lacked power to identify hybrids with sufficient confidence, in 495 particular later-generation backcrossed genotypes.

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496 We detected genetic subdivision within species, with two clusters in *M. lagerheimii* 497 and four clusters in *M. silenes-inflatae*. Given their weak genetic differentiation (as shown by 498 ITS sequences and distribution in the PCA), the genetic clusters within M. silenes-inflatae 499 likely do not represent further cryptic species. Within M. lagerheimii, the genetic subdivision 500 corresponded to separation in geography, host and environmental conditions and it remains to 501 be determined if these might be cryptic species. *Silene vulgaris* and *S. uniflora* were allopatric 502 during the last glaciation (Prentice et al. 2011), with S. uniflora likely persisting in northern 503 refugia from the Baltic/Scandinavian region and S. vulgaris in southern European refugia. 504 This suggests that the differentiation between the two M. lagerheimii clusters found on S. 505 vulgaris and S. uniflora, respectively, may result from past separation in disjunct glacial 506 refugia with contrasting climates rather than host adaptation.

507 Subdivisions within *M. silenes-inflatae* had less obvious biological interpretation as 508 the clusters were broadly overlapping in geography. Indeed, all four M. silenes-inflatae 509 clusters were found in the Alps, the UK and the Pyrenees on S. vulgaris. However, only the 510 green cluster was found regularly on S. uniflora in the UK. Additionally, this green cluster 511 was more frequent in the western Alps than in the Pyrenees, while the opposite was found for 512 the orange cluster, suggesting possible allopatric or host-related differentiation of the M. 513 silenes-inflatae genetic clusters. The geographic co-occurrence of the four M. silenes-inflatae 514 clusters in the Alps may result from recent movements and secondary contact. Footprints of a 515 glacial refugia in Europe have been found in M. lychnidis-dioicae parasitizing S. latifolia 516 (Badouin et al. 2017; Feurtey et al. 2016; Gladieux et al. 2011; Vercken et al. 2010) and M. 517 silenes-acaulis parasitizing S. acaulis (Bueker et al. 2016). The genetic clusters within M. 518 silenes-inflatae have not homogenised despite sympatry, which is likely due to the selfing 519 mating system of these fungi. All three *Microbotryum* species indeed had high estimated

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520 selfing levels, being highly homozygous, especially M. violaceo-irregulare and M. 521 lagerheimii, as has been found for other Microbotryum species previously studied (e.g., those 522 on S. latifolia, S. dioica, S. acaulis, Saponaria officinalis and Dianthus spp.; Bucheli et al. 523 2001; Bueker et al. 2016; Delmotte et al. 1999; Fortuna et al. 2016; Giraud 2004; Gladieux et 524 al. 2011; Hood & Antonovics 2000; Hood & Antonovics 2004; Petit et al. 2017). Such high 525 selfing rates may be the result of mating among sporidia from one diploid individual (a spore 526 load from a single infected flower). Additionally, it may be the result of mating between 527 sporidia from single teliospores, a process also promoted by intra-tetrad mating that is present 528 in many Microbotryum species as a result of ordered segregation of the mating-type loci 529 (Hood & Antonovics 2000; Hood et al. 2015). Interestingly, M. silenes-inflatae had lower 530 inferred selfing rates than the other species, and it would be interesting informative to 531 investigate in future studies if this is due to ecological factors, such as more frequent co-532 dispersal of spores from different fungal individuals by pollinators, or to intrinsic factors, 533 such as a lower propensity of intra-tetrad mating.

The three *Microbotryum* species had large range overlaps on *S. vulgaris*, with frequent co-occurrence within sampling sites, indicating that allopatry may not be a major factor currently allowing the persistence of these distinct pathogen species on the same host. The distribution of *M. violaceo-irregulare* and *M. lagerheimii* was nevertheless significantly different between the local regions in the Pyrenees and Alps, suggesting they may have a history of past allopatry. However, their current broad range overlap indicates that their coexistence must either be transient or promoted by niche specialization.

