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Vertical and horizontal photobiont transmission within populations of a lichen symbiosis

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Abstract

Lichens are widespread symbioses and play important roles in many terrestrial ecosystems. The genetic structure of lichens is the result of the association between fungal and algal populations constituting the lichen thallus. Using eight fungus- and seven alga-specific highly variable microsatellite markers on within-population spatial genetic data from 62 replicate populations across Europe, North America, Asia and Africa, we investigated the contributions of vertical and horizontal transmission of the photobiont to the genetic structure of the epiphytic lichen *Lobaria pulmonaria*. Based on pairwise comparisons of multilocus genotypes defined separately for the mycobiont and for the photobiont, we inferred the transmission mode of the photobiont and the relative contribution of somatic mutation and recombination. After constraining the analysis of one symbiont to pairs of individuals with genetically identical symbiotic partners, we found that 77% of fungal and 70% of algal pairs were represented by clones. Thus, the predominant dispersal mode was by means of symbiotic vegetative propagules (vertical transmission), which dispersed fungal and algal clones co-dependently over a short distance, thus shaping the spatial genetic structure up to distances of 20 m. Evidence for somatic mutation generating genetic diversity was found in both symbionts, accounting for 30% of pairwise comparisons in the alga and 15% in the fungus. While the alga did not show statistically significant evidence of recombination, recombination accounted for 7.7% of fungal pairs with identical algae. This implies that, even in a mostly vegetatively reproducing species, horizontal transmission plays a role in shaping the symbiotic association, as shown in many coral and other symbioses in nature.

Keywords: algae, ecological genetics, empirical, fungi, *Lobaria pulmonaria*, microsatellite, population genetics

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Introduction

Lichens are symbiotic organisms composed of a fungal partner (mycobiont) and a population of algae and/or cyanobacteria (photobiont). Mycobionts express their symbiotic phenotype only in association with compatible photosynthetic partners, and the tight morphological integration and physiological dependence of the symbionts result in a distinct lichen body called thallus (Ahmadjian 1993). In lichens, the mechanism for symbiotic contact and thallus formation in nature is only partially understood. Reproduction and dispersal of lichens is a complex process because both partners have to be present for the successful development of a new lichen thallus (Honegger 1998, 2008; Dobson 2003). A vast majority of lichens have a sexual and asexual life cycle. In the sexual life cycle, fungal spores are released from specialized structures on the thallus (ascomata). Upon

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germination, fungal spores must obtain a compatible algal or cyanobacterial partner, which may be free living (Etges & Ott 2001; Sanders & Lücking 2002; Sanders 2005; Handa *et al.* 2007; Hedenås *et al.* 2007; Macedo *et al.* 2009) or obtained through capture from another lichen (Friedl 1987; Ott 1987a; Ott 1987b; Stenroos 1990; Rambold & Triebel 1992; Ott *et al.* 1995; Gaßmann & Ott 2000; Lücking & Grube 2002). In the vegetative life cycle, mycobiont and photobiont are simultaneously dispersed within specialized asexual propagules (e.g. corticated protuberances called isidia or non-corticated clumps called soredia) or through thallus fragmentation.

The genetic structure of a lichen population will be strongly influenced by the manner in which photobionts are dispersed and transmitted to the fungus (Hill 2009). Vertical (or co-dependent) transmission occurs when the photobiont disperses as part of the vegetative propagule of the lichen, thus presumably representing the predominant process in exclusively or nearly exclusively asexual lichen species (Werth & Sork 2010). The vegetative propagules produce physically separate but genetically identical thalli, that is, thalli with fungal and algal components genetically identical to the mother thallus (Paulsrud *et al.* 1998; Doering & Piercey-Normore 2009). On the other hand, horizontal (or independent) transmission usually occurs when the fungus reproduces sexually. The sexual life cycle is considered to reshuffle the genetic composition of the lichen, generating new combinations of fungal and algal genotypes (i.e. genetically different thalli). Horizontal transmission may also depend on the dispersal ability of the photobiont. The ability of green algal photobionts to move is very restricted, as they usually do not disperse (either sexually or asexually) while embedded in the lichen thallus (Sluiman *et al.* 1989; Nash 1996). However, many green algal photobionts can occur in free-living populations on soil, rocks or tree stems (Mukhