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Summary

Cold-adapted organisms with current arctic-alpine distributions have persisted during the last glaciation in multiple ice-free refugia, leaving footprints in their population structure that contrast with temperate plants and animals. However, pathogens that live within hosts having arctic-alpine distributions have been little studied. Here, we therefore investigated the geographical range and population structure of a fungus parasitizing an arctic-alpine plant. A total of 1437 herbarium specimens of the plant *Silene acaulis* were examined, and the anther smut pathogen *Microbotryum silenes-acaulis* was present throughout the host's geographic range. There was significantly greater incidence of anther smut disease in more northern latitudes and where the host locations were less dense, indicating a major influence of environmental factors and/or host demographic structure on the pathogen distribution. Genetic analyses with seven microsatellite markers on recent collections of 195 *M. silenes-acaulis* individuals revealed three main genetic clusters, in North America, northern Europe and southern Europe, likely corresponding to differentiation in distinct refugia during the last glaciation. The lower genetic diversity in northern Europe indicates postglacial recolonization northwards from southern refugia. This study combining herbarium surveys and population genetics thus uniquely reveals the effects of climate and environmental factors on a plant pathogen species with an arctic-alpine distribution.

INTRODUCTION

The geographic distribution and genetic structure of present-day species result both from dispersal ability and from historical processes such as continental drift and climate changes (Hewitt 1996), as changes in sea level and extensions of ice sheets have affected the availability and connectivity of species´ habitats (Andersen & Borns 1997; Ehlers & Gibbard 2007). In particular, studies using molecular markers have revealed that the genetic structure of present-day species has been strongly influenced by the oscillations in temperature of the Quaternary (Hewitt 2004).

During glacial periods of the Pleistocene, cold-sensitive species persisted in ice-free "refugia" in southern regions. Long-term isolation of populations in separate refugia fostered population differentiation (Bennett *et al.* 1991; Hewitt 1996, 2004). Increasing temperatures and decline of ice sheets during interglacial periods enabled range expansions and migration northwards (Comes & Kadereit 1998; Abbott & Brochmann 2003). Expansions as part of these recolonization processes were often associated with founder effects, resulting in low levels of genetic diversity in northern regions, while southern regions remained reservoirs of genetic diversity (Hewitt 2004). In contrast, cold-adapted species were able to survive glacial maxima in more northern areas, at the edge of the ice-sheets, and also in mountains of southern areas (Murray 1995; Abbott *et al.* 2000; Brochmann *et al.* 2003). Molecular studies have supported the existence of additional, smaller refugia in northern Europe and North America, *e.g.,* in "nunataks", that were mountains completely surrounded by glacial ice (e.g. Stehlik *et al.* 2002; Westergaard *et al.* 2011), from which species dispersed when the climate became warmer. The range of arctic-alpine species was thus also fragmented during the ice age, which left footprints

as strong genetic population structures (Eidesen *et al.* 2013). Unlike species adapted to temperate climates however, the postglacial recolonization of many cold-adapted species was likely broad-fronted, with admixtures between different genetic pools, and therefore with little loss of overall genetic variation (Brochmann & Brysting 2008).

Recent studies on arctic-alpine species have shown that, beside the impact of post-glacial migration, the genetic structure of present day species varies greatly depending on species-specific factors, *e.g.,* dispersal ability and mating system (Taberlet *et al.* 1998; Abbott & Brochmann 2003; Stewart *et al.* 2010). In pathogenic organisms, population structure may depend in addition on the distribution of the host (Nieberding *et al.* 2008). Although a wealth of studies on the genetic diversity of arctic-alpine organisms has been accumulated, they virtually all focus on plant and animal taxa, leaving a striking lack of knowledge in other groups. For instance, only few studies have focused on fungal pathogens or fungi in symbiosis, *e.g*., mycorrhizae (Cripps & Eddington 2005); yet such research is important if we are to understand the effects of climate change on disease emergence and spread and broader issues of impact from warming temperatures on biodiversity.

We therefore studied here the population structure of the arctic-alpine distributed pathogen *Microbotryum silenes-acaulis* for understanding its population history in relation to climate changes during and after the last glaciation. This specialized basidiomycete fungal pathogen, *M. silenes-acaulis,* is found on the plant *Silene acaulis* (Lutz *et al.* 2008), which occurs in arctic-alpine environments in the northern hemisphere (Körner 2003). *Silene acaulis* forms large, long-lived cushions with hundreds of short stems (Jones & Richards 1962). A well-studied relative of *M. silenes-acaulis*, *Microbotryum lychnidis-dioicae*, causes anther smut on the temperate host *Silene latifolia*, and comparable population genetics studies indicate a clear pattern of

recolonization from southern European refugia (Spain, Italy, Balkans) after the last glaciation (Vercken *et al.* 2010). The *M. lychnidis-dioicae* recolonization history is mostly congruent with that of *Silene latifolia* (Taylor & Keller 2007), although that pathogen seemed to have persisted in more numerous and smaller refugia than its host (Vercken *et al.* 2010; Gladieux *et al.* 2011). Infections by multiple pathogen genotypes have been reported in *M. lychnidis-dioicae* and can have important consequences on disease dynamics (Lopez-Villavicencio *et al.* 2007, 2011; Buono *et al.* 2014; Susi & Laine 2015).

