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

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Abstract
The competitive exclusion principle postulates that different species can only coexist in sympathy if they occupy distinct ecological niches. The goal of this study was to understand the geographical distribution of three species of Microbotryum anther-smut fungi that are distantly related but infect the same host plants, the sister species Silene vulgaris and S. uniflora, in Western Europe. We used microsatellite markers to investigate pathogen distribution in relation to host specialization and ecological factors. Microbotryum violaceo-irregulare was only found on S. vulgaris at high elevations in the Alps. Microbotryum lagerheimii could be subdivided into two genetically differentiated clusters, one on S. uniflora in the UK and the second on S. vulgaris in the Alps and Pyrenees. The most abundant pathogen species, M. silenes-inflatae, could be subdivided into four genetic clusters, co-occurring in the Alps, the UK and the Pyrenees, and was found on both S. vulgaris and S. uniflora. All three fungal species had high levels of homozygosity, in agreement with the selfing mating system generally observed in anther-smut fungi. The three pathogen species and genetic clusters had large range overlaps, but occurred at sites with different elevations, temperatures and precipitation levels. The three Microbotryum species thus do not appear to be maintained by host specialization or geographic allopatry, but instead may occupy different ecological niches in terms of environmental conditions.

KEYWORDS
altitude, biogeography, endemicity, fungi, hybrid zones, Microbotryum violaceum, population structure, Silene maritima, speciation

1 INTRODUCTION

The competitive exclusion principle postulates that two species occupying the same niche in sympathy cannot coexist indefinitely.
(Gause, 1934; Hardin, 1960). In pathogens, this means that species coexisting in sympatry in association with a common host species are likely to differ in other components of their ecological niches. The coexistence of multiple pathogens on the same crop species is in fact often associated with separation by time, resource use, abiotic conditions or geographical area (Fitt, Huang, van den Bosch, & West, 2006; Giraud, Koskella, & Laine, 2017). Closely related pathogens can also specialize on either the early or the late season part of a given plant life cycle (Hamelin, Bisson, Desprez-Loustau, Fabre, & Mailleret, 2016; Mailleret, Castel, Montaray, & Hamelin, 2012). For example, two eyespot wheat pathogens, O. yallundae and O. acuformis, differ in temperature optima for growth and fungicide resistance (Fitt et al., 2006). In oilseed rape, the pathogens Leptosphaeria maculans and L. biglobosa differ in their location in stem and leaf tissues and in their maturation rates under low temperatures (Fitt et al., 2006). In malaria, variation in host resistance to different Plasmodium species has been invoked to explain pathogen coexistence (Snounou & White, 2004). Furthermore, some Plasmodium species are specialized to attack only the youngest or oldest red blood cells, leading to complex within-host dynamics during coinfection (McQueen & McKenzie, 2004a, 2004b). Pathogens with different transmission modes, for example, sexual versus nonsexual transmission, can also stably coexist (Thrall & Antonovics, 1997).

The goal of this study was to delineate and understand the coexistence of different species of anther-smut fungi on two closely related host plants, Silene vulgaris and S. uniflora. Microbotryum is a species complex of basidiomycete fungi responsible for anther-smut disease in many plants in the Caryophyllaceae. Microbotryum anther-smut fungi are obligate pathogens that sterilize their hosts. Infected plants produce fungal teliospores in place of pollen, and female structures do not mature; in dioecious or gynodioecious species, infected female plants also develop spore-bearing anthers. Within Microbotryum, there are numerous phylogenetically divergent lineages many of which have been given species status (Denchev, Giraud, & Hood, 2009; Kemler, Goker, Oberwinkler, & Begerow, 2006; Le Gac, Hood, Fournier, & Giraud, 2007). These typically show intersterility (de Vienne, Refrégier et al., 2009; Le Gac, Hood, & Giraud, 2007) and strong host specificity (de Vienne, Hood, & Giraud, 2009). There is usually only one endemic Microbotryum species per host plant species (Le Gac, Hood, Fournier, et al., 2007; Refrégier et al., 2008), although transient host shifts are not uncommon (Antonovics, Hood, & Partain, 2002; Gladieux et al., 2011; Hood, Antonovics, & Heishman, 2003).

A conspicuous exception to this pattern is the presence of three divergent Microbotryum species on S. vulgaris and its closely related sister species S. uniflora (Figure 1). These are as follows: (a) M. lagerheimii (Denchev, 2007; MvSv1 in Le Gac, Hood, Fournier et al., 2007); (b) M. silenes-inflatae (MvSv2 in Le Gac, Hood, Fournier et al., 2007); and (c) M. violaceo-irregulare (Kemler et al., 2006; Lutz, Pia Tek, Kemler, Chleibicki, & Oberwinckler, 2008). These three Microbotryum species are not particularly closely related to one another (Figure 1) and show differences in spore morphology (colour and ornamentation; Figure 1; Denchev, 2007; Gold, Giraud, & Hood, 2009; Lutz et al., 2005, 2008). Microbotryum lagerheimii has lighter spore colour, while verrucose spore ornamentation is unique to M. violaceo-irregulare (Figure 1; Lutz et al., 2005; Vánky, 1994). All three species castrate host flowers in the same way, replacing the pollen by their spores and aborting ovaries, and are sexually transmitted (the spores are dispersed by pollinators), suggesting that it is unlikely they can have distinct ecological niches within host individuals. None of the three species has ever been found to persist in a host species other than S. vulgaris or S. uniflora, indicating that they are maintained endemically on these host plants.