541 The co-existence of the three *Microbotryum* species is clearly not maintained by host 542 specialization, as all three species occurred on *S. vulgaris*. Moreover, it has been shown that 543 resistance of *S. uniflora* to *M. lagerheimii* and *M. silenes-inflatae* was significantly positively

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544	correlated among plant families (Chung et al. 2012), which indicates that the plant resistance	Field Code Changed
545	is not specific to any one Microbotryum species. This further supports the inference that host	
546	specialization on S. uniflora versus S. vulgaris is not a major factor contributing to the co-	
547	occurrence of these Microbotryum species. However, because the host was not genotyped in	Formatted: English (U.S.)
548	our study and no infection trials were carried out, we cannot be ruled out that the influence of	Formatted: English (U.S.)
549	different host genotypes influence on Microbotryum species distribution and coexistence.	Formatted: English (U.S.)
550	The situation of the anther-smut species co-occurring on S. vulgaris is in some	Formatted: Font: Italic
551	respects similar to that of anther-smut disease on the genus Dianthus where multiple	
552	Microbotryum species co-occur in the same sampling sites on multiple Dianthus species (Petit	Field Code Changed
553	et al. 2017). In this case, however, the Microbotryum species on Dianthus hosts are closely	
554	related phylogenetically and hybrids, likely facilitated by the proximal relationships between	
555	the Microbotryum species, are frequently found (Petit et al. 2017). The situation on Dianthus	Field Code Changed
556	spp. and the S. vulgaris/S. uniflora sister pair contrasts with the pattern found in all	
557	Caryophyllaceae studied to date, where each host species has been found to harbor only one	
558	endemic Microbotryum species in natural populations (Le Gac et al. 2007a; Refrégier et al.	Field Code Changed
559	2010). Silene vulgaris also occasionally harbors Microbotryum species as a result of transient	Field Code Changed
560	cross-species disease transmission from other Silene hosts, such as S. latifolia and S. dioica	
561	(Antonovics et al. 2002; Hood et al. 2003). It is unclear if this is due to a particular	Field Code Changed
562	susceptibility of S. vulgaris to anther smut or to its very wide geographic and environmental	Field Code Changed
563	ranges that allow contact with the disease on many other Silene hosts.	
564	The three anther-smut species on S. vulgaris occurred at significantly different	
565	elevations, suggesting that they may occupy different ecological niches in terms of abiotic	
566	conditions. This was supported by some association of M. lagerheimiii occurrence with	
567	climatic variables related to temperature and precipitation. Within M. silenes-inflatae, the	

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568	relative frequency of some of the genetic clusters also varied with elevation and climatic		
569	factors. These effects may reflect environmental tolerances of the fungal species and clusters,		
570	or ecotypic differences among host populations. It is well known that the host S. vulgaris has		
571	different ecotypes adapted to contrasting elevations (Marsden-Jones & Turrill 1957), and	[Field Code Changed
572	these may have different anther-smut specificities. In the present study, just one host		
573	population was identified as S. vulgaris var. prostrata, the high elevation form of the host		
574	(Site 22, in which all infections were caused by <i>M. violaceo-irregulare</i>). However, a previous		
575	study of Microbotryum on S. vulgaris in the small region of the south-eastern French Alps		
576	where Site 22 is located showed that none of the three fungal species were confined to any		
577	specific host ecotype (Abbate & Antonovics 2014). Furthermore, phenotypic traits including	[Field Code Changed
578	those that mark differences between the ecotypes change gradually over elevation, and the		
579	differentiation between populations is relative to their geographic distance (Berardi et al.	[Field Code Changed
580	2016). The much wider sampling in the present study confirms that <i>Microbotryum</i> on <i>S</i> .		
581	vulgaris is largely confined to higher elevations (79% of the samples, 88% of the sites, were		
582	found above 1300m), even though populations of the host plant are abundant at lower		
583	elevations across its range. More generally, elevation and abiotic conditions have been shown		
584	to impact the presence of fungal pathogens in other systems (Cordier et al. 2012; Desprez-	< [Field Code Changed
585	Loustau et al. 1998; Enjalbert et al. 2005; Gange et al. 2007; Mboup et al. 2012; Vacher et al.	 	Field Code Changed Field Code Changed
586	2008). This study thus begs important questions for future work with Microbotryum		Field Code Changed
587	pathogens investigating how the genetics of adaptation to elevation in fungi interacts with the	`\	Field Code Changed
588	adaptation to, or of, host plants. For example, one question is whether Microbotryum is rare at		
589	low elevations because the fungus cannot adapt to low-elevation climatic conditions, or		