To investigate the phylogeography of *M. silenes-acaulis* in relation to its host distribution, we used a combination of herbarium surveys and population genetic analyses using microsatellite markers. Our specific questions were the following: 1) What is the distribution of *M. silenes-acaulis* as compared to its host plant *S. acaulis*? 2) What is the population structure of *M. silenes-acaulis* across its geographical range, and does it allow retracing migration histories in relation to glacial cycles? 3) What is the spatial distribution of the genetic variation at finer scales, including whether infections by multiple pathogen genotypes occur in the same *S. acaulis* individuals?

MATERIAL AND METHODS

Herbarium surveys for assessing species distribution

To study the distribution of *M. silenes-acaulis* and its host *S. acaulis,* a total of 1437 herbarium specimens of *S. acaulis* were examined. Several previous studies have shown that anther-smut fungi can be detected among herbarium specimens of its hosts; because there is little evidence

that the original collectors or subsequent annotator recognized the plants as being diseased (Rabeler 1993; Antonovics *et al.* 2003; Hood *et al.* 2010), this allows assessment of the pathogen distribution as well. Specimens of *S. acaulis* were examined in the general collections from the following herbaria: U.S. Forest Service Herbarium (USFS), New York Botanical Garden (NY), Museum National d'Histoire Naturelle (P), Oregon State University (OSC), University of California, Berkeley (UC), University of Iowa and Iowa State University (ISC), University of Minnesota (MIN), University Washington (WTU), Washington State University (WS), British Museum of Natural History (BM). The location and date of collection for healthy and diseased specimens were recorded.

The distributions of healthy and diseased specimens were compared to the previously published *S. acaulis* map by Hultén and Fries (1986). Disease incidence among herbarium specimens was analysed in the context of collection site latitude and density of nearby collection sites. The effects of latitude were analysed by separating the samples into equal sized groups based on quartiles from southern to northern latitudes; quartile latitude boundaries of <44, 49, 62, and the maximum observed site of 82. For defining local densities, latitude and longitude information were translated into pairwise linear distances between sites. The number of collection sites near diseased sites (within radii of 50, 100, or 200 km) was compared to an equal number (N = 36) of randomly selected healthy collection sites. One thousand randomly assembled sets of 36 healthy collection sites were used to assess statistical significance of differences between average densities of collection sites near to diseased and healthy localities. Latitude and disease status, and their interaction term, were analysed for the response variable of density of host collection sites using the generalized linear model approach with Poisson log link function in SPSS

STATISTICS 19 (SPSS, Inc, Chicago, Illinois); this analysis included the disease sites and an equal number of randomly selected healthy sites.

Sample collection of *M. silenes-acaulis* in living populations for population genetic analyses

The analyses of *M. silenes-acaulis* population structure were performed on a sample of individuals collected from natural, living populations across its range (Fig. 1, Table S1) from 2001 to 2013. Diploid teliospores from host plants within an area of 2 km^2 were considered as a geographic population and spores from a given flower were treated as a single diploid individual (Lopez-Villavicencio *et al.* 2007).

Genotyping populations of the global dataset

DNAs from all 255 sampled individuals (from populations in North America, Iceland, southern and northern Europe, including Norway and Svalbard), hereafter called the global dataset, were extracted using chelex (Giraud *et al.* 2004) and genotyped using 16 microsatellite markers previously available (Table S2) (Bucheli *et al.* 1998; Giraud *et al.* 2002; Giraud *et al.* 2008). Only individuals with genotype data for more than 70 % of the markers were retained for analyses, which represented 195 individuals from 39 populations.

Development of microsatellite markers and genotyping populations of the northern-European dataset

Because of higher sampling density in Norway and Svalbard, we also analyzed these populations separately, and with markers specifically developed from local genotypes, to increase the power of the population structure analyses in these regions.

For this goal, spores from 137 flowers were germinated on Maltose-Yeast-Peptone medium (MYP) with kanamycin (100 µg/ml) for six days at room temperature and DNA from cultures was extracted by the method of Hoffman & Winston (1987). The genome of a *M. silenes-acaulis* strain (CE008) from Norway (Jotunheimen, population 16) was sequenced (3X coverage) on a Roche GS FLX+I using the 454 sequencing method (GATC-Biotech, Konstanz, Germany). The program MIRA 3.4.0.1 (Chevreux *et al.* 1999) was used to assemble single reads into contigs. Only contigs larger than 3 kb were retained for the subsequent detection of microsatellites using PHOBOS 3.3.12 (Mayer 2010). We retained microsatellite loci only when: 1) their repeat motif length was between two and nine bp, which is typically considered as the optimal length range for obtaining variable and scorable markers, 2) they had a minimum length of 20 bp and/or at least four repeats, for increasing the probability that the loci would be polymorphic, and 3) they were perfect repeats of the motif, for decreasing the probability of wrong allele scoring and for maximizing the probability of polymorphism (Leese & Held 2011).