One explanation for coexistence of these three fungal species could be that they are allopatric, although they have been found to co-occur in the same sites (Abbate & Antonovics, 2014; Chung, Petit, Antonovics, Pedersen, & Hood, 2012; Le Gac, Hood, Fournier et al., 2007); however, sampling has been limited thus far, and it remains possible that contact zones of allopatric distributions were sampled. An alternative hypothesis is that the species display differential specialization towards S. vulgaris versus S. uniflora. Previous studies further suggest that this hypothesis is unlikely, given that both host species have been found to be parasitized in nature by at least two of the Microbotryum species also parasitizing S. vulgaris: M. lagerheimii and M. silenes-inflatae (Chung et al., 2012; Smith, Lutz, Ziegler, & Piattek, 2017).

A more plausible explanation for the species coexistence could be adaptation to different abiotic conditions. All three species affecting S. vulgaris were found almost exclusively in host populations at higher elevations in the French Alps, where overall temperatures were lower and precipitation more consistently higher than conditions experienced by lower-elevation host populations (Abbate & Antonovics, 2014). Here, we investigated the generality of this pattern with a larger and broader sampling in order to further understand their coexistence. Differentiation in disjunct refugia during the last glaciation in Europe may also have shaped differential adaptation to abiotic conditions and/or generated allopatry. Strong population differentiation has been found in other Microbotryum species that reflects footprints of persistence in glaciation refugia, for example, in M. lychnidis-dioicae parasitizing S. latifolia (Badouin et al., 2017; Furtéy et al., 2016; Gladieux et al., 2011; Vercken et al., 2010) and M. silenes-acaulis parasitizing S. acaulis (Bueker et al., 2016). High selfing rates were inferred for previously studied Microbotryum species (Bueker et al., 2016; Giraud, 2004; Gladieux et al., 2011; Hood & Antonovics, 2000, 2004), and such a closed mating system may contribute to generate and maintain strong population structure and genetic isolation between species (Gibson, Hood, & Giraud, 2012; Vercken et al., 2010).

To assess the wider geographical and elevational distributions of the three different 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: (a) had contrasting geographical or elevational distributions; (b) showed different frequencies on the two sibling host species S. vulgaris and S. uniflora; (c) co-occurred in the same
sampling sites, and if so, whether hybrids could be detected; (d) exhibited within-species geographical population subdivision; and (e) displayed high selfing rates like other Microbotryum species.

2 | MATERIALS AND METHODS

2.1 | Teliospore collection

The Microbotryum samples analysed in this study were collected across Western Europe (Supporting Information Figure S1, Table S1) from 51 sites with *S. vulgaris* (*n* = 407) and 12 sites with *S. uniflora* (*n* = 59). A sampling site was defined as a location in which samples were collected a few metres apart. Sampling was performed by numerous investigators throughout Europe and was carried out as part of general disease surveys, other field trips, or as part of more formal investigations (Chung et al., 2012; Le Gac, Hood, Fournier et al., 2007; J. Abbate & J. Antonovics, unpublished studies). Within sampling sites, a single diseased flower was collected per individual plant, stored in silica gel, and DNA from the diploid teliospores was extracted for genetic analyses. Multiple infections by different genotypes are not uncommon in *Silene-Microbotryum* systems (Bueker et al., 2016; Buono, Lopez-Villavicencio, Shykoff, Snirc, & Giraud, 2014; Chung et al., 2012; Hood, 2003; López-Villavicencio et al., 2011), but teliospores within a single flower originate from a single diploid genotype (Gold et al., 2009; López-Villavicencio et al., 2007).

Teliospores sampled from a given flower were therefore assumed to be from a particular pathogen 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.

2.2 | Microsatellite, ITS genotyping and spore morphology

DNA was extracted and genotyped using 11 microsatellite loci as described previously (SVG1, SVG2, SVG5, SVG6, SVG8, SVG15, SN2, SN5, SN11, DC2 and DC5; Giraud, 2004; Giraud et al., 2008). Microsatellite scoring was performed as described previously (Vercken et al., 2010). Analyses using GENCLONE (Arnaud-Haond & Belkhir, 2007) showed that the eleven markers were sufficient to discriminate multilocus genotypes in the data set as a plateau was reached in terms of number of genotypes detected (Supporting Information Figure S2).

In order to assign the genetic clusters from microsatellite analyses to the three *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 ITS1 and ITS4 (White, Bruns, Lee, & Taylor, 1990) and matched these to published ITS sequences from *M. silenes-inflatae*, *M. lagerheimii*, *M. violaceo-irregulare* (Kemler et al., 2006; Lutz et al., 2008). Additionally, we verified in 15 individuals spore colour (light vs. dark purple) under the binocular and spore ornamentation (i.e., verrucose vs. reticulate) under light microscopy. The samples chosen for ITS sequencing and for spore morphology assessment were picked at random within each cluster, and there was complete agreement in the species assignments between ITS, spore morphology and genetic clusters.

### 2.3 Data analyses

#### 2.3.1 ITS tree

A maximum-likelihood phylogeny of ITS region sequenced was inferred using PhyML 3.0 (Guindon et al., 2010).

