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Lichenicolous fungi show population subdivision by host species but do not share population history with their hosts

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ABSTRACT

Lichenicolous fungi are a species-rich biological group growing on lichen thalli. Here, we analyze the genetic structure of the lichenicolous basidiomycete *Tremella lobariacearum* and three host species (*Lobaria pulmonaria*, *Lobaria macaronesica*, and *Lobaria immixta*) in Macaronesia. We used ordination and analysis of molecular variance to investigate the structuring of genetic variation, and a simulation test to investigate whether rDNA haplotypes of *T. lobariacearum* were significantly associated with host species. To investigate the evolutionary and demographic history of the lichenicolous fungus and its hosts, we used coalescent samplers to generate trees, and Bayesian skyline plots. We found that the hosts were most important in structuring populations of the lichenicolous species. Despite their wide geographic distribution, the same haplotypes of *T. lobariacearum* consistently associated with a given host species. Our results suggest that the *Lobaria* hosts create a selective environment for the lichenicolous fungus. Both the pathogen and the host populations exhibited substantial genetic structure. However, evolutionary and demographic histories differed between the parasite and its hosts, as evidenced by different divergence times and tree topologies.

Introduction

Lichenicolous fungi are a taxonomically heterogeneous group forming obligate associations with lichens. More than 1500 species are known to date, while a total of 5000–7500 species have been predicted to exist in nature (Lawrey & Diederich 2003). The diversity of interactions in these fungi is very high, spanning from autotrophic lichenicolous lichens, commensals that do not damage their hosts ('parasymbionts'), parasites that may be weakly or strongly pathogenic, to saprotrophs colonizing dead lichens (Poelt 1958; Hafellner 1979; Hawksworth 1982b; Rambold & Triebel 1992). The virulence of lichenicolous fungi is inversely correlated to host-specialization – the more pathogenic fungi are generally less host-specific (Hawksworth 1982b). Conversely, highly host-specific lichenicolous fungi are often benign, and have been suggested to coevolve with their hosts (Hawksworth 1982a; Stone & Hawksworth 1986; Lawrey & Diederich 2003).

The only study to date investigating the intraspecific genetic variability of a lichenicolous fungus was conducted on the basidiomycete *Marchandiomyces corallinus* (Agaricomycetes), a fungus parasitizing a wide range of lichen fungi

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(mycobionts) (Molina et al. 2005). This study suggested that geographic structure predominates by far over any host-species related genetic differences, but this pattern has never been tested in a host-specific lichenicolous species.

The great majority of lichenicolous fungi – 95 % – belong to the ascomycetes (Lawrey & Diederich 2003), but basidiomycetes are also represented. Among the latter, *Tremella* (Tremellales, Basidiomycota, Fungi) is a morphologically variable genus, which includes numerous mycoparasitic species growing on a wide taxonomic range of fungal hosts, including lichenized fungi (Diederich 1996; Chen 1998; Lawrey & Diederich 2003; Sérusiaux et al. 2003; Millanes et al. 2011; Zamora et al. 2011; Millanes et al. 2012).

Lichenicolous *Tremella* species are generally very host-specific and among them, *Tremella lobariacearum* is an interesting taxon to test the influence of host-specialization *vs.* geographic isolation on the genetic structure of its populations. Being a widespread species, *T. lobariacearum* has known occurrences in continental Europe (France), Macaronesia (Azores, Canary Islands, and Madeira), Asia (Russia, Japan, Papua New Guinea, Philippines), South America and Africa (Madagascar, La Réunion) (Diederich 1996). Across its range, *T. lobariacearum* associates with multiple species in the lichen-forming fungal genera *Lobaria* and *Pseudocyphellaria* (Diederich 1996). We will restrict our study area to Macaronesia (Fig 1), where the geographic isolation of archipelagos is expected to be an important factor determining population structure in both *T. lobariacearum* and its hosts. In Macaronesia, the lichenicolous fungus occurs frequently on three host species (Fig 1): on the widespread *Lobaria pulmonaria*, and on two closely related Macaronesian endemics, *Lobaria immixta* (Diederich 1996) and *Lobaria macaronesica* (Cornejo & Scheidegger 2010; Werth et al. 2010); the species has also been reported from *Lobaria sublaevis* on Madeira, but during our field work, we did not find *T. lobariacearum* on this host species. Infections are easily identified as they form conspicuous galls on thalli of *Lobaria* spp., either developed on the upper or lower surface of the thallus, or on vegetative symbiotic propagules (Fig 1). *Tremella lobariacearum* does not kill its host thalli, and the interaction appears to be either commensalistic or mildly pathogenic. In galls, the fungus forms basidia producing haploid basidiospores that are released and, after germination and mating with a compatible fungal strain, can infect a new host. Additionally, *T. lobariacearum* forms conidia (Diederich 1996), which probably function as vegetative propagules. Codispersal of *T. lobariacearum* with vegetative propagules of *Lobaria* spp. seems likely, as the vegetative propagules of the hosts are frequently infected.

The evolutionary and demographic history of a pathogen should resemble that of its host, because in interacting species, coevolutionary processes such as coadaptation act at the level of genes and may create non-random associations between haplotypes (Wade 2007). In some cases, the genetic structure of a pathogen may provide valuable information on the population history and demography of its host (Nieberding & Olivieri 2007). Moreover, we would expect similar demographic histories because a pathogen's population dynamics is tied to that of its host. In highly host-specific lichenicolous fungi, which are often restricted to grow on hosts belonging to the same genus, such specificity may translate to congruent genetic structures among host lichens and lichenicolous fungi. If only certain haplotypes of the pathogen could infect a particular host species, we expect to find substantial genetic structure among the lichenicolous fungi sampled from different hosts, irrespective of geographic location. This pattern of host-associated haplotypes would prevail even if gene flow were high, as long as gene flow occurred only within groups with different host preference. However, whether the genetic structure of a lichenicolous fungus corresponds to that of its lichen fungal hosts also depends on the relative spatial extent of gene flow in the hosts *vs.* the pathogens, and on the transmission mode of the lichenicolous fungus. Similar to the case of photobiont transmission in lichens (Werth & Sork 2008; Werth & Sork 2010; Dal Grande et al. 2012), genetic structures would be expected to be similar if both fungi were predominantly codispersed, which is the case if the lichen fungus reproduces vegetatively with symbiotic propagules infested by the lichenicolous fungus. In contrast, in the case of frequent horizontal transfer of the lichenicolous species, the genetic structures would get uncoupled and may then differ markedly, unless hosts and parasites are co-adapted (Werth & Scheidegger 2012). In the latter case, host–parasite compatibility mechanisms could prevent the infection of certain host strains or species, and non-random associations of host and parasite genotypes could thus be expected, corresponding to host-associated structure in the lichenicolous fungus despite of horizontal transmission mode and widespread gene flow.

Here, first, we determine the degree to which geography and host-specificity shape the genetic structure of *T. lobariacearum*. Second, by estimating divergence time and population demographic parameters, we assess if the lichenicolous fungi and their hosts share similar evolutionary and demographic history. Our study is among the first to report the population genetic structure of a lichenicolous fungal species, and is the first to compare demographic histories among lichenicolous fungi and their lichen hosts.

Material and methods

Study area and sampling

Our study area included the Macaronesian archipelagos with their typical laurisilva vegetation and a single site situated on the Iberian Peninsula (Fig 1; Electronic Supplementary Material, Table S1). Macaronesia has a well-known geological history, and includes the volcanic archipelagos of Madeira (5.6–14 Myr, Borges et al. 2008), the Azores (0.25–8 Myr, Borges & Hortal 2009), and the Canary Islands (1.12–35 Myr, Carracedo et al. 1998). The archipelagos are several hundred km apart (440–860 km), and the one closest to the European continent is Madeira (880 km). The easternmost Canary Islands are situated at a distance of 100 km from West-Sahara, but neither *Tremella lobariacearum* nor any of its lichen hosts have been reported from there or adjacent southwestern Morocco.