Primer binding sites were chosen so that the amplified fragment would range between 100 and 350 bp (Table S3). Primer pairs were designed using PRIMER3 0.4.0 (Rozen & Skaletsky 2000) and prepared by *Sigma-Aldrich Life Science* (Taufkirchen, Germany). Variation for the designed microsatellite markers was tested by genotyping four specimens from four distinct regions, respectively: Alps, Norway, Svalbard and Alaska. To reduce the occurrence of stutter bands in fragment analyses, the final elongation time in the PCRs was set to 30 minutes (Leese & Held 2011). Markers with multiple amplified fragments were not retained for further genotyping. After testing different microsatellite combinations using gel electrophoresis, we pooled two markers at most using multiplex PCR, with different fragment lengths and adding the fluorescent markers 5-Carboxytetramethyl-rhodamine (TAMRA™), 5-Tetrachloro-Fluorescein (TET™) and

6-Fluorescein-phosphoramidite (6-FAM™) (*Sigma-Aldrich Life Science*) to the 5'-end forward primers. Fragment analysis was performed on an ABI3130xl sequencer (*Applied Biosystems*), using the standard ladder GeneScan- 500 ROX™.

After genotyping northern European populations with these markers, we checked for large allele drop out using MICROCHECKER 2.2.3 (Van Oosterhout *et al.* 2004) and removed loci displaying extremely large among-population variance using ANIMALFARM 1.0 (Landry *et al.* 2002). The program LOSITAN 1.0 (Beaumont & Nichols 1996) was used to detect markers evolving under positive selection, and GENEPOP 4.1 (Rousset 2008) was used to detect markers in linkage disequilibrium, which were then excluded from further analyses. We eventually retained seven of the newly developed markers for the population structure analysis of Norway and Svalbard populations (Table S3). Finally, we retained 110 individuals from 18 populations with no more than 30 % missing data.

Investigation of the occurrence of multiple infections

To investigate the occurrence of multiple infections in *S. acaulis*, we genotyped spores from three to five different stems per diseased host plant in four of the populations from southern Europe (Table S1). This subset represented 64 flowers from 16 plants, genotyped with the 16 microsatellite markers used for the global dataset.

Genetic diversity

For the global dataset, the degree of genetic variability within the geographic groups North America, southern Europe and northern Europe was assessed by allelic richness (A_r) (Petit *et al.*

1998), unbiased expected heterozygosity (H_E), observed heterozygosity (H_O), and Nei´s standardized fixation index ($G´_{ST}$) (Nei 1987; Meirmans & Hedrick 2011). The R package "hierfstat" (Goudet 2014) was used to estimate A_r, and GENALEX 6.5 (Peakall & Smouse 2005) to estimate H_E, H_O, and $G´_{ST}$. The significance of the heterozygosity deficit compared to the expected heterozygosity under Hardy-Weinberg assumptions was tested in GENEPOP 4.2 (Rousset 2008) and G_{IS} coefficient was estimated with the correction of Nei and Chesser (1983) using GENALEX 6.5 (Peakall & Smouse 2005). Differences in genetic diversity (H_E, A_r) between geographic groups were tested using paired Wilcoxon signed rank tests. The relationship between the level of multiple infections and population genetic diversity was assessed by testing the significance of the correlation between the number of genotypes detected per plant and population diversity (H_E, A_r) using the Spearman´s rank coefficient. All statistical tests were performed using RSTUDIO 3.0.2 (RStudio Team 2015).

Population structure

Population genetic structures were investigated with the Bayesian assignment method implemented in STRUCTURE 2.3 (Pritchard *et al.* 2000) using the diploid datasets. Ten independent runs were performed for each value of K (number of clusters), with K ranging from one to 20. We used 500,000 iterations after a burn-in period of 100,000. STRUCTURE outputs were processed using STRUCTURE HARVESTER (Earl & von Holdt 2012). CLUMPP 1.1.2 (Jackobsson & Rosenberg 2007) was used to align independent runs with the same clustering mode, with random input order, 10,000 permutations, and the Greedy algorithm for $K \leq 9$ and the Large-Greedy algorithm for $K > 9$. Barplots with cluster-membership coefficients for all individuals and geographic populations were generated with the program DISTRUCT 1.1