#### 2.3.2 Map

The sample map (Supporting Information Figure S2) was generated using the GGMAP package in R environment (Kahle & Wickham, 2013).

#### 2.3.3 Descriptive statistics

Microsatellite variability was quantified by the unbiased gene diversity (*H*<sub>o</sub>), allelic richness (*A*<sub>r</sub>) and the fixation index (*F*<sub>IS</sub>) using FSTAT 2.9.3.2 (Goudet, 2001). Observed and unbiased expected heterozygosities (*H*<sub>o</sub> and *H*<sub>e</sub>) were computed using a custom python script. Analyses of variance (ANOVA) and Kruskal–Wallis tests were performed using JMP v7.0 (SAS Institute).

#### 2.3.4 Population subdivision

We assessed population subdivision using Bayesian clustering implemented in STRUCTURE 2.3.4 (Falush, Stephens, & Pritchard, 2003; Pritchard, Stephens, & Donnelly, 2000). The method partitions multilocus genotypes into clusters while minimizing departure from expected frequencies and linkage equilibrium among loci. For this analysis, we used a haploid setting because *Microbotryum* individuals were highly homozygous (Table 1), which could bias structure inferences based on Hardy–Weinberg expectations in a diploid setting. We conducted independent runs with different number of clusters (*K* = 1–15). Each run of 400,000 iterations and 100,000 burn-in iterations was repeated ten times, using a model allowing for admixture and correlated allele frequencies. We used CLUMPP 1.1.1 (Jakobsson & Rosenberg, 2007) to identify potential distinct solutions among the results of independent replicate runs for each *K* (“Greedy” algorithm; 100 random input sequences; G’ statistic) and to average individual assignment probabilities (*q*) over replicated runs with identical clustering solution.

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

<table>
<thead>
<tr>
<th>Species/Cluster</th>
<th>N</th>
<th><em>H</em>&lt;sub&gt;o&lt;/sub&gt;</th>
<th><em>A</em>&lt;sub&gt;r&lt;/sub&gt;</th>
<th><em>H</em>&lt;sub&gt;o&lt;/sub&gt;/<em>H</em>&lt;sub&gt;e&lt;/sub&gt;</th>
<th><em>F</em>&lt;sub&gt;IS&lt;/sub&gt;</th>
<th><em>s</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Microbotryum silenes-inflatae</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green</td>
<td>79</td>
<td>0.346</td>
<td>1.72</td>
<td>0.24/0.33</td>
<td>0.355</td>
<td>0.52</td>
</tr>
<tr>
<td>Yellow</td>
<td>64</td>
<td>0.468</td>
<td>2.01</td>
<td>0.18/0.44</td>
<td>0.577</td>
<td>0.73</td>
</tr>
<tr>
<td>Pink</td>
<td>75</td>
<td>0.243</td>
<td>1.49</td>
<td>0.20/0.23</td>
<td>0.199</td>
<td>0.33</td>
</tr>
<tr>
<td>Orange</td>
<td>40</td>
<td>0.178</td>
<td>1.35</td>
<td>0.10/0.17</td>
<td>0.387</td>
<td>0.56</td>
</tr>
<tr>
<td><em>Microbotryum violaceo-irregulare</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species-wide</td>
<td>7</td>
<td>0.594</td>
<td>2.07</td>
<td>0.08/0.44</td>
<td>0.852</td>
<td>0.92</td>
</tr>
<tr>
<td><em>Microbotryum lagerheimii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue</td>
<td>24</td>
<td>0.492</td>
<td>2.03</td>
<td>0.12/0.45</td>
<td>0.760</td>
<td>0.86</td>
</tr>
<tr>
<td>Light blue</td>
<td>12</td>
<td>0.491</td>
<td>1.98</td>
<td>0.13/0.43</td>
<td>0.662</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Note. *A*<sub>r</sub>; allelic richness, corrected for sample size using rarefaction (standardized sample size of two diploid individuals); *F*<sub>IS</sub>; fixation index; *H*<sub>o</sub>; gene diversity; *H*<sub>o</sub>/*H*<sub>e</sub>; observed/expected heterozygosity; N; number of pure representatives of the species/clusters, as determined using Bayesian clustering analyses; *s*; selfing rate estimated as 2*F*<sub>IS</sub>/(1 + *F*<sub>IS</sub>).

Because *M. lagerheimii* and *M. violaceo-irregulare* were minority components of the data set, they could not be differentiated in the overall analysis. We therefore also ran STRUCTURE on a sub-data set encompassing only genotypes assigned to these species based on ITS sequencing. We retained for this sub-data set the genotypes with total memberships *q* ≥ 0.8 in the cluster corresponding to both *M. lagerheimii* and *M. violaceo-irregulare* in the analysis on the whole data set (blue cluster at *K* = 5, see results). The 0.8 threshold is arbitrary but is typically used for assigning individuals, and the genotypes with main assignments below 0.8 had assignment probabilities balanced among the different clusters, indicating they likely corresponded to individuals with low assignment power (see Results).