Thalli of *Lobaria pulmonaria*, *Lobaria macaronesica*, and *Lobaria immixta* were sampled for a phylogeographic study of these three lichen species (Werth S. and Scheidegger C.,

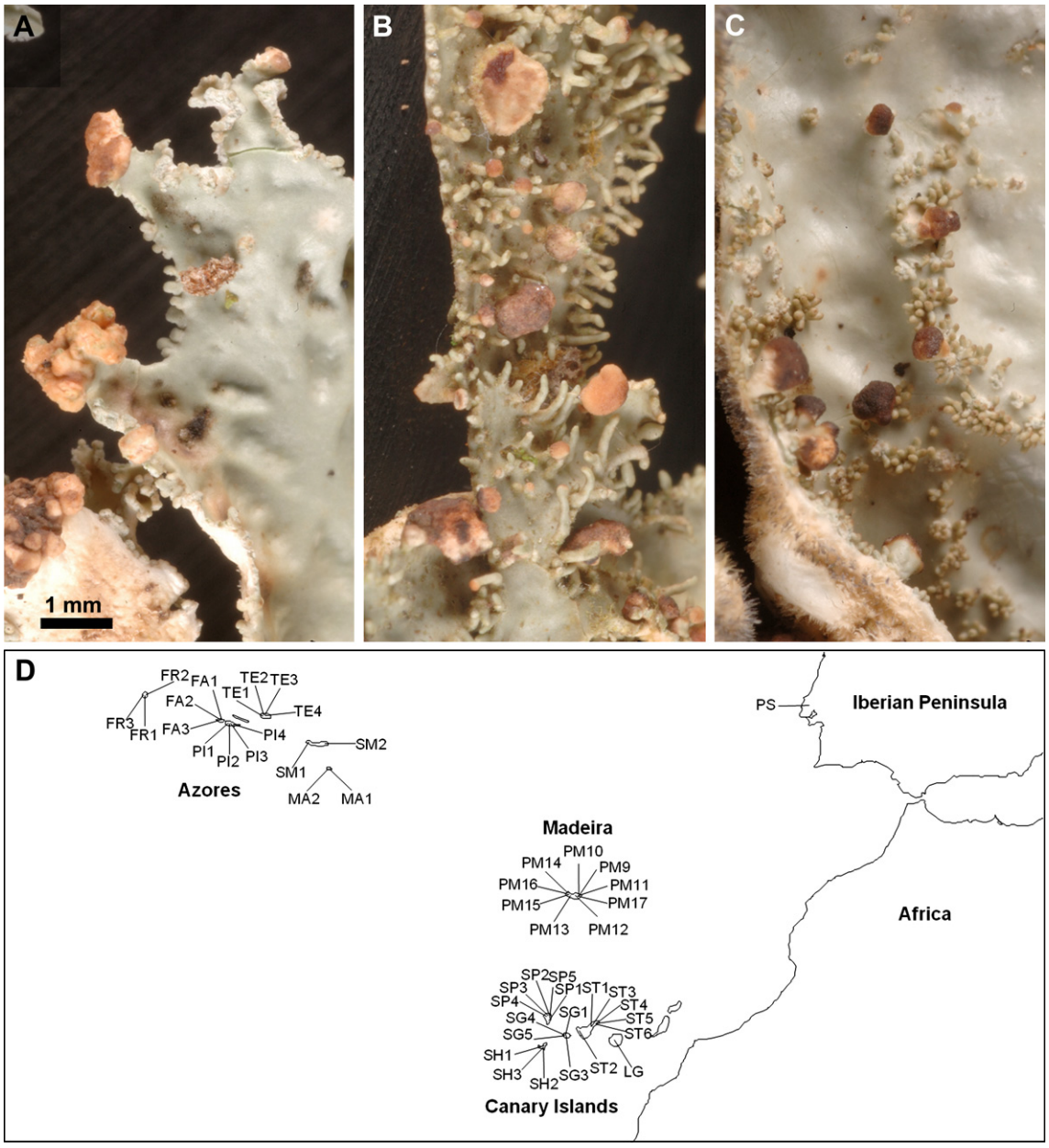

Fig 1 – Study species and study area. (A–C) *Tremella lobariacearum* infecting lichen hosts; all pictures were taken with the same magnification. (A) Typical brown apical and marginal galls of *T. lobariacearum* on symbiotic propagules (phyllidia) of the lichen *Lobaria immixta*. (B) Galls of *T. lobariacearum* on *L. macaronesica*, infesting its cylindrical symbiotic propagules (isidia), and causing laminal and marginal brown swellings. (C) On *L. pulmonaria*, galls are typically laminal on the symbiotic propagules (soredia and isidioid soredia). (D) Spatial location of the sampling sites. Map source: ArcWorld Supplement, ESRI, Redlands, California, USA, 1996.

unpublished data). We sampled 30–50 thalli per site and species, or less if fewer were found. Thalli were inspected with a stereomicroscope for infection with *T. lobariacearum*, i.e., the presence of orange-brown or blackish galls on isidia, phyllidia, thallus upper and lower surface (Fig 1). From each infected thallus, a single gall of *T. lobariacearum* was excised and used to isolate DNA.

To investigate geographic and host-species related genetic structure in *T. lobariacearum*, we attempted to sample 1–2 specimens per host species and site. In some sites, not all host species were colonized by *T. lobariacearum*, and our sample is thus not completely balanced. In total, 90 specimens of *T. lobariacearum* were included in the analyses, sampled from 28 sites (Table S1, Fig 1). Nine sites were sampled on the Azores, comprising 27 specimens; 13 sites on the Canary Islands ($N = 37$); five sites were sampled on Madeira ($N = 19$); and one site on the Iberian Peninsula ($N = 2$; *T. lobariacearum* only on *L. pulmonaria*). Specimens are deposited in the cryopreserved herbarium of CS at Swiss Federal Research Institute WSL.

As we did not intend to perform a direct comparison of genetic distances between *Lobaria* hosts and their *T. lobariacearum* pathogen, we did not require matching pathogen–host samples. For the *Lobaria* hosts, our main goal was to infer whether they shared evolutionary and demographic history with *T. lobariacearum*. One of the basic assumptions of coalescent analysis is random sampling. Hence, we randomly picked one to two thalli per host species and sampling site for DNA sequencing. For the analyses, host individuals were retained that had DNA sequence data for both loci.

Laboratory procedures

Lichenicolous fungus

DNA was isolated from air-dried thallus material using the DNeasy 96 well plant kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR and Sanger DNA sequencing of several parts of the nuclear ribosomal gene cluster (rDNA) were performed following Millanes *et al.* (2011): the primers ITS1F (Gardes & Bruns 1993), BasidLSU3-3 and BasidLSU1-3 (Millanes *et al.* 2011) were used to amplify the internal transcribed spacer (ITS) I, the 5.8S rDNA gene, the ITS II and a short fragment of approximately 400 bp in the nLSU rDNA gene (rDNA). The PCR primers selectively amplified *Tremella* sp., and had been designed to discriminate against lecanoromycetalean hosts. PCR amplifications used the Jump Start Red Taq polymerase kit (Sigma–Aldrich, Buchs, Switzerland), using PCR settings described in Millanes *et al.* (2011), but reducing initial denaturation to 2 min. Cycle sequencing was performed using BigDye 3.1 (Life Technologies, Rotkreuz, Switzerland) according to the manufacturer's protocol, but in a reaction volume of 10 μL.

We used Sephadex G50, fine DNA-grade (GE Healthcare, Buckinghamshire, UK) in combination with 96 well filtration plates (Millipore, Zug, Switzerland) to clean up PCR products and cycle sequencing reactions. The DNA sequences were run on an automated ABI 3130 capillary sequencer using a 36 cm array and the polymer Pop7 (Applied Biosystems, Rotkreuz, Switzerland). All unique DNA sequences were deposited in GenBank (Electronic Supplementary Material, Table S1).