(Rosenberg 2003). The geographic distributions of genetic clusters were visualized using R package "RgoogleMaps"(Loecher 2015). Genetic diversity and selfing rates in genetic populations were estimated using GENALEX 6.5 (Peakall & Smouse 2005) and INSTRUCT (Gao *et al.* 2007), respectively. F_{ST} values among genetic clusters were calculated at K = 3 to K = 8 and visualized with neighbour-joining trees using the R package "ape" (Paradis *et al.* 2015). In addition to these model-based methods, we performed a principal component analysis (PCA) to visualize genetic patterns without any assumption on panmixia (R packages "vegan" (Oksanen *et al.* 2015), "rgl" (Adler *et al*. 2015) and "scatterplot3d" (Ligges *et al.* 2015). For each value of K from two to ten, individuals were assigned to a cluster if their membership coefficient in this cluster was higher than 0.7.

RESULTS

Distribution and demographic data

Herbarium specimens of *S. acaulis* were examined from throughout the species range and largely conformed to the species map provided by Hultén and Fries (1986) (Fig. 1), *i.e.,* an alpine/circum-polar distribution, including more southern extensions in mountain ranges, such as the Rocky Mountains, and a gap in the northern Eurasia regions of the Sakha Republic.

A total of 1,437 herbarium specimens were examined, including 41 (2.9%) that were found to be affected by the anther-smut disease. Peak collection period of the plant *S. acaulis* was in the 1930s, consistent with previous studies on other plant species (Antonovics *et al.* 2003). There was no significant difference in the distribution of collection times (years) between diseased and

healthy samples (Kolmogorov-Smirnov two sample test, p-value = 0.545), indicating no recent change in disease incidence. As previously observed for other *Silene* herbarium specimens (Antonovics *et al.* 2003), there was no indication that collectors had been aware of the disease status of the plants and would have biased collection towards healthy or diseased plants. In particular, no written mention of diseased was recorded on herbarium sheets. Excluding specimens with illegible or ambiguous locality information, 790 distinct collection localities were identified, including 36 with anther smut infection.

Anther-smut disease was found to be widespread within the host's geographic range, with no major region being free of infections. However, the distribution of anther smut disease showed significant patterns among the collection sites in two ways. First, the disease tended to be found more often in regions with low densities of *S. acaulis* collection sites (Fig. 2); none of the 1,000 randomly sampled sets of healthy sites had average densities of nearby sites, in radii at 50, 100 or 200 km, as low as that observed for the set of diseased sites (p-value < 0.001). Second, the proportion of sites that contained diseased plants increased in more northern regions (Fig. 3). This pattern of greater disease incidence in the northern latitudes was despite the occurrence of greater numbers of samples per site in more southern localities (1.7 samples/site for each of the two southern latitudinal quartiles, and 1.4 and 1.3 samples/site for the two more northern quartiles, respectively).

While host collection site density had a strong latitudinal component (Fig. 3), the number of nearby collection sites was significantly determined by disease status independently of the effect of latitude, with disease being found in areas of lower host site density. The number of nearby sites within a radius of 100km was indeed predicted from the generalized linear model by

latitude (Wald X^2 = 113.32, p-value < 0.001) and disease status (Wald X^2 = 5.66, p-value = 0.017), while the interaction term was non-significant (Wald X^2 = 0.43, p-value = 0.514).

Genetic variation in populations and within individual host plants

We detected 99 different alleles overall across the 16 microsatellite markers used on the global dataset, including *M. silenes-acaulis* samples from 195 different plants across 42 populations. Typically, three to nine alleles per locus were observed, except for the E18 marker, which displayed 24 alleles (Table S2). For all markers, observed heterozygosity (H_O) showed significantly lower values than the expected heterozygosity (H_E) ($p < 0.001$); this tendency of heterozygote deficiency was also indicated by positive and high G_{IS} values (Table 1, Table S2).

Expected heterozygosity (H_E) and allelic richness (A_r) were highest in southern European populations ($H_E = 0.45$, $A_r = 4.89$), lowest in northern European populations ($H_E = 0.18$, $A_r = 2.65$) and intermediate in North America ($H_E = 0.35$, $A_r = 3.76$) (Table 1). Paired Wilcoxon signed rank tests indicated significant differences between southern Europe and northern Europe (H_E: W = 4, p-value < 0.001, A_r: W = 136, p-value = 0.031) and between North America and northern Europe (H_E: W = 4, p-value = 0.009; A_r: W = 129, p-value < 0.001), while the values of southern Europe and North America were significantly different only for A_r (H_E: W = 81, p-value = 0.244, A_r: W = 118, p-value = 0.008).

Multiple infections were detected in the 16 plants from populations in the Alps for which spores from multiple stems had been genotyped (mean number of stems analyzed = 4). A mean of 3.25 genotypes were detected per plant, *i.e.,* nearly every stem analyzed bore a specific genotype. There was no significant correlation between the average number of genotypes per individual

and genetic diversity (H_E and A_r) of a population (H_E: r = 0.80, p-value = 0.333; A_r: r = 0.63, p-value = 0.368).