590 because the costs of resistance in the host to the pathogen decline under those conditions.

591 In conclusion, using a molecular ecology approach we showed that we can assign 592 samples to the three *Microbotryum* anther-smut pathogens parasitizing *S. vulgaris* and *S.* 593 uniflora using microsatellite markers. Furthermore, we revealed the existence of four genetic 594 clusters within M. silenes-inflatae. We also showed that the different species and genetic 595 clusters had large range overlaps and were all found on S. vulgaris. These findings suggest 596 that the coexistence of multiple species and genetic clusters of *Microbotryum* pathogens on S. 597 vulgaris and S. uniflora cannot be explained by differences in host or geographic distributions 598 alone, although recent secondary contacts cannot be entirely excluded. Instead, we found that 599 the different species and genetic clusters appeared to occupy different ecological niches in 600 terms of abiotic conditions, in particular elevation, temperature and precipitation. This study 601 highlights the importance of including not only species differences and geography but also 602 abiotic conditions when considering the distribution and co-existence of pathogenic fungi on 603 similar hosts.

604

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622 Data accessibility

- 623 The genotypes and elevation data are available on Dryad : doi:10.5061/dryad.627fm05.
- 624 GenBank accession numbers for ITS sequences: MH491551-MH491577.
- 625 The genotypes and elevation data will be submitted to Dryad upon acceptance.

626 Author contributions

- TG, JAn and MEH designed the study; AS, JAb and DdV genotyped samples; PG, JAn, JAb
- and TG analysed data; PG, JAb and TG wrote the manuscript with contributions by all
- authors.
- 630

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- 816

Species/Cluster	N	H_{d}	$\mathbf{A}_{\mathbf{r}}$	H_O/H_E	$F_{IS} \\$	S
Microbotryum st	ilenes	s-inflata	е			
Green	79	0.346	1.72	0.24/0.33	0.355	0.52
Yellow	64	0.468	2.01	0.18/0.44	0.577	0.73
Pink	75	0.243	1.49	0.20/0.23	0.199	0.33
Orange	40	0.178	1.35	0.10/0.17	0.387	0.56
Microbotryum violaceo-irregulare						
Species-wide	7	0.594	2.07	0.08/0.44	0.852	0.92
Microbotryum lagerheimii						
Blue	24	0.492	2.03	0.12/0.45	0.760	0.86
Light blue	12	0.491	1.98	0.13/0.43	0.662	0.80

818 Table 1. Summary statistics of genetic variation and estimates of selfing rates in the identified
 819 *Microbotryum* species and clusters.

N, number of pure representatives of the species/clusters, as determined using Bayesian
 clustering analyses; H_d, gene diversity; A_r, allelic richness, corrected for sample size
 using rarefaction (standardized sample size of two diploid individuals); H_o/H_E,