Host species

DNA of the haploid *Lobaria* hosts of *Tremella lobariacearum* was isolated using the same method as for the lichenicolous species. The three species of *Lobaria* were identified using a species-specific RealTime PCR assay according to the methods given in Werth *et al.* (2010). PCR and Sanger sequencing of the RNA polymerase II (RPB2) and ITS regions of *Lobaria immixta*, *Lobaria macaronesica* and *Lobaria pulmonaria* followed Cornejo & Scheidegger (2010); all unique sequences were deposited in GenBank (Electronic Supplementary Material, Table S2).

Data analyses

Multiple sequence alignment and polymorphism

DNA sequences were aligned manually in MEGA version 4 (Tamura *et al.* 2007) because the alignment was straightforward due to the presence of only few and short (2 bp) indel polymorphisms. The rDNA of *Tremella lobariacearum* included two indels ('CC' *vs.* 'C-' and 'TT' *vs.* 'T-'). The RPB2 region of the hosts contained no indels. The ITS region of the hosts contained five short indels (1. 'TG' or '–', 2. 'CC' or '-T', 3. 'TT' or '-T', 4. 'AA' or 'A-', 5. '–','TC' or 'CC'). Nucleotide and haplotype diversity were calculated for the rDNA data of *T. lobariacearum* and for the ITS and RPB2 loci of the *Lobaria* hosts using DNAsp version 4.10.6 (Rozas *et al.* 2003). One individual of *T. lobariacearum*, the only individual carrying haplotype H11, sampled from a *Lobaria immixta* was excluded from the analysis because of an ambiguous site, as identified by a mixed base in the DNA sequence electropherogram.

Population subdivision & specificity

We performed hierarchical analyses of molecular variance (AMOVA) in R using the library 'ade4' (function 'amova'), nesting archipelagos (Madeira, Canary Islands, Azores) within lichen-forming fungi (*Lobaria pulmonaria*, *Lobaria macaronesica*, and *Lobaria immixta*). Identical hierarchical AMOVA models were used for the lichenicolous fungus (rDNA data set) and its host species (ITS and RPB2 sequences). The single site on the Iberian Peninsula was excluded from the AMOVAs because of its low sample size for *Tremella lobariacearum* ($n = 2$). The AMOVAS were performed using raw distances between haplotypes computed with the library 'ape' in R. Statistical significance of variance components was assessed using a randomization test (function 'randtest.amova') with 999 permutations.

To identify if host species and/or geography explained significant portions of the variance in the *T. lobariacearum* DNA sequence data, and to quantify the amount of covariance between the two, we performed redundancy analysis (RDA) with variance partitioning (Borcard *et al.* 1992; Peres-Neto *et al.* 2006) using the functions 'rda' and 'varpart' implemented in the library 'vegan' in R (Oksanen *et al.* 2010). For the RDA, we used a binary coded matrix containing rDNA haplotype data of *T. lobariacearum*. As constraining matrices, we used a binary matrix of the host species and a second of the geographic location (presence/absence on islands). The significance of individual fractions of variance was tested in R using the function 'anova.cca' with 2000 permutations.

Last but not least, we were interested in examining whether the observed number of occurrences of each rDNA haplotype of *T. lobariacearum* on the three host species deviated from the expectation under random association. Thus, we devised a simulation test where we randomly drew rDNA haplotypes with replacement based on their observed frequencies, keeping the host species constant (Werth & Scheidegger 2012). Using the function 'sample' in R, 1000 resamples of the data were performed. Next, we calculated the average number of occurrences of each rDNA haplotype of *T. lobariacearum* on the hosts based on the resampled data, i.e., the expected number of rDNA haplotype occurrences on each host under random association. Based on the values of the 1000 resamples, we computed the probability P_{SE} that the observed value was smaller or equal to the resampled value, and the probability P_L that the observed value was larger than the resampled value.

Demography & biogeography

First, used neutrality tests to tests whether the populations were in mutation–drift equilibrium. Tajima's D (Tajima

1989a,b) is based on the number of segregating sites. A significantly positive D indicates either the presence of diversifying selection, or a recent population bottleneck. A significantly negative D indicates either purifying selection, or recent range expansion, as there are many recent mutations relative to the number of segregating sites. If D is not significantly different from zero, a population is in mutation–drift equilibrium. Fu's F_S is based on the observed number of alleles (Fu 1997) and can be interpreted along the same lines as Tajima's D. A significantly negative Fu's F_S indicates an excess number of alleles relative to the expectation under neutrality, and can be interpreted as a sign of demographic expansion. A significantly positive Fu's F_S indicates a deficiency of alleles that may have been caused either by a recent bottleneck or by divergent selection.

To fulfil the assumptions of coalescent analysis of no recombination within loci, we ran IMgc (Woerner *et al.* 2007) to retrieve the largest non-recombining block of sequence for the rDNA of *Tremella lobariacearum*, as well as the ITS and RPB2 data of the three *Lobaria* species. Subsequently, coalescent analyses were performed on the data sets comprising the largest non-recombining blocks of sequence.

To assess whether tree topologies were similar for the lichenicolous fungus and its host species, we constructed maximum clade credibility trees in BEAST v. 1.6.1, sampling from a coalescent prior under constant population size (Drummond & Rambaut 2007). The analysis was run for 200 000 000 generations, logging trees and parameters every 10 000 generations. Trees were annotated in TreeAnnotator v. 1.4.8 and 2001 of a total of 20 001 trees were discarded as burn-in. Effective sample sizes were >>100 for all parameters, as calculated with the program Tracer version 1.4.1 (Rambaut & Drummond 2007), indicating that runs had converged. For *T. lobariacearum*, trees were rooted using a specimen growing on an Asian *Lobaria isidiosa*. The tree topology remained the same if the trees were midpoint rooted (data not shown). For the lichen hosts, the closely related species *Lobaria tuberculata* served as an outgroup. Trees were drawn in FigTree version 1.2 (Rambaut 2008). If the lichenicolous fungus and its hosts diverged in allopatry, and spread from the archipelago of origin to others after the establishment of reproductive barriers, the genetic signature of an increase in population size would be expected in the data. To assess such a possible population size change, we constructed Bayesian Skyline Plots in BEAST (Drummond *et al.* 2005) by sampling trees under a Bayesian skyline tree prior. The analysis was performed with five groups and a piecewise-constant skyline model. As starting tree, a random tree was used. The analysis was performed using the same number of generations and burn-in as above. Effective sample size values were inspected and skyline plots were constructed using Tracer version 1.4.1 (Rambaut & Drummond 2007). Skyline plots were also constructed using an empty alignment to see whether the results could be due to the initial configuration of the samples.

To infer whether the timing of the divergence of populations in *T. lobariacearum* happened simultaneously to the speciation of its hosts, we analyzed migration rate and divergence time between populations of *T. lobariacearum* from the three host species, and among the *Lobaria* species with coalescent analysis performed in MDIV (Nielsen & Wakeley 2001). Using maximum likelihood approaches, MDIV uses Markov chain Monte Carlo (MCMC) coalescent simulations to estimate the population size parameter θ, the migration rate M, and the divergence time T. The Hasegawa, Kishino and Yano (HKY) model was used because it allowed the inclusion of the one ambiguous site in the analysis, and thus the retention of haplotype H11. After a burn-in phase of 5×10^5, 2×10^6 trees were sampled. The maximum divergence time *T* was set to ten because it included the mode of the posterior distribution in preliminary runs, and a maximum migration rate *M* of six was used. To convert the parameter θ to the effective population size N_e, θ was divided by twice the mutation rate. The mutation rate was not known for our species, but overall neutral mutation rates of 3.296×10^{-9} and 1.391×10^{-9} substitutions per site per year have previously been reported for the ITS and RPB2 regions of parmeloid lichen-forming fungi based on a concatenated gene tree approach (Leavitt *et al.* 2012). We used these rates along with the minimum and maximum rates reported to convert our estimates of population size and divergence time. First, we multiplied the substitution rates from the literature with the number of sites. Then, we used the thus adjusted substitution rates for parameter conversion. These rates were 2.91×10^{-6} (minimum 2.2×10^{-6}, maximum 3.63×10^{-6}) for the rDNA of *T. lobariacearum*, 1.57×10^{-6} (minimum 1.18×10^{-6}, max 1.95×10^{-6}) for ITS of *Lobaria* spp., and 1.09×10^{-6} (minimum 8.47×10^{-7}, maximum 1.37×10^{-6}) for RPB2 of *Lobaria* spp.