Population structure

We first used the Bayesian methods implemented in the STRUCTURE software to investigate the population structure in the global dataset. There was a constant increase of the likelihood Ln(Pr(X|K)) for the number of clusters (K) ranging from two to 20 clusters; nevertheless, after K = 8 the rate of increase in probability became markedly smaller. In addition, the barplots showed that for K > 8 each new cluster appeared completely admixed and therefore did not correspond to a genuine population structure. Together this indicated that K = 8 was the finest genetic structure we could detect with our dataset.

At K = 3, a clear separation was observed between North America, northern Europe and southern Europe. Populations from North America, Norway and the Pyrenees were mostly assigned to a single cluster each (blue, yellow and green clusters, respectively), while individuals from the Alps were assigned to either one of these three clusters (Figs. 4 and 5).

At K = 8 the North American populations were mainly assigned to two clusters (blue and orange) (Figs. 4 and 5). The blue cluster was exclusively found in North America, and mostly in Colorado, while the orange cluster occurred mostly in Alaska but also in the Alps. In Norway, two clusters were found (yellow and brown), scattered across all Norwegian regions. Samples from central Alps were mainly assigned to three clusters (red, orange and violet), also with some individuals from the western Alps. Individuals from southern Alps were assigned to two clusters, one being restricted to this area (light green), while the other cluster could be also found in Iceland and Pyrenees (dark green). Pyrenees and Iceland populations were highly admixed, with

substantial membership proportions in northern and southern European populations.

INSTRUCT, which jointly estimates selfing rates and population structure, inferred a similar population structure as the STRUCTURE software. For K = 5, i.e., the highest K-value for which cluster assignment was clear, the lowest selfing rates were found in the dark green cluster (0.37), in the southern Alps, while the highest selfing rate was inferred in the red cluster (0.67), in central Alps. Allelic richness (A_r) was lowest in the Norway yellow cluster (A_r = 1.69) and highest in the dark green cluster in the southern Alps (A_r = 3.51) (Table 2).

PCA analyses displayed patterns largely consistent with the Bayesian clustering (Fig. S2). PCA and pairwise F_{ST} values (Table 3; Figs. 5 and S2) further revealed a close relationship between the two clusters identified in Norway/Svalbard, which also appeared genetically close to the red cluster in central Alps. The blue cluster, which occurred mostly in the North American populations, appeared genetically the most distant.

Genetic structure of Norway/Svalbard populations

For the populations from Norway and Svalbard, the set of the seven new microsatellite markers showed similar levels of genetic variability of *M. silenes-acaulis* as the previously developed markers. The average number of alleles per population indeed ranged from 1.6 to 2.14 across the 110 individuals collected in the 18 populations from Norway and Svalbard populations. Observed heterozygosity (H_O) was lower than expected heterozygosity (H_E) for all seven loci, and the tendency of homozygosity excess was also indicated by high G_{IS} values, with an average of 0.65 (Table 1, Table S2).

STRUCTURE results displayed an increase of the likelihood Ln(Pr(X|K) for increasing K up to K = 9, but the increase became smaller above K = 4. The Evanno-method thus indicated that the

subdivision in four genetic clusters was the strongest structure (Fig. S1). Results of the structure analysis supported the existence of four genetic clusters, that were however not strongly associated with geographical regions (Figs. S3 and S4). Each cluster was indeed present in all regions, although the yellow cluster was mainly restricted to Svalbard populations (Figs. S3 and S4). This cluster appeared to be the most genetically distant based on F_{ST} values (Table S4; Fig. S4). The selfing rates inferred by INSTRUCT varied from 0.72 to 0.81 for the four genetic clusters (Table 4).

DISCUSSION

The present study combined herbarium surveys and population genetics for understanding the effects of climate and environmental factors on the distribution and population subdivision a plant pathogen species with an arctic-alpine distribution.

Distribution of *Silene acaulis* and anther-smut disease

Herbarium surveys allowed mapping the distribution of the plant *S. acaulis* and of its anther smut pathogen *M. silenes-acaulis* at a broad geographic scale. The geographical range inferred for *S. acaulis* was largely in agreement with the herbarium and records-based species map provided by Hultén and Fries (1986). This supports the utility of herbarium collections as resources for estimating worldwide species ranges, including for fungal pathogens preserved with their hosts (Antonovics *et al.* 2003; Hood *et al.* 2010). The absence of notes about anther-smut disease on specimens in herbarium surveys further confirmed that collectors do not appear to detect infections on the plants, which has been also shown by other studies (Hood & Antonovics 2003),

suggesting that collection bias has likely been minimal. Thus, herbaria surveys are useful for inferring disease range and rates of disease in nature (Antonovics *et al.* 2003).

Anther-smut pathogen was found throughout the geographic range of *S. acaulis* with an incidence of 2.9%, which is twice the average rate compared to other *Silene* hosts of anther smuts estimated from broader herbarium surveys (Hood *et al.* 2010). Part of the explanation for the high levels of disease on *S. acaulis* may come from the very long-lived nature of the plants (several hundred years; Forbis & Doak 2004) in combination with a systemic infection from which *S. acaulis* typically rarely recovers (Marr *et al.* 2005). Using a model that incorporates dynamics of anther smut transmission, Bruns *et al.* (2015) recently showed that host longevity is associated with higher equilibrium disease prevalence (*i.e.,* proportion of host individuals that are diseased within populations), which would result in the disease being more likely to be sampled when herbarium materials are collected.

In addition, there was significantly greater incidence of anther-smut disease in more northern latitudes and where the host locations were less dense, indicating an influence of environmental factors and/or host demographic structure on the pathogen distribution. While there is a possibility that far northern regions have lower densities of collection sites because of biases in sampling efforts, previous herbarium surveys have suggested the importance of host demography on disease prevalence, where the disease was more common in host species exhibiting higher levels of localized endemicity (Hood *et al.* 2010) or in more isolated localities (Antonovics *et al.* 2003). A negative effect of host site density on anther smut incidence may appear surprising, as higher host density is usually thought to promote sustainability of pathogen populations (Anderson & May 1979). However, field studies of *Microbotryum* on the plant *Viscaria alpine* also found higher disease prevalence in regions of more rarefied host populations (Carlsson-

Granér & Thrall 2006; Carlsson-Granér *et al.* 2014). This was explained by lower population connectedness preventing the maintenance and selection for resistant host genotypes. In *S. acaulis*, additional losses of resistant alleles might have occurred due to founder effects during northward recolonization after the last glaciation. Resistance is indeed known to display extensive genetic variation within *Silene* hosts (Biere & Honders 1996; Cafuir *et al.* 2007) while infection ability varies little within *Microbotryum* species (Kaltz *et al.* 1999); it would therefore be expected that founder effects in northern areas would have less of an impact on infection ability in the pathogen than on resistance within the host. In addition to these possible influences on resistance polymorphisms, climatic effects of latitude may play a direct role in determining anther smut distributions, through a sensitivity of the pathogen to warmer conditions. *Microbotryum* as a genus tends to exhibit an association between high frequencies of disease and mountainous regions (Hood *et al.* 2010), and it has been assumed that, like many fungal diseases, it is favored by cooler temperatures. The recent study by Abbate and Antonovics (2014) actually demonstrated that *Microbotryum* species affecting *S. vulgaris* were more likely to be found in host populations where overall temperatures were lower.

Population structure of *M. silenes-acaulis* in comparison to other non-arctic-alpine species

The genetic diversity of *M. silenes-acaulis* was low overall compared to other *Microbotryum* species, such as *M. lychnidis-dioicae* and *M. silenes-dioicae* (*Vercken et al.* 2010). This could be due to *M. silenes-acaulis* having a relatively small effective population size, due to a high degree of endemicity for arctic and alpine environments. In comparison, population size and connectedness in the widespread *Silene* hosts *S. latifolia* and *S. dioica* have much more generalist

habitats that may facilitate the exchange and maintenance of allelic diversity in their anther smuts.

Additional host-specific features of *S. acaulis* may also be expected to affect pathogen genetic diversity within host populations and even within individual host plants through multiple infections, as is known to occur in *M. lychnidis-dioicae* (Hood 2003; Lopez-Villavicencio *et al.* 2007). In particular, this study shows that for some samples from the Alps most stems within a given plant carry different *M. silenes-acaulis* genotypes. The long lifespan and the high number of stems of the host plant in particular are expected to favor the occurrence of multiple infections, as it increases the probability that different genotypes are deposited on a given host individual plant and that different genotypes occupy different stems. Marr & Delph (2005) estimated that their particular *S. acaulis* population under-study could have persisted for approximately 2000 years based on past geological data, and *S. acaulis* cushions can bear a multitude of perennial stems (Jones & Richards 1962).

Our results suggested a predominantly selfing mating system in *M. silenes-acaulis*. Although selfing is typical of *Microbotryum* species (Giraud *et al.* 2008), estimated selfing rates were not as severe as previously observed in *M. lychnidis-dioicae* and *M. silenes-dioicae*, for example (Vercken *et al.* 2010; Gladieux *et al.* 2011). Opportunities to encounter a mating partner for outcrossing may be higher in *M. silenes-acaulis* than in other *Microbotryum* species due to the long life span, high number of stems per plant, persistence of host populations, and low disease recovery rates that characterize *S. acaulis* (Marr & Delph 2005). Alternatively, arctic alpine environment and long generation time (Bruns *et al.* 2015) may affect mating system and outcrossing rates in fungal systems in a similar way as described for plants. In plants, artic-alpine environments have been suggested to increase selfing rates due to inadequate pollinator services

in harsh environments, stress-induced increase in levels of self-compatibility and a paucity of self-incompatibility alleles within smaller populations (Karron *et al.* 2012; Levin *et al.* 2012).