#### 2.3.5 Hybrid detection

To assess whether individuals with intermediate assignment probability to clusters were high-confidence intercluster hybrids or may be simply genotypes with low power of assignment, possibly carrying alleles frequent in all species, we used a two-step procedure. In the first step, we used the membership proportions of individual genotypes in different clusters (*q* values) inferred using the admixture model of STRUCTURE to identify “pure” representatives of the different species or of clusters within species. Based on the STRUCTURE outputs of the full data set at *K* = 5 (identified as the most relevant subdivision, see Results), pure representatives of a given species were identified as genotypes for which the sum of *q* values in the different clusters assigned to this species was above 0.8; genotypes not meeting this requirement were classified as putative hybrids. Pure representatives of the various clusters within a given species were defined as genotypes displaying *q* values above 0.8 within one of the clusters identified within this species; genotypes not meeting this requirement were considered as putative admixed genotypes among clusters.
In the second step, we used pure representatives of the species or clusters as “learning samples,” allowing estimation of the membership proportions for the putative hybrid samples in “learning clusters,” using STRUCTURE, options USEPOPINFO and UPDATEPFROMPOPFLAGONLY. In assignment tests of putative hybrids between M. silenes-inflatae and either M. lagerheimii or M. violaceo-irregulare, genotypes were considered as hybrids if they had q values above 0.2 and below 0.8 in learning clusters. For M. lagerheimii and M. violaceo-irregulare, genotypes were considered as hybrids if they had a membership proportion q above 0.2 and below 0.8 in the two learning clusters corresponding to M. lagerheimii and M. violaceo-irregulare for differentiating these two species. Because clustering with STRUCTURE can be strongly influenced by unbalanced sample sizes (Kalinowski, 2011), and because sample size for M. silenes-inflatae was one order of magnitude higher than for the other species, data sets of pure representatives were randomly resampled ten times to the size of the smallest species sample (n = 7, M. violaceo-irregulare), and STRUCTURE analyses were run on each resampled data set.

To check the validity of the genetic subdivision retrieved from the STRUCTURE analysis, using a method not assuming outcrossing or lack of genetic disequilibrium, we also carried out a principal component analysis (PCA) on the microsatellite data using the “dudi.pca” function in the R-package ade4 (Dray & Dufour, 2007). We then used a discriminant analysis of the principal components (DAPC), as implemented in the R-package adegenet (Jombart, 2008; Jombart, Devillard, & Balloux, 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.

2.3.6 | Host specificity, geographical and climatic distributions

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 distribution of the Microbotryum species and their genetic clusters to assess their degree of allopatri. Because there was large variance in sample size at each site, with sites often having only one individual sample, we used analyses based on the presence and absence of a species or cluster at a site. Analyses based on raw numbers or relative frequencies often violated model assumptions. The associations between host species and pathogen species or genetic clusters were tested using the “CATMOD” procedure in the SAS Statistical Computing Environment (SAS Institute Inc. 2013). Associations between species or genetic clusters and geographic regions were tested using Fisher’s exact tests with the SAS procedure “FREQ” due to small 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 (Supporting Information Table S2) were obtained from the WorldClim database (means across years 1950–2000) recorded from a network of weather stations and interpolated over a 30-arc second-resolution grid (www.worldclim.org; Hijmans, Cameron, Parra, Jones, & Jarvis, 2005). For each sampling site, values of the 19 variables were extracted using nearest-neighbour interpolation with the Spatial Analyst Tool in ArcMap 9.2 software (ESRI (Environmental Systems Resource Institute), 2009). We used a principal components analysis to analyse and reduce the variation to three major axes using the SAS procedure PRINCOMP (based on the correlation matrix). We then carried out a logistic regression to examine the relationship between species presence and the first three components generated by the PCA, using the “glm” function in the R stats package with a quasi-binomial link.

3 | RESULTS

3.1 | Species identification, species distributions and population subdivision

The STRUCTURE analysis on the whole data set, modelling K = 2–15 clusters, revealed several levels of strong genetic differentiation, with well-defined Microbotryum clusters appearing up to K = 5 (i.e., clusters encompassing multiple genotypes with membership coefficients near the maximum; Figure 2 and Supporting Information Figure S3). The genetic clusters were overall broadly distributed among sampled regions, although a few clusters appeared geographically more restricted (Figure 2 and Supporting Information Figure S3). Some genetic differentiation was observed among samples collected on S. uniflora and S. vulgaris, with some genetic clusters being specific to either host species, but also with other genetic clusters 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 tree, also including published ITS sequences from M. silenes-inflatae, M. lagerheimii, M. violaceo-irregulare and other Microbotryum species (Supporting Information Figure S4; GenBank Accession nos MH491551–MH491577). Of the 20 sequences in the fully supported clade containing the published M. silenes-inflatae ITS sequences, 19 were genotypes assigned to the largest STRUCTURE cluster in analyses assuming K = 2 clusters (pink cluster; Supporting Information Figure S4), indicating that this cluster corresponds to M. silenes-inflatae. Analyses at K > 2 only further subdivided M. silenes-inflatae into several clusters (Figure 2). The ITS sequences falling in the fully supported clades containing the published M. lagerheimii (n = 5) and M. violaceo-irregulare (n = 2) sequences all belonged to genotypes in the second (blue) cluster of the K = 2 STRUCTURE model (Supporting Information Figure S4).
The log-likelihood plateaued at $K > 5$ with only a single clustering solution (Supporting Information Figure S5a); the additional clusters appearing at $K$ values above 5 mainly introduced additional subdivision within *M. silenes-inflatae*, produced many genotypes with intermediate assignment probability and never clearly separated the genotypes assigned to *M. lagerheimii* and *M. violaceo-irregulare* using ITS into distinct clusters. $K = 5$ was therefore considered as the most relevant $K$ value, with the blue cluster corresponding to both *M. lagerheimii* and *M. violaceo-irregulare*, and the four other clusters (green, orange, pink and yellow) to different lineages within *M. silenes-inflatae*.