- 823 observed/expected heterozygosity; F_{IS} , fixation index; s, selfing rate estimated as 824 $2F_{IS}/(1+F_{IS})$.
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829	Figure Legends
830	Figure 1. Maximum-likelihood tree showing the phylogenetic placement of Microbotryum
831	lagerheimii (HQ832090), M. violaceo-irregulare (AY588104), and M. silenes-inflatae
832	(JN223404), all parasitizing Silene vulgaris, among other Microbotryum species, based on
833	internal transcribed spacer (ITS) sequences. Microbotryum from Polygonum bistorta was
834	used as an outgroup. Bootstraps are indicated. Pictures show differences in teliospore color
835	between the dark purple of <i>M. silenes-inflatae</i> and <i>M. violaceo-irregulare</i> versus the lighter
836	lavender of <i>M. lagerheimii</i> , as well as differences in teliospore ornamentation (verrucose in
837	M. violaceo-irregulare, fully reticulated in M. silenes-inflatae and M. lagerheimii) under
838	1000x light-microscope magnification.
839	Figure 2. Proportions of ancestry in K (from 2 to 10) clusters of Microbotryum spp.
840	genotypes inferred with the STRUCTURE program. Each genotype is represented by a
841	vertical bar, partitioned into K segments representing the amount of ancestry of its genome in
842	K clusters. When several clustering solutions ("modes") were found within replicate runs,
843	only the major mode is shown with its corresponding proportion of runs. IS: Iceland, NL:
844	Netherlands, UK: United Kingdom, CH: Switzerland, IT: Italy, F: France. For each region,
845	genotypes are ordered by sampling elevation (represented at bottom). See Supplementary
846	Figure S3 for a sorting by membership coefficient.
847	Figure 3. Proportions of ancestry in <i>K</i> (from 2 to 5) clusters of the <i>Microbotryum violaceo</i> -
848	irregulare and M. lagerheimii genotypes inferred with the STRUCTURE program, using a
849	sub-dataset excluding <i>M. silenes-inflatae</i> . In the sub-dataset we kept only the genotypes
850	assigned to the blue cluster at $K=5$ in the output of the Structure analysis on the whole dataset
851	(Figure 4). A single clustering solution ("mode") was found among replicate runs. Each

- 852 genotype is represented by a vertical bar, partitioned into *K* segments representing the amount

853	of ancestry of its genome in K clusters. NL: Netherlands, UK: United Kingdom, CH:
854	Switzerland, IT: Italy, F: France. For each region, samples are ordered by sampling elevation
855	(represented at bottom; see Figure S5 for a sorting by assignment coefficient within regions).
856	Figure 4. Principal component analysis (PCA) on multilocus microsatellite genotypes for the
857	dataset including the three Microbotryum species (A: principal component 1 vs principal
858	component 2; B: principal component 1 vs principal component 3) for the sub-dataset
859	including only M. lagerheimii and M. violaceo-irregulare (C: principal component 1 vs
860	principal component 2; D: principal component 1 vs principal component 3). Scatterplots for
861	the first three principal components are shown using a colour labelling of genotypes defined
862	according to the assignment of multilocus genotypes to three species and six clusters within
863	species using Bayesian clustering analyses. Colors indicate clusters as in Figures 2 and 3.
864	Black outlines indicate the samples for which the ITS region has been sequenced.
865	Figure 5. Distribution maps of the different genetic clusters and species of anther-smut
866	Microbotryum fungi on Silene vulgaris and S. uniflora, and maps of their co-occurrence. Pie
867	charts are proportional to the number of samples. Colors correspond to those used in other
868	figures.
869	Figure 6. Violin plots showing elevational distribution of species and clusters within species.
870	All samples belonging to Microbotryum silenes-inflatae at K=5 (including all genotypes with
871	low assignment power to the green, orange, pink and yellow M. silenes-inflatae clusters) are
872	included in the leftmost M. silenes-inflatae column. Otherwise, only samples with high-

- 873 confidence cluster assignment are shown. Samples belonging to *M. lagerheimii* and *M.*
- 874 *violaceo-irregulare* were determined using the K=3 output from the sub-dataset excluding *M*.
 875 *silenes-inflatae*.



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Silene hosts



Molecular Ecology

Regions

Microbotryum species





M. silenes-inflatae



Silene hostsecular Egolferbbotryant species







Molecular Ecology