Genetic subdivision and migration in *M. silenes-acaulis*

Long-standing physical barriers (*e.g.* oceans or mountains) are known to limit dispersal and induce differentiation of populations (Abbott & Brochmann 2003; Eidesen *et al.* 2013). Our results revealed strong differentiation in *M. silenes-acaulis* between populations from North America and Europe, as previously reported in other arctic-alpine distributed species (Abbott *et al.* 2003; Alsos *et al.* 2007; Westergaard *et al.* 2011). Genetic footprints of long-distance dispersal were nevertheless detected, as North American individuals assigned to European clusters. This feature is shared with several arctic-alpine plant species, for which genetic data have provided ample evidence for extreme colonization abilities, in many cases despite the lack of obvious adaptations to long-distance dispersal (Brochmann & Brysting 2008). It remains speculation whether this is human-mediated by importing plants as ornamentals (*e.g.,* Farr *et al.* 1989) or if long-distance dispersal of seeds of *S. acaulis* occurs more regular e.g. by seed transfer in feathers of birds (*e.g.,* Lira-Noriega *et al*. 2015).

The biological features of *Microbotryum* concerning long-distance dispersal similarly raise questions, because they are obligate, specialized pathogens that are neither transmitted by the seeds nor persistent as contaminants of the environment (Begerow *et al.* 2014). However, the case of the temperate species *M. lychnidis-dioicae* – that was introduced in North America – shows that even a vector-borne pathogen can successfully invade a new continent multiple times independently (Fontaine *et al.* 2013; Gladieux *et al.* 2015).

Moreover the present study revealed a clear genetic differentiation between southern and

northern European populations, similar to the population structure of its host *S. acaulis* (Mikhailova *et al.* 2010). During the last glaciation, conditions in central Europe were not suitable even for cold-adapted organisms, so that populations have remained separated for long in distinct refugia, which has fostered population differentiation. Multiple previous studies have similarly revealed footprints of distinct glacial refugia in Europe (Hewitt 1996, 2004), including in artic-alpine plants (Abbott & Brochmann 2003; Eidesen *et al.* 2013). Only few studies have investigated population genetic structures at large scales in natural plant pathogens (*i.e.,* non-crop disease), and some of them have found subdivision between continents, *e.g.,* in *Melampsora larici-populina* (Barrès *et al.* 2008).

The differentiation in southern Europe of several distinct genepools in separated refugia also explains the highest levels of genetic variation in *M. silenes-acaulis* in southern Europe. Post-glacial migration from several peripheral refugia or even "nunataks" would then have resulted in the observed co-occurrence of several clusters within small areas as well as admixture (Schönswetter *et al.* 2006). High genetic diversity and cluster richness in southern Europe are features shared with several other arctic-alpine species i.e. *Arabis alpina* (Koch *et al.* 2006) and *Ranunculus glacialis* (Schönswetter *et al.* 2003).

The lower genetic diversity and the occurrence of distinct clusters in the more northern populations of *M. silenes-acaulis* in Europe would result from demographic bottlenecks following recolonization from more southern refugia. This is supported by the northern clusters being derived from clusters occurring in southern Europe in the NJ trees. Similar patterns have been observed for the host species *S. acaulis* (Abbott 1995; Mikhaylova *et al.* 2010) and other organisms (Abbott & Brochmann 2003).

CONCLUSION

This study combined surveys of natural history collections and population genetics to infer the global biogeography of a fungal pathogen with arctic-alpine distribution. The pathogen appears to have a nearly ubiquitous presence in regions occupied by the hosts *S. acaulis*. The significantly greater incidence of the pathogen in more northern and more rarefied host locations reveals a major within-species variation in disease risk that has been rarely observed in non-agricultural or domesticated systems. This may be due to lower genetic variation for resistance in northern regions due to post-glacial recolonization and a paucity of host population connectedness. We additionally found evidence for strong population structure within *M. silenes-acaulis,* suggesting persistence in distinct glacial refugia, followed by recolonization from multiple differentiated populations and recent migration events. These overall patterns are consistent with those reported in the host *S. acaulis* (Abbott 1995; Mikhaylova *et al.* 2010). This supports the very close and long-term association of the obligate and specialized pathogen on a very long-lived herbaceous host throughout integrated processes of recolonization after the last glaciation.