Results

Lichenicolous fungus

Polymorphism and haplotype sharing

The rDNA sequences of *Tremella lobariacearum* were 883 bp long and contained 12 polymorphic sites. We found 13 haplotypes in the nuclear ribosomal gene cluster for *T. lobariacearum*. Figure S1 presents the number of haplotypes overlapping with, or unique to geographic regions, indicating that the Azores and Madeira had most private haplotypes, i.e., haplotypes that were unique to these regions. In general, few haplotypes were shared among geographic regions. Genetic diversity is presented in Table 1. For *T. lobariacearum* sampled from *Lobaria immixta* and *Lobaria macaronesica*, genetic diversity was highest in the Azores and Madeira archipelagos, respectively, despite lower sample sizes than in the remaining areas. *T. lobariacearum* from *Lobaria pulmonaria* showed the highest diversity in the Canary Islands and Madeira. Four haplotypes of *T. lobariacearum* were shared among *L. pulmonaria* and *L. macaronesica* hosts, three haplotypes were unique to the host *L. pulmonaria* and one haplotype was solely found on *L. macaronesica* hosts. No haplotype sharing occurred between *L. immixta* and the other hosts, and *T. lobariacearum* had four unique haplotypes on *L. immixta* (Table S3).

Population subdivision & specificity

Analysis of molecular variance (Table 2) indicated that most of the genetic structure of the lichenicolous fungus was due to genetic differentiation among host species (39.8 %, $\Phi_{RT} = 0.3979^{+}$), and a slightly smaller amount of variance

Table 1 – Polymorphism and sample size of the lichenicolous heterobasidiomycete *Tremella lobariacearum* and its three host species in Macaronesia. Samples of *T. lobariacearum* were grouped by host species. The geographic region with highest diversity is printed in bold.

Species or lineage	Sample size (N)	No. sites (s)	No. haplotypes (h)	Haplotype diversity H_d (variance)	Nucleotide diversity (π)
rDNA of *T. lobariacearum*					
Host: *L. immixta*	38	2	3	0.286 (0.0082)	0.00034
Azores	**8**	**1**	**2**	**0.536 (0.0152)**	**0.00061**
Canary Islands	16	0	1	0.000 (0.0000)	0.00000
Madeira	14	1	2	0.363 (0.0170)	0.00041
Host: *L. macaronesica*	22	6	4	0.333 (0.0155)	0.00109
Azores	6	1	2	0.533 (0.0296)	0.00061
Canary Islands	13	4	2	0.154 (0.0159)	0.00070
Madeira	**3**	**5**	**2**	**0.667 (0.0988)**	**0.00378**
Host: *L. pulmonaria*	30	9	7	0.796 (0.0019)	0.00324
Azores	12	2	3	0.545 (0.0206)	0.00069
Canary Islands	**8**	**6**	**4**	**0.750 (0.0194)**	**0.00308**
Madeira	**8**	**6**	**3**	**0.679 (0.0149)**	**0.00332**
Iberian Peninsula	2	0	1	0.000 (0.0000)	0.00000
Total	90	12	13		
ITS of *Lobaria* spp.					
L. immixta	45	4	4	0.209 (0.0062)	0.00090
Azores	16	1	2	0.125 (0.0113)	0.00027
Canary Islands	**15**	**3**	**3**	**0.448 (0.0181)**	**0.00207**
Madeira	13	0	1	0.000 (0.0000)	0.00000
Iberian Peninsula	1	0	1	0.000 (0.0000)	0.00000
L. macaronesica	47	10	10	0.766 (0.0019)	0.00185
Azores	**15**	**9**	**6**	**0.743 (0.0089)**	**0.00318**
Canary Islands	12	6	3	0.318 (0.0268)	0.00214
Madeira	11	1	2	0.182 (0.0206)	0.00039
Iberian Peninsula	9	0	1	0.000 (0.0000)	0.00000
L. pulmonaria	50	2	3	0.079 (0.0027)	0.00017
Azores	10	0	1	0.000 (0.0000)	0.00000
Canary Islands	15	1	2	0.133 (0.0126)	0.00028
Madeira	10	0	1	0.000 (0.0000)	0.00000
Iberian Peninsula	15	1	2	0.133 (0.0126)	0.00028
Total	142	32	16		
RPB2 of *Lobaria* spp.					
L. immixta	45	7	7	0.642 (0.0042)	0.00124
Azores	16	1	2	0.125 (0.0113)	0.00016
Canary Islands	**15**	**6**	**6**	**0.762 (0.0092)**	**0.00143**
Madeira	13	2	3	0.500 (0.0186)	0.00078
Iberian Peninsula	1	0	1	0.000 (0.0000)	0.00000
L. macaronesica	47	9	8	0.639 (0.0038)	0.00133
Azores	**15**	**6**	**5**	**0.752 (0.0058)**	**0.00168**
Canary Islands	12	3	4	0.561 (0.0237)	0.00081
Madeira	11	2	3	0.564 (0.0180)	0.00079
Iberian Peninsula	9	0	1	0.000 (0.0000)	0.00000
L. pulmonaria	50	8	7	0.582 (0.0050)	0.00140
Azores	10	0	1	0.000 (0.0000)	0.00000
Canary Islands	15	6	5	0.695 (0.0119)	0.00209
Madeira	10	5	4	0.778 (0.0082)	0.00181
Iberian Peninsula	15	5	4	0.600 (0.0127)	0.00129
Total	142	40	22		

was due to geographic location, i.e., archipelago (23.3 %, $\Phi_{SR} = 0.3874^{*}$), as expected from Figure S1. If we switched the grouping and grouped host species within archipelagos, no significant variance was associated with archipelago (0 %, $\Phi_{RT} = -0.19154^{NS}$), but substantial variation was associated with host species nested within archipelago (76.2 %, $\Phi_{SR} = 0.6399^{*}$).

The results from RDA with variance partitioning showed that host species explained most of the variance in the data of the lichenicolous fungus (52 %, $p = 0.0005$), followed by geography (6 %, $p = 0.004$); the covariance of geography and host contributed 3 %. The residual amounted to 39 % of the total variance.

The simulation test where *Tremella lobariacearum* rDNA haplotypes were randomly assigned to host species demonstrated that for several of the haplotypes, the observed number of occurrences on specific hosts differed significantly from the expectation under random association (Table 3).

Table 2 – Analysis of molecular variance for the rDNA sequence haplotypes in *Tremella lobariacearum* and the nuclear ribosomal ITS region and the nuclear RPB2 gene in *Lobaria pulmonaria*, *L. macaronesica*, and *L. immixta*. Both AMOVAs were hierarchical, with archipelagos nested within three *Lobaria* species, *Lobaria pulmonaria*, *L. macaronesica*, and *L. immixta*. The table gives the tested genetic structure (Structure), the degrees of freedom (df), sum of squares (SS), mean square (MS), percentage of variation (Perc), the label (Stat) and value (Value) of the Φ statistic, and the significance of the Φ statistic (*p*).

Structure	df	SS	MS	Perc	Stat	Value	*p*
***Tremella lobariacearum*, rDNA**							
Between host species	2	151.5	75.7	39.8	Φ_{RT}	0.3979	0.050
Between archipelagos within host species	6	80.3	13.4	23.3	Φ_{SR}	0.3874	0.001
Within archipelagos	75	152.9	2.0	36.9	Φ_{ST}	0.6311	0.001
Total	83	384.6	4.6	100.0			
***Lobaria species*, RPB2**							
Between species	2	6709.4	3354.7	83.5	Φ_{RT}	0.8352	0.005
Between archipelagos within species	6	181.4	30.2	1.1	Φ_{SR}	0.0666	0.046
Within archipelagos	108	1703.2	15.8	15.4	Φ_{ST}	0.8462	0.001
Total	116	8594.0	74.1	100.0			
***Lobaria species*, ITS**							
Between species	2	4582.2	2291.1	95.6	Φ_{RT}	0.9564	0.001
Between archipelagos within species	6	134.5	22.4	2.7	Φ_{SR}	0.6246	0.001
Within archipelagos	108	108.0	1.0	1.6	Φ_{ST}	0.9836	0.001
Total	116	4824.7	41.6	100.0			

The observed number of rDNA haplotype occurrences was significantly lower than expected for nine haplotypes on *Lobaria immixta* hosts, eight haplotypes on *Lobaria macaronesica*, and five haplotypes on *Lobaria pulmonaria*. Conversely, four haplotypes were found more frequently than expected on one of the host species.