The species *M. lagerheimii* and *M. violaceo-irregulare* could not be separated into distinct clusters at any $K$ values using the full data set, despite their large phylogenetic distance (Figure 1). The lack of ability to distinguish these species is likely due to their small sample sizes compared to that collected for *M. silenes-inflatae*. Such difficulties in recovering species differentiation in cases of unbalanced sample sizes have been shown by simulations (Neophytou, 2014). We therefore ran *structure* on a sub-data set with the genotypes from *M. lagerheimii* and *M. violaceo-irregulare* only. At $K = 3$ in this sub-data set (Figure 3; Supporting Information Figures S5b and S6), one cluster (brown) corresponded to *M. violaceo-irregulare* genotypes while the other two clusters (dark and light blue) represented *M. lagerheimii* genotypes, mainly separating isolates collected on *S. uniflora* in the UK and the Netherlands (light blue) versus genotypes collected on *S. vulgaris* in the Pyrenees and Alps (dark blue).

Spore morphology (colour and ornamentation) of 15 individuals, chosen at random within each species, provided further evidence for cluster assignment to species: all five strains assigned to *M. silenes-inflatae* had dark purple spores with reticulate ornamentation, all five strains assigned to *M. lagerheimii* had light purple spores with reticulate ornamentation, and all strains assigned to *M. violaceo-irregulare* had dark purple spores with verrucose ornamentation (Supporting Information Table S3).

**FIGURE 2** Proportions of ancestry in $K$ (from 2 to 10) clusters of *Microbotryum* spp. genotypes inferred with the *structure* program. Each genotype is represented by a vertical bar, partitioned into $K$ segments representing the amount of ancestry of its genome in $K$ clusters. When several clustering solutions (“modes”) were found within replicate runs, only the major mode is shown with its corresponding proportion of runs. IS: Iceland, NL: the Netherlands, UK: United Kingdom, CH: Switzerland, IT: Italy, F: France. For each region, genotypes are ordered by sampling elevation (represented at bottom). See Supporting Information Figure S3 for a sorting by membership coefficient.
The principal component analysis (PCA) on the full data set separated the three species, with M. lagerheimii and M. silenes-inflatae in separate corners of the parameter space and M. violaceo-irregulare appearing intermediate between them (Figure 4a). The ITS sequence variants particular to M. silenes-inflatae were not confined to any of its four genetic clusters (Supporting Information Figure S3; Figures 4a,b), providing evidence that they constitute within-species genetic subdivision. The M. lagerheimii and M. violaceo-irregulare species and two M. silenes-inflatae clusters were found on both S. vulgaris and S. uniflora, while the two other M. silenes-inflatae clusters occurred only on S. vulgaris (Figure 2). A PCA run on the sub-data set of the two less frequent Microbotryum species also discriminated them well (Figures 4c,d).

Clustering using DAPC supported the clustering patterns found in STRUCTURE analyses (Supporting Information Figure S7 and Figure 2). The DAPC Bayesian information criterion reached a minimum value at K = 28 (Supporting Information Figure S8). However, visual inspection of membership probability patterns across K values indicated that DAPC and STRUCTURE identified the same five main clusters (Supporting Information Figure S7 and Figure 2). The DAPC on the full data set 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 intermediate assignment probabilities in the blue cluster occurring only on S. vulgaris (Figure 2). A single clustering solution assigned to the blue cluster at K = 5 in the output of the Structure value at K = 28 (Supporting Information Figure S8). However, visual inspection of membership probability patterns across K values indicated that DAPC and STRUCTURE identified the same five main clusters (Supporting Information Figure S7 and Figure 2). The DAPC on the full data set 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 (Supporting Information Figure S9).

Clustering using DAPC supported the clustering patterns found in STRUCTURE analyses (Supporting Information Figure S7 and Figure 2). The DAPC Bayesian information criterion reached a minimum value at K = 28 (Supporting Information Figure S8). However, visual inspection of membership probability patterns across K values indicated that DAPC and STRUCTURE identified the same five main clusters (Supporting Information Figure S7 and Figure 2). The DAPC on the full data set 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 (Supporting Information Figure S9).

**FIGURE 3** Proportions of ancestry in K (from 2 to 5) clusters of the Microbotryum violaceo-irregulare and M. lagerheimii genotypes inferred with the STRUCTURE program, using a sub-data set excluding M. silenes-inflatae. In the sub-data set, we kept only the genotypes assigned to the blue cluster at K = 5 in the output of the Structure analysis on the whole data set (Figure 4). A single clustering solution (“mode”) was found among replicate runs. Each genotype is represented by a vertical bar, partitioned into K segments representing the amount of ancestry of its genome in K clusters. NL: the Netherlands, UK: United Kingdom, CH: Switzerland, IT: Italy, F: France. For each region, samples are ordered by sampling elevation (represented at bottom; see Figure S5 for a sorting by assignment coefficient within regions)

### 3.2 | Hybridization and admixture checking

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

We identified 7, 38 and 400 pure genotype representatives of M. violaceo-irregulare, M. lagerheimii and M. silenes-inflatae, respectively, that we used as learning samples. In assignment tests for interspecific hybrid identification, all genotypes had substantial membership in all learning samples considered. Therefore, no genotype could be identified as a high-confidence hybrid, neither between species nor between lineages within M. silenes-inflatae (Supporting Information Table S5), and they may instead represent genotypes with low assignment power. This inference was supported by the heterozygosity values of the genotypes with intermediate assignment probabilities, whose mean (Hs = 0.18) was not higher than that in other samples (Table 1), in contrast to what would be expected for hybrids.

### 3.3 | Host specificity, geographical and climatic distributions

Pathogen species and several clusters within M. silenes-inflatae and M. lagerheimii were differentially distributed among host species. Because pathogens were sampled without prior knowledge of their genotype, their differential distribution was not due to sampling bias. The rarest species, M. violaceo-irregulare, was found only on S. vulgaris in the western and central Alps (Figure 5h and Supporting Information Figure S10). The two other species, M. lagerheimii and M. silenes-inflatae, were both found in the Alps, the Pyrenees and the UK (Figure 5h and Supporting Information Figure S10). However, the two genetic clusters within M. lagerheimii were well-separated both geographically and with regard to host species, with the light blue cluster occurring only on S. uniflora in the UK and the dark blue cluster only on S. vulgaris in the Alps and the Pyrenees (Figure 5f,g). While all Microbotryum species were found on S. vulgaris, M. silenes-
inflatae was found in 90% of S. vulgaris populations but just 50% of S. uniflora populations, the difference in frequency being significant (Fisher’s 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 5a–e). The green cluster of M. silenes-inflatae was found commonly on S. uniflora in the UK (Figure 5b), 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 of 32 displayed sympatry among at least two genetic clusters (Supporting Information 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 M. silenes-inflatae clusters were in 33 of 40 (82%) sites with a single cluster, and the genotypes with intermediate assignment

**FIGURE 4** Principal component analysis (PCA) on multilocus microsatellite genotypes for the data set including the three Microbotryum species (a: principal component 1 vs. principal component 2; b: principal component 1 vs. principal component 3) for the sub-data set including only M. lagerheimii and M. violaceo-irregulare (c: principal component 1 vs. principal component 2; d: principal component 1 vs. principal component 3). Scatterplots for the first three principal components are shown using a colour labelling of genotypes defined according to the assignment of multilocus genotypes to three species and six clusters within species using Bayesian clustering analyses. Colours indicate clusters as in Figures 2 and 3. Black outlines indicate the samples for which the ITS region has been sequenced.
probabilities in different *M. lagerheimii* clusters were in 13 of 17 (76%) sites with a single cluster (grey genotypes in Supporting Information 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. silenes-inflatae* (37 sites with unambiguous assignments) were associated with local geography, elevation and/or climate. The occurrence of *M. silenes-inflatae* did not differ between the Pyrenees, western Alps or central Alps (Fisher’s exact test, \( p = 0.168 \)). The occurrence of *M. violaceo-irregulare* and *M. lagerheimii* differed significantly among regions (Fisher’s exact tests, \( p = 0.031 \) and \( p = 0.001 \), respectively), with the former being absent in the Pyrenees and the latter absent in the Western Alps (Figure 5 and Supporting Information Figure S10). Within *M. silenes-inflatae*, the green cluster showed significant differences in occurrence among regions (Fisher’s exact test, \( p = 0.025 \)), being more common in the western Alps. The analysis approached significance for the orange cluster (Fisher’s exact test, \( p = 0.052 \)) which was more common in the Pyrenees (Figure 5 and Supporting Information Figure S10). The other two clusters showed no significant differences in occurrence among regions (Fisher’s exact tests, \( p = 0.634 \) for pink and \( p = 0.408 \) for yellow).

The three species occurred at significantly different elevations (Figure 6; ANOVA, \( df = 2 \), Sum of squares = 4486478, F-ratio = 8.7, \( p = 0.0002 \)). The elevational distribution was not normal, but ANOVAs are robust to such assumption violations (Lindman, 1974) and a nonparametric test also indicated significant differences in elevation among species (Kruskal-Wallis nonparametric test, \( df = 2 \), \( \chi^2 = 16.7 \), \( p = 0.002 \)). The species *M. violaceo-irregulare* was found only at high elevations, *M. lagerheimii* at the lowest elevations, while *M. silenes-inflatae* was found across a greater range of elevations (Figure 6). The two clusters within *M. lagerheimii* were also found at significantly different elevations (Figure 6; ANOVA, \( df = 1 \), Sum of squares = 21620792, F-ratio = 167.9, \( p < 0.0001 \); Kruskal-Wallis test, \( df = 1 \), \( \chi^2 = 24.1 \), \( p < 0.0001 \)), as well as the four clusters within *M. silenes-inflatae* (Figure 6; ANOVA, \( df = 3 \), Sum of squares = 10011015, F-ratio = 24.3, \( p < 0.0001 \); Kruskal-Wallis test, \( df = 2 \), \( \chi^2 = 56.6 \), \( p < 0.0001 \)). In *M. lagerheimii*, the contrast in elevation between its two clusters was associated with the differences in host species, as described above, that is, coastal *S. uniflora* in the UK versus broad elevational distribution of *S. vulgaris* elsewhere. The Figure 2 also illustrates the differences in elevation between species and clusters within each geographical region: Clusters remain well separated when genotypes are sorted by elevation, in particular at \( K = 5 \).