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DATA ACCESSIBILITY

- Sampling locations: Online Supporting Information

- Dryad: Microsatellite data, Structure Input files, Herbaria-Collections: doi:10.5061/dryad.5pt70

AUTHOR CONTRIBUTIONS

MEH analysed herbarium specimens. MEH and TG performed the sampling in the global dataset. CE developed the second set of markers and performed the samling in Norway and Svalbard. ASch helped with the design of the second marker set and designed the sampling strategy in Norway and Svalbard. DB coordinated the sampling and analysis of the second set of markers. DJB and AS genotyped the global dataset. PG and DJB carried out preliminary analyses of a first version of the dataset. BB performed the final analyses of population genetics for both datasets. PG supervised population genetics analyses. BB, MEH, PG and TG wrote the manuscript, with contributions by all authors. TG, MEH and DB supervised the whole project.

TABLES

Table 1. Polymorphism in *Microbotryum silenes-acaulis* from different geographic regions.

	Southern Europe[1]	Northern Europe[1]	North America[1]	Norway/Svalbard[2]
N	53	121	21	110
A_r [+]	4.89	2.65	3.76	2.87
H_O [+]	0.19	0.11	0.18	0.08
H_E [+]	0.45	0.18	0.35	0.33
G_{IS} [+]	0.32**	0.09**	0.13**	0.65**

N: Number of individuals analyzed, A_r: Allelic richness, H_E: Expected heterozygosity, H_O: Observed heterozygosity, G_{IS}: Inbreeding coefficient, [1]: using sixteen microsatellite markers developed in Giraud *et al.* 2008 and Bucheli *et al.* 1998, [2]: using the seven microsatellite markers developed in this study. [+]Average across markers. ** p-value < 0.05; ** p-value < 0.01.

Table 2. Number of *Microbotryum silenes-acaulis* individuals, diversity (allelic richness A_r and expected heterozygosity H_E) and selfing rates for the STRUCTURE clusters with K = 5 in the whole dataset of *Microbotryum silenes-acaulis.*

	N	A_r	H_E	Selfing rate
Blue cluster/ `North America´	16	2.31	0.54	0.63
Yellow cluster/ `Norway´	106	1.69	0.18	0.67
Dark Green cluster/ `South Europe I´	22	3.51	0.58	0.37
Red cluster/ `South Europe II´	22	2.04	0.08	0.69
Orange cluster/ `South Europe III´	13	2.10	0.42	0.46

[+]Average across the sixteen microsatellite markers developed in Giraud *et al.* 2008 and Bucheli *et al.* 1998; N: Number of individuals, A_r: Allelic richness; H_E: Expected heterozygosity

Table 3. Genetic differentiation (F_{ST}) values between *Microbotryum silenes-acaulis* genetic clusters at K = 5.

	Blue	Yellow	Dark Green	Red	Orange
Blue cluster/ `North America´	0				
Yellow cluster/ `Norway´	0.43	0			
Dark Green cluster/ `South Europe I´	0.31	0.23	0		
Red cluster/ `South Europe II´	0.45	0.09	0.16	0	
Orange cluster/ `South Europe III´	0.35	0.15	0.25	0.20	0

Table 4. Number of individuals, diversity (allelic richness A_r and expected heterozygosity H_E) and selfing rates for the genetic clusters at K=4 in the Norway/Svalbard populations of *Microbotryum silenes-acaulis.*

	N	A_r	H_E	Selfing rate
Red cluster	28	2.02	0.22	0.81
Blue cluster	22	2.04	0.18	0.72
Yellow cluster	23	1.77	0.16	0.74
Green cluster	25	2.12	0.19	0.77

[+]Average across seven microsatellite markers developed in the present study; N: Number of individuals analyzed, Number of different alleles per locus; A_r: Allelic richness; H_E: Expected heterozygosity.

FIGURE LEGENDS

Figure 1. Distribution map of healthy (green points) and diseased (red points) Silene acaulis specimen observed in herbaria. The distribution map of S. acaulis after Hulten Fries (1986) is shown in green. Blue points represent regions from where populations for present study were sampled.

Figure 2. Density of Silene acaulis localities near diseased and healthy herbarium collection sites. Sites are sorted by the number of other collections sites within a 100 km radius around diseased sites (red line) and around randomly selected healthy sites (grey lines; first 40 random healthy sites shown).

Figure 3. Demographic and disease incidence variation in Silene acaulis across southern to northern latitudes. Herbarium localities (N = 790) were divided into latitude quartiles for analysis and density of herbarium collection sites was based upon 100km radii.

Figure 4. Genetic clustering of Microbotryum silenes-acaulis genotypes for the worldwide data set (N = 195) under the assumptions from K = 3 to K = 8 clusters. Each vertical bar represents an individual and the colors indicate the proportion of its genotype assigned to the different clusters.

Figure 5. Maps showing the mean assignment proportions per locality estimated from the STRUCTURE analyses at K = 3 (a) and K = 8 (b). The square at the bottom is a zoom on the three localities in the Alps. Neighbour-joining trees illustrate the genetic distances among clusters based on FST distances.