Samples of the lichenicolous fungus mainly grouped by host species, thus forming three host-associated clades (Fig 2B). The tree of the lichenicolous species did not mirror the tree topology of the hosts: *T. lobariacearum* from *L. immixta* clustered with samples isolated from *L. pulmonaria* (rather than with samples from the sister species of *L. immixta*, *L. macaronesica*). In the clade containing *T. lobariacearum* sampled mainly from *L. macaronesica* hosts, seven specimens were found in association with *L. pulmonaria*. In the clade of *T. lobariacearum* associated mainly with *L. pulmonaria* hosts, a single specimen originated from a thallus of *L. macaronesica*.

Demography & evolutionary history

All but one groups of samples were in mutation–drift equilibrium, as indicated by a non-significant Tajima's D (Table 5). Only *Tremella lobariacearum* sampled from the Canary Islands had a significantly positive Tajima's D, indicating the occurrence of either a recent population bottleneck or of divergent selection. In the Bayesian Skyline plots, no population size changes were evident in *T. lobariacearum* (Fig 3). However, the data contained little information on population size changes and only a short time period was covered. The likelihood estimates obtained from MDIV for *T. lobariacearum* indicated that the scaled migration rate $2N_em$ between *Lobaria*

Table 3 – Observed and expected association of rDNA haplotypes of *Tremella lobariacearum* with host species. The table shows the rDNA haplotype of *T. lobariacearum* (rDNA), the observed number of occurrences on a host species (obs), the expected number of occurrences under randomness of the association based on the average from 1000 resamples of the data (exp), the standard error of the expected number of occurrences based on 1000 resamples (SE), the probability that the observed value is larger than the value expected under random association (P_L), the probability that the observed value is smaller or equal to the expected value under randomness (P_{SE}). Probabilities larger than 0.95 are printed in bold.

	Lobaria immixta					*Lobaria macaronesica*					*Lobaria pulmonaria*				
rDNA	obs	exp	SE	P_L	P_{SE}	obs	exp	SE	P_L	P_{SE}	obs	exp	SE	P_L	P_{SE}
H1	3	1.3	0.035	0.872	0.128	0	0.7	0.027	0.000	**1.000**	0	0.9	0.030	0.000	**1.000**
H2	0	0.9	0.029	0.000	**1.000**	0	0.5	0.023	0.000	**1.000**	2	0.7	0.026	0.861	0.139
H3	0	10.4	0.086	0.000	**1.000**	17	5.9	0.065	**0.999**	0.001	7	7.6	0.073	0.328	0.672
H4	0	0.5	0.022	0.000	**1.000**	0	0.2	0.016	0.000	**1.000**	1	0.3	0.018	0.706	0.294
H5	0	0.9	0.030	0.000	**1.000**	1	0.5	0.023	0.601	0.399	1	0.6	0.026	0.531	0.469
H6	0	0.8	0.029	0.000	**1.000**	2	0.5	0.022	0.921	0.079	0	0.7	0.026	0.000	**1.000**
H7	0	4.3	0.060	0.000	**1.000**	0	2.5	0.047	0.000	**1.000**	10	3.2	0.053	**0.999**	0.001
H8	0	0.9	0.028	0.000	**1.000**	1	0.5	0.022	0.616	0.384	1	0.7	0.024	0.497	0.503
H9	0	2.6	0.049	0.000	**1.000**	0	1.4	0.037	0.000	**1.000**	6	2.0	0.043	**0.986**	0.014
H10	0	0.9	0.031	0.000	**1.000**	1	0.5	0.022	0.625	0.375	1	0.7	0.026	0.517	0.483
H11	1	0.4	0.020	0.642	0.358	0	0.2	0.016	0.000	**1.000**	0	0.3	0.018	0.000	**1.000**
H12	32	13.9	0.096	**0.999**	0.001	0	7.8	0.072	0.000	**1.000**	0	10.4	0.081	0.000	**1.000**
H13	3	1.3	0.034	0.865	0.135	0	0.7	0.027	0.000	**1.000**	0	0.9	0.030	0.000	**1.000**