Differences in *Microbotryum* distributions among both elevations and geographic regions could indicate impacts of climate. To test whether species or clusters were associated with different elevations and/or climates among the most heavily sampled regions (*S. vulgaris* populations in the Pyrenees, western Alps and central Alps), we carried out a two-step analysis to first reduce climatic variables to uncorrelated principal components (PCs), and then test for the relative ability of climate, elevation and geographical location to explain species and cluster distributions. For both species and cluster data sets, the 19 bioclimatic variables (Supporting Information Table S2) were reduced to three axes using PCA. All PC axis loadings were very similar for both species and cluster analyses (Supporting Information Tables S2 and S6). Axis 1 (explaining 63% and 66% of the

**Figure 5** Distribution maps of the different genetic clusters and species of anther-smut *Microbotryum* fungi on *Silene vulgaris* and *S. uniflora*, and maps of their co-occurrence. Pie charts are proportional to the number of samples. Colours correspond to those used in other figures.
Figure 6 Violin plots showing elevational distribution of species and clusters within species. All samples belonging to Microbotryum silenes-inflatae at K = 5 (including all genotypes with low assignment power to the green, orange, pink and yellow M. silenes-inflatae clusters) are included in the leftmost M. silenes-inflatae column. Otherwise, only samples with high-confidence cluster assignment are shown. Samples belonging to M. lagerheimii and M. violaceo-irregulare were determined using the K = 3 output from the sub-data set excluding M. silenes-inflatae.

3.4 Levels of genetic variation and mating system

Summary statistics were computed for clusters identified at K = 5 in analyses of the full data set for M. silenes-inflatae, and at K = 3 in analyses of the M. lagerheimii-M. violaceo-irregulare data set (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).

4 Discussion

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

We detected genetic subdivision within species, with two clusters in M. lagerheimii and four clusters in M. silenes-inflatae. Given their weak genetic differentiation (as shown by ITS sequences and
distribution in the PCA), the genetic clusters within *M. silenes-inflatae* likely do not represent further cryptic species. Within *M. lagerheimii*, the genetic subdivision corresponded to separation in geography, host and environmental conditions and it remains to be determined if these might be cryptic species. *Silene vulgaris* and *S. uniflora* were allopatric during the last glaciation (Prentice, Andersson, & Mансby, 2011), with *S. uniflora* likely persisting in northern refugia from the Baltic/Scandinavian region and *S. vulgaris* in southern European refugia. This suggests that the differentiation between the two *M. lagerheimii* clusters found on *S. latifolia*, *S. dioica*, *S. acaulis*, *Saponaria officinalis* and *M. violaceo-irregulare* especially indeed had high estimated selfing levels, being highly homozygous, have not homogenized despite sympatry, which is likely due to the host and environmental conditions and it remains to be determined if the genetic subdivision corresponded to separation in geography, likely do not represent further cryptic species. Within *S. vulgaris*, host was not genotyped in our study and no infection trials were carried out, we cannot rule out the influence of host genotypes on *Microbotryum* species distribution and coexistence.

Subdivisions within *M. silenes-inflatae* had less obvious biological interpretation as the clusters were broadly overlapping in geography. Indeed, all four *M. silenes-inflatae* clusters were found in the Alps, the UK and the Pyrenees on *S. vulgaris*. However, only the green cluster was found regularly on *S. uniflora* in the UK. Additionally, this green cluster was more frequent in the western Alps than in the Pyrenees, while the opposite was found for the orange cluster, suggesting possible allopatric or host-related differentiation of the *M. silenes-inflatae* genetic clusters. The geographic co-occurrence of the four *M. silenes-inflatae* clusters in the Alps may result from recent movements and secondary contact. Footprints of a glacial refugia in Europe have been found in *M. lychnidis-dioicae* parasitizing *S. latifolia* (Badouin et al., 2017; Feurtey et al., 2016; Gladieux et al., 2011; Vercken et al., 2010) and *M. silenes-acaulis* parasitizing *S. acaulis* (Bueker et al., 2016). The genetic clusters within *M. silenes-inflatae* have not homogenized despite sympatry, which is likely due to the selfing mating system of these fungi. All three *Microbotryum* species indeed had high estimated selfing levels, being highly homozygous, especially *M. violaceo-irregulare* and *M. lagerheimii*, as has been found for other *Microbotryum* species previously studied (e.g., those on *S. latifolia*, *S. dioica*, *S. acaulis*, Saponaria officinalis and Dianthus spp.; Bucheli, Gautschi, & Shykoff, 2001; Bueker et al., 2016; Delmotte, Bucheli, & Shykoff, 1999; Fortuna et al., 2016; Giraud, 2004; Gladieux et al., 2011; Hood & Antonovics, 2000, 2004; Petit et al., 2017). Such high selfing rates may be the result of mating among sporidia from one diploid individual (a spore load from a single infected flower). Additionally, it may be the result of mating between sporidia from single teliospores, a process also promoted by intrategrad mating that is present in many *Microbotryum* species as a result of ordered segregation of the mating-type loci (Hood & Antonovics, 2000; Hood, Scott, & Hwang, 2015). Interestingly, *M. silenes-inflatae* had lower inferred selfing rates than the other species, and it would be informative to investigate in future studies if this is due to ecological factors, such as more frequent codispersal of spores from different fungal individuals by pollinators, or to intrinsic factors, such as a lower propensity of intrategrad 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.