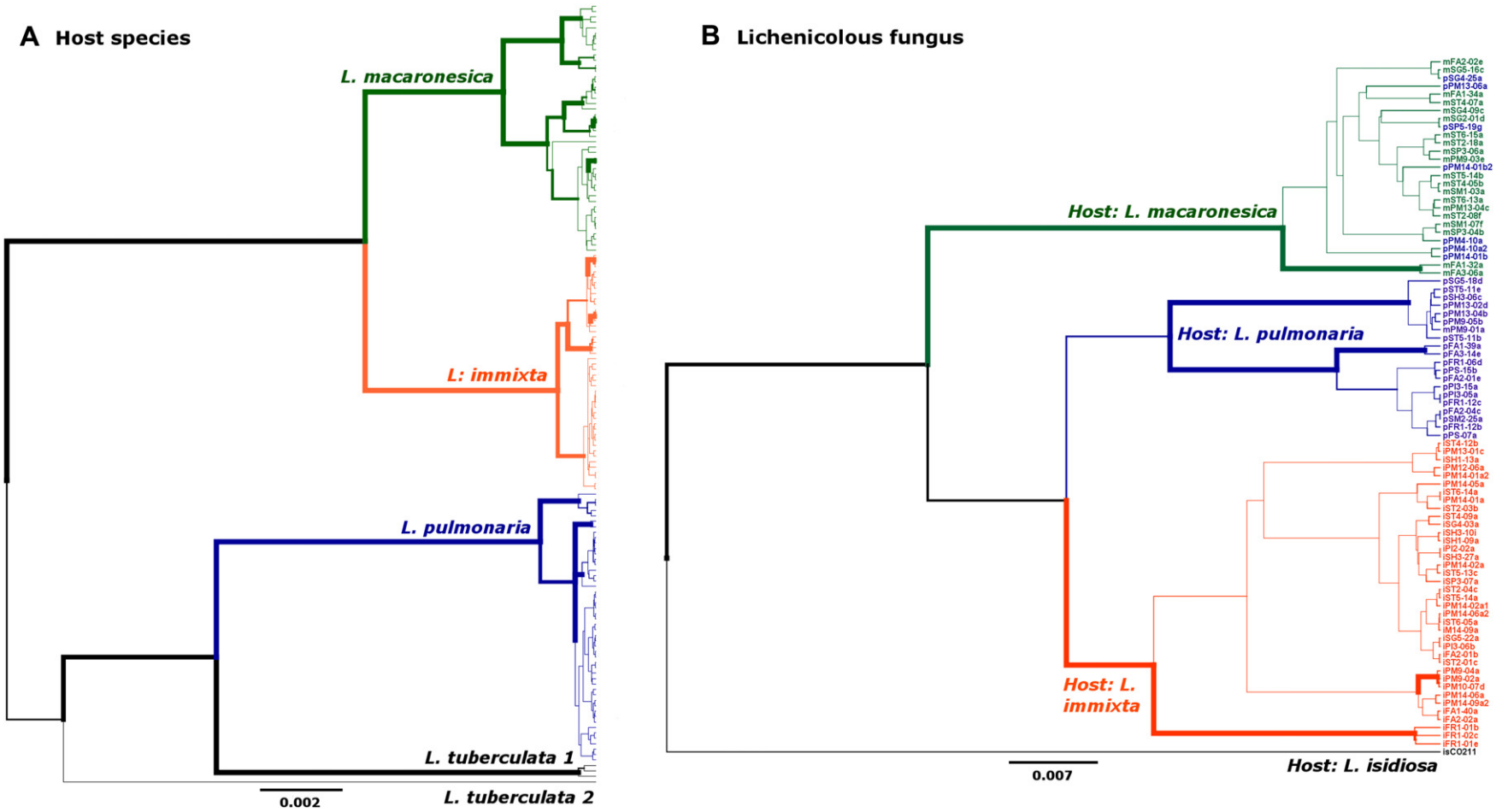

Fig 2 – Maximum clade credibility coalescent trees. The colour-coding of clades indicates the identity of lichen and host species; nodes with high posterior probabilities are indicated by thick lines. (A) Combined coalescent tree from the rDNA and RPB2 loci of the host lichens *Lobaria immixta*, *L. macaronesica*, *L. pulmonaria*, and the outgroup species *Lobaria tuberculata* 2. (B). Coalescent tree of rDNA genes of the lichenicolous fungus *Tremella lobariacearum* sampled on *L. immixta*, *L. macaronesica*, *L. pulmonaria*, and *L. isidiosa* (outgroup). The colour of taxon labels indicates the host species that *T. lobariacearum* was associated with.

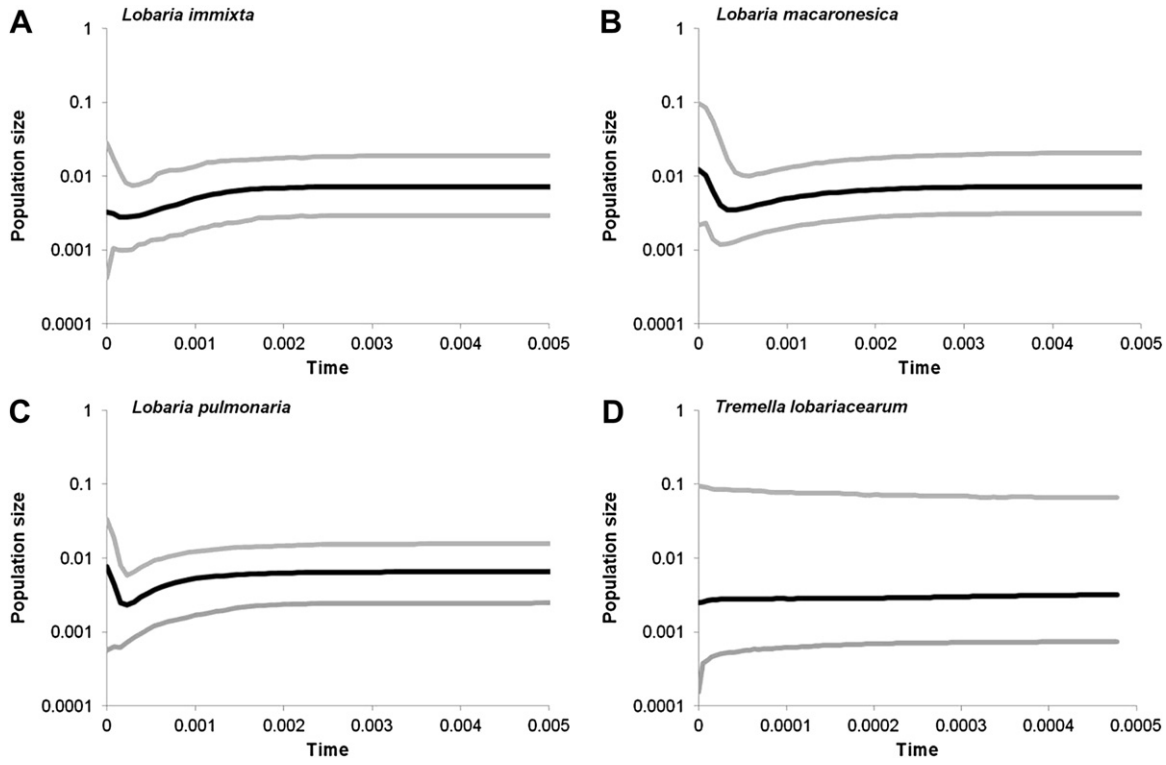

Fig 3 – Bayesian skyline plots showing changes in population size over time. The *x*-axis depicts coalescent time, with the present at $T = 0$ and going back in time towards the right. The y-axis gives the product of effective population size N_e and generation time in years. The thick solid line is the median estimate, and the grey enveloping lines show the 95 % highest posterior density intervals; (A) *Lobaria immixta*, (B) *Lobaria macaronesica*, (C) *Lobaria pulmonaria*, (D) *Tremella lobariacearum*. The plots show a slight recent increase of population size in the three host lichens, but not in the lichenicolous fungus *T. lobariacearum*. Note that the scaling of the *x*-axis differs in *T. lobariacearum*.

pulmonaria and *Lobaria macaronesica* hosts was close to one, whereas the other two migration rates were close to zero (Table 4). Results were similar when migrate-n was used in ten Bayesian runs to infer bidirectional migration rates (results not shown). The effective population size of the lichenicolous species sampled from *Lobaria immixta* and *L. macaronesica* hosts was low relative to the population size of the lichenicolous fungus on *L. pulmonaria* hosts (Table 4). Divergence time in *T. lobariacearum* from *L. macaronesica vs. L. immixta* hosts, and from *L. immixta vs. L. pulmonaria* hosts were similar; the curve for *L. macaronesica vs. L. pulmonaria* was not informative.

Host species

Polymorphism & haplotype sharing

Our analyses of the hosts are based on a nuclear data set comprising 475 bp of the ITS region and 784 bp of the nuclear RPB2 gene. The host *Lobaria immixta* exhibited the highest diversity and largest number of private haplotypes in ITS and RPB2 sequences on the Canary Islands, whereas *Lobaria macaronesica* was the most diverse and rich in private haplotypes on the Azores (Table 1; Figure S1). *Lobaria macaronesica* showed no variability in the site on the Iberian Peninsula. In *Lobaria pulmonaria*, none of the geographic regions showed an obvious maximum of genetic diversity for both genes and both haplotype and nucleotide diversity, but the sites on the Canary Islands and the Iberian Peninsula hosted most of the variability including private haplotypes (Table 1; Figure S1). *Lobaria pulmonaria* showed no DNA sequence variation on the Azores archipelago, and in Madeira, only the RPB2 region was polymorphic.

Population subdivision & specificity

In the hosts, a high amount of genetic structure was due to genetic differences among species (ITS: 95.6 %, $\Phi_{RT} = 0.9564^{*}$; RPB2: 83.5 %, $\Phi_{RT} = 0.8352^{*}$), and a smaller amount (ITS: 2.7 %, $\Phi_{SR} = 0.6246^{*}$; RPB2: 1.1 %, $\Phi_{SR} = 0.0666^{*}$) was due to genetic differences among populations on different archipelagos. If we switched the hierarchical order and grouped host species within archipelagos (data not shown), no significant variance was associated with archipelago (ITS: $\Phi_{RT} = -0.4574^{NS}$; RPB2: $\Phi_{RT} = -0.1169^{NS}$), the negative values indicating a Wahlund effect (Wahlund 1928).

The maximum clade credibility coalescent trees (Fig 2A) indicated that the phylogenetic pattern of the lichenicolous fungus did not follow that of its lichen hosts: *Lobaria immixta* and *Lobaria macaronesica* were sister species and well supported (posterior probability 0.99), whereas *Lobaria pulmonaria* grouped with the outgroup species *Lobaria tuberculata* 1. The outgroup was heterogeneous, most likely because *L. tuberculata* 2 represents a different phylogenetic species than *L. tuberculata* 1.

Table 4 – Divergence time estimates for the lichenicolous fungus *Tremella lobariacearum* and its host lichens from analysis of migration with isolation. Time estimates were obtained for the timing of splits of lineages associated with three host species, *Lobaria immixta*, *L. pulmonaria*, and *L. macaronesica*, and the divergence between host species. Given are the time T in coalescent units, the mutation-scaled population size θ, the scaled migration rate ($2N_em$ for the dikaryotic, N_em for the haploid species), the effective population size N_e, divergence time t in numbers of generations, and divergence time t in years assuming a generation time of 2 y for *T. lobariacearum* and 10 y for the *Lobaria* hosts. For rDNA of *T. lobariacearum* and ITS of *Lobaria*, the divergence time in generations was calculated assuming a mutation rate of 3.296×10^{-9} (minimum 2.49×10^{-9}, maximum 4.11×10^{-9}) per site and year estimated for the ITS region of parmelioid lichen fungi (Leavitt *et al.* 2012). For the RPB2 region of *Lobaria*, we used 1.391×10^{-9} (minimum 1.08×10^{-9}, maximum 1.75×10^{-9}) mutations per site and year (Leavitt *et al.* 2012). Note that for *T. lobariacearum* from *L. pulmonaria vs. L. macaronesica* hosts, the curve of T showed no obvious mode, and values for divergence time were thus omitted. Values in parenthesis are the estimates assuming maximum and minimum substitution rates reported for parmelioid lichens.

Organism and marker	Statistic	*L. immixta vs. L. macaronesica*	*L. immixta vs. L. pulmonaria*	*L. macaronesica vs. L. pulmonaria*
T. lobariacearum	T	2.92	1.72	–
rDNA	θ	0.869	1.451	1.169
	$2N_em$	0.036	0.012	0.912
	N_e	74,604 (59,829–98,753)	124,682 (99,988–165,041)	132,921
	t [gen]	435,688 (349,399–576,718)	428,906 (343,960–567,741)	–
	t [years]	871,376 (698,797–1,153,436)	857,813 (687,920–1,135,482)	–
Lobaria spp. ITS	T	2.3	11.4	5.7
	θ	2.604	1.412	2.146
	N_em	0.08	0.02	0.04
	N_e	831,727 (667,001–1,100,953)	451,009 (361,685–596,998)	685,318 (549,589–907,152)
	t [gen]	2,166,070 (1,737,072–2,867,216)	636,914 (510,771–843,080)	1,470,604 (1,179,345–1,946,630)
	t [years]	19,129,724 (15,341,015–25,321,916)	51,415,012 (41,232,088–68,057,783)	39,063,149 (31,326,555–51,707,687)
Lobaria spp. RPB2	T	7.2	9.6	12.1
	θ	1.091	1.750	0.959
	N_em	0.04	0.04	0.02
	N_e	500,125 (397,528–644,143)	802,504 (637,876–1,033,595)	439,752 (349,540–566,384)
	t [gen]	3,600,901 (2,862,202–4,637,827)	7,704,035 (6,123,607–9,922,511)	5,321,000 (4,229,435–6,853,251)
	t [years]	36,009,012 (28,622,020–46,378,274)	77,040,347 (61,236,070–99,225,113)	53,210,003 (42,294,351–68,532,513)

Demography & evolutionary history

Results from neutrality tests indicated that most of the populations were in mutation–drift equilibrium. There were two exceptions: *Lobaria macaronesica* from the Canary Islands had a significantly negative Tajima's D, indicating either a recent expansion or purifying selection. Moreover, *Lobaria immixta* sampled from the Canary Islands had a significantly negative Fu's F_S, implying a recent expansion. The Bayesian skyline plots constructed in BEAST showed a slight recent increase in population size after a slight decline in two of the host species, *Lobaria pulmonaria* and *L. macaronesica* (Fig 3). In *L. immixta*, after a slight decline, a minor recent increase was visible. No trends in population size over time were visible in the analyses that included empty alignments (data not shown).

In the *Lobaria* hosts, all migration rates among species were close to zero in the MDIV analysis, consistent with a model of speciation in the absence of gene flow (Table 4). These results are similar to those of the analysis of demographic parameters using ten Bayesian runs of migrate-n (data not shown). The effective population sizes varied between loci but were about one order of magnitude higher than those of the lichenicolous fungus. The divergence time estimates indicated that the most recent split occurred between *L. immixta* and *L. macaronesica*, followed by divergence among *L. pulmonaria* and *L. macaronesica*, with the oldest divergence in *L. immixta* and *L. pulmonaria* (Table 4).

Table 5 – Results from neutrality tests. F_S, Fu's F_S; D, Tajima's D. Significant values marked by bold print.

Population	ITS *Lobaria*		RP2 *Lobaria*		rDNA *T. lobariacearum*[b]	
	F_S[a]	D	F_S	D	F_S	D
IM	−1.3	−1.3	−2.2	−1.1	−0.8	−0.7
IM_Azores	−0.7	−1.2	−0.7	−1.2	0.2	0.9
IM_Canary	0.9	0.2	**−2.5***	−1.4	3.8	**2.1***
IM_Madeira	—	—	−0.2	−0.1	1.9	0.8
MA	−1.3	0.2	−2.9	−1.4	0.1	−1.8+
MA_Azores	−1.6	−1.7+	−0.8	−1.0		
MA_Canary	0.7	**−1.9***	−1.6+	−1.2		
MA_Madeira	−0.4	−1.1	−0.3	−0.3		
MA_Sintra	—	—	—	—		
PU	−3.0	−1.5	−1.7	−1.0	0.8	0.9
PU_Azores	—	—	—	—		
PU_Canary	−0.6	−1.2	−0.3	−1.2		
PU_Madeira	—	—	−0.1	−0.8		
PU_Sintra	−0.6	−1.2	−0.3	−0.4		

a Significant results are indicated by an asterisk (D: $p < 0.05$; F_S: $p < 0.02$) or by a plus (D: $0.05 < p < 0.10$; F_S $0.02 < p < 0.05$) (Fu 1997). '—', analysis not possible due to lack of polymorphism.

b For *T. lobariacearum*, the sample site within hosts was too small to test for neutrality within archipelagos. Hence, samples were grouped by archipelago (but not host species) for this test.

Discussion

Polymorphism in Tremella lobariacearum *and its hosts*

Our data showed that (i) specialization on host species was the main factor driving the population subdivision of *T.*

lobariacearum in Macaronesia, and (ii) pathogen and hosts did not show similarities in their evolutionary or demographic histories.

The pathogen and its *Lobaria* hosts showed moderate levels of polymorphism in DNA sequences. On the endemic Macaronesian hosts *Lobaria immixta* and *Lobaria macaronesica*, the genetic diversity of *T. lobariacearum* was lowest, as evidenced by haplotype numbers and haplotype diversity; about twice as many haplotypes were found on the widespread lichen *Lobaria pulmonaria*. This difference was not due to sample size: *T. lobariacearum* from *L. immixta* had the highest sample size, but this species hosted the lowest number of *T. lobariacearum* haplotypes. It is not straightforward to compare the polymorphism in *T. lobariacearum* with that of *Marchandiomyces corallinus* studied by Molina & Coworkers (2005) because of differences in sample size, marker choice, geographic and taxonomic representation of samples. A total of four ITS haplotypes were found in *M. corallinus*, but when using a combination of five random amplification of polymorphic DNA markers and DNA sequences of the ITS region, all but one of the 12 samples studied in *M. corallinus* differed genetically. Each of the samples had been collected from a different lichen species, but other than in *T. lobariacearum*, the samples of the lichenicolous fungus did not show a tendency to group according to the taxonomy of their hosts. While in *M. corallinus*, the majority of samples were heterozygous, we found no evidence of heterozygosity in our rDNA data, suggesting that recombination occurs seldom in *T. lobariacearum*. However, we caution that markers with higher resolution such as microsatellites would be better suited to determine whether this is indeed the case.