The coexistence of the three *Microbotryum* species is clearly not maintained by host specialization, as all three species occurred on *S. vulgaris*. Moreover, it has been shown that resistance of *S. uniflora* to *M. lagerheimii* and *M. silenes-inflatae* was significantly positively correlated among plant families (Chung et al., 2012), which indicates that the plant resistance is not specific to any one *Microbotryum* species. This further supports the inference that specialization on *S. uniflora* versus *S. vulgaris* is not a major factor contributing to the co-occurrence of these *Microbotryum* species. However, because the host was not genotyped in our study and no infection trials were carried out, we cannot rule out the influence of host genotypes on *Microbotryum* species distribution and coexistence.

The situation of the anther-smut species co-occurring on *S. vulgaris* is in some respects similar to that of anther-smut disease on the genus *Dianthus* where multiple *Microbotryum* species co-occur in the same sampling sites on multiple *Dianthus* species (Petit et al., 2017). In this case, however, the *Microbotryum* species on *Dianthus* hosts are closely related phylogenetically and hybrids, likely facilitated by the proximal relationships between the *Microbotryum* species, are frequently found (Petit et al., 2017). The situation on *Dianthus* spp. and the *S. vulgaris/S. uniflora* sister pair contrasts with the pattern found in all Caryophyllaceae studied to date, where each host species has been found to harbour only one endemic *Microbotryum* species in natural populations (Le Gac, Hood, Fournier et al., 2007; Refrégier, Hood, & Giraud, 2010). *Silene vulgaris* also occasionally harbours *Microbotryum* species as a result of transient cross-species disease transmission from other *Silene* hosts, such as *S. latifolia* and *S. dioica* (Antonovics et al., 2002; Hood et al., 2003). It is unclear if this is due to a particular susceptibility of *S. vulgaris* to anther smut or to its very wide geographic and environmental ranges that allow contact with the disease on many other *Silene* hosts.

The three anther-smut species on *S. vulgaris* occurred at significantly different elevations, suggesting that they may occupy different ecological niches in terms of abiotic conditions. This was supported by some association of *M. lagerheimii* occurrence with climatic variables related to temperature and precipitation. Within *M. silenes-inflatae*, the relative frequency of some of the genetic clusters also varied with elevation and climatic factors. These effects may reflect environmental tolerances of the fungal species and clusters, or ecotypic differences among host populations. It is well known that the host *S. vulgaris* has different ecotypes adapted to contrasting elevations (Marsden-Jones & Turrill, 1957), and these may have different anther-smut specificities. In the present study, just one host population was identified as *S. vulgaris* var. *prostrata*, the high elevation form of the host (Site 22, in which all infections were caused by *M. violaceo-irregulare*). However, a previous study of *Microbotryum* on *S. vulgaris* in the small region of the southeast-French Alps where Site 22 is located showed that none of the three
fungal species were confined to any specific host ecotype (Abbate & Antonovics, 2014). Furthermore, phenotypic traits including those that mark differences between the ecotypes change gradually over elevation, and the differentiation between populations is relative to their geographic distance (Berardi, Fields, Abbate, & Taylor, 2016). The much wider sampling in the present study confirms that Microbotryum on S. vulgaris is largely confined to higher elevations (79% of the samples, 88% of the sites, were found above 1,300 m), even though populations of the host plant are abundant at lower elevations across its range. More generally, elevation and abiotic conditions have been shown to impact the presence of fungal pathogens in other systems (Cordier et al., 2012; Desprez-Loustau, Capron, & Dupuis, 1998; Enjalbert, Duan, Leconte, Hovmöller, & De Vallieville-Pope, 2005; Gange, Gange, Sparks, & Boddy, 2007; Mboup et al., 2012; Vacher, Vile, Helion, Piou, & Desprez-Loustau, 2008). This study thus begs important questions for future work with Microbotryum pathogens investigating how the genetics of adaptation to elevation in fungi interacts with the adaptation to, or of, host plants. For example, one question is whether Microbotryum is rare at low elevations because the fungus cannot adapt to low-elevation climatic conditions, or because the costs of resistance in the host to the pathogen decline under those conditions.

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

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DATA ACCESSIBILITY

The genotypes and elevation data are available on Dryad: https://doi.org/10.5061/dryad.627fm05. GenBank Accession nos for ITS sequences: MH491551–MH491577.

AUTHOR CONTRIBUTIONS

T.G., J.An. and M.E.H. designed the study, A.S., J.Ab. and D.V. genotyped samples. P.G., J.An., J.Ab. and T.G. analysed data. P.G., J.Ab. and T.G. wrote the manuscript with contributions by all authors.

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