The host *L. immixta* exhibited the highest haplotype and nucleotide diversities on the Canary Islands, whereas *L. macaronesica* was most diverse on the Azores. For *L. pulmonaria*, two geographic regions had high genetic diversity (Iberian Peninsula, Canary Islands). Haplotype numbers of the three species of *Lobaria* (three to ten haplotypes in 45–50 samples) were comparable to the number found in other temperate and boreal lichen fungi (Werth 2010) with comparable sample size such as *Letharia vulpina* (Högberg *et al.* 2002), *Cavernularia hultenii* (Printzen & Ekman 2002), *Cladonia arbuscula* (Robertson & Piercey-Normore 2007) and other species of *Cladonia* (Piercey-Normore 2004). However, tropical lichen fungi had a higher haplotype diversity, including epiphyllous lichens of the genus *Porina* (Baloch & Grube 2009) and the crustose lichen *Trapeliopsis glaucolepidea* (Palice & Printzen 2004).

Population subdivision & specificity

Host species was the main factor driving population subdivision of Macaronesian *Tremella lobariacearum*. This finding is supported by analysis of molecular variance and RDA with variance partitioning, both showing that host species explains most of the variance in the data, while geography explains less variation. Moreover, the results from the simulation test indicated that the association of *T. lobariacearum* haplotypes with hosts deviated markedly from the expectation under randomness. Thus, *T. lobariacearum* is structured mainly by host species and, to a lesser degree, by archipelago (Table 2). Also in the host species, significant genetic differentiation due to geography was found, similar to the results in other lichen fungi (Lindblom & Ekman 2006; Werth *et al.* 2006; Yahr *et al.* 2006; Werth 2010). In marked contrast with our results, geography (rather than host species) was the most important factor explaining the genetic constitution of 12 isolates of *Marchandiomyces corallinus* (Molina *et al.* 2005). This may suggest that generalist lichenicolous fungi could be structured by geographic location, whereas specialists could be structured mainly by their host species. However, it has to be noted that among the lichenicolous *Tremellas*, *T. lobariacearum* is not the most host-specific species, as it can grow on multiple species of *Lobaria* and *Pseudocyphellaria*. Other species of *Tremella* are found on a single host. Before we can draw general conclusions, more systems of both types of strategies (generalists and specialists) should be studied.

The pattern of genetic differentiation according to host species found in our data is similar to what has been observed in some phytopathogenic fungi (Bucheli *et al.* 2000; Stukenbrock *et al.* 2007). Host-driven, sympatric divergence was the mode of speciation in several phytopathogenic fungi, but sympatric speciation is notoriously hard to prove (Kohn 2005; Giraud *et al.* 2008). Our data show unambiguously that specialization of the pathogen on lichen hosts is a major theme in the evolutionary history of the lichenicolous fungus *T. lobariacearum*. Host species may create different selective environments for the pathogen that only specific strains of the pathogen are able to infect. The haplotypes of *T. lobariacearum* infecting *Lobaria immixta*, the only host producing gyrophoric acid, were not found on any other host species, while the other two chemically indistinguishable hosts shared haplotypes of *T. lobariacearum*. These results indicate that a selective environment could be created by the hosts' secondary metabolites that only adapted pathogen strains are able to tolerate. Results from other studies give support to this hypothesis: experiments with non-host-specific lichenicolous fungi – *Marchandiomyces corallinus* and *Nectria parmeliae* – suggested that the degree of host-specialization depends on the ability of the lichenicolous species to tolerate the hosts' secondary metabolites (Lawrey *et al.* 1999; Torzilli *et al.* 1999; Lawrey 2000). Lichens contain metabolites some of which have antibiotic properties (Lawrey 1986; Torzilli *et al.* 1999; Boustie & Grube 2005). Some lichenicolous species can only attack a lichen thallus if its secondary compounds have been removed, or have been degraded by a fungal species (Lawrey *et al.* 1999; Lawrey 2000). The host's secondary metabolism provides a possible explanation for the observed segregation of lichenicolous haplotypes on host species. An alternative explanation is that pathogen–host compatibility mechanisms at the cellular level are responsible for the observed segregation of lichenicolous haplotypes on host species.

It is not known whether *T. lobariacearum* is a homothallic or a heterothallic fungus. Both strategies occur among the non-lichenicolous *Tremellas*, but heterothallism is more frequent (Fox & Wong 1990; Hanson & Wells 1991; Boekhout *et al.* 2011). Heterothallic species require a compatible partner differing in two loci for sexual reproduction. In lichenicolous *Tremellas*, it is not known at what distances mating may occur. For fertilization to occur, a secondary infection of the same host by the two compatible yeast stages of *Tremella* would be

required if the species is heterothallic, but only a single infection if the species is homothallic.

Demography & evolutionary history

Divergence times differed markedly between the pathogen and its hosts, thus rejecting the hypothesis that *Tremella lobariacearum* evolved synchronously with its host lichens – the lichenicolous fungus is much younger than its hosts, even when we assume conservatively that generation times are equal in *Tremella* and *Lobaria* (Table 4). Most pathogens have short generation times; from a population dynamics viewpoint, it seems unlikely that a highly specific pathogen could have a much longer generation time than its host (Greischar & Koskella 2007). If we assume a generation time of 2 y for *T. lobariacearum*, our estimates of divergence times are in the mid Pleistocene (0.85–1.0 Ma). The divergence in *Tremella* among hosts hence occurred after island formation.

The host *Lobaria pulmonaria* is reported to have a long generation time (17–25 y) (Scheidegger & Walser 1998; Scheidegger & Werth 2009; Larsson & Gauslaa 2011), though its generation time is likely to be much shorter in oceanic areas such as Macaronesia. If we assume a likely generation time of 10 y, the split between *L. pulmonaria* and either endemic species predates the first appearance of the archipelagos (8–35 Ma, Table 4). There is some ambiguity about the split between the endemic species. In RPB2, it predates island formation (36 Ma), but not ITS (19 Ma). If we assume the generation time to equal that of *L. pulmonaria* in oceanic parts of the European continent (17 y, Larsson & Gauslaa 2011) to transform our divergence time estimates to time in years, all divergence times predate island formation. These results suggest that the endemics represent palaeoendemics that have arisen on the mainland and have secondarily colonized the islands, unless they evolved on Macaronesian palaeoislands that no longer exist. After initial colonization of the archipelagos, the 'endemic' species must have gone extinct on the continent (with the exception of the one mainland site). This scenario is different from what has been reported for Macaronesian endemics of the lichen-forming ascomycete genus *Nephroma*, which evolved on the Islands (Sérusiaux *et al.* 2011).

Results from neutrality tests were not in accordance across different genes for the *Lobaria* hosts. Nevertheless, for two species, neutrality tests were significant for the same archipelago (Canary Islands), indicating that the populations *Lobaria immixta* and *Lobaria macaronesica* on this archipelago might have undergone recent expansion. Neutrality tests often fail to reveal demographic events, unless they happened recently. Thus, it is not surprising that several of the neutrality tests failed to detect the signature of population expansion evident in the Bayesian skyline plots of the *Lobaria* hosts, such as the strong pattern of a recent expansion in *L. pulmonaria*.

Several observations suggest that the lichenicolous pathogen and its hosts have different biogeographic patterns: (*i*) The divergence times differed between both partners. (*ii*) There was no evidence for a common tree topology in the coalescent trees between pathogens and hosts. (*iii*) The divergence of host species followed a model of isolation without gene flow, whereas *T. lobariacearum* was in accordance with an isolation with migration model in one case (migration was non-zero and directed from *L. macaronesica* to *L. pulmonaria*). These migration rates indicated that there was recent asymmetric gene flow in the pathogen from one host species to the other, but no evidence for gene flow between the host species, as expected in taxa that have established reproductive barriers a long time ago and are unable to interbreed. (*iv*) Also the spatial location of centres of genetic diversity differed slightly between the pathogens and hosts. Taken together, our results indicate that the biogeographic histories of hosts and pathogen have few common features despite of the species' tight antagonistic interaction, indicating that these taxa do not coevolve tightly. Future studies should seek to investigate this enigmatic pathogen–host system in detail with highly polymorphic markers such as microsatellites or SNPs.

This study is one of the first exploring the genetic variability in a lichen-lichenicolous system, and as such, it contributes significantly to our increased knowledge of these fascinating fungi and their lichen hosts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2012.11.007.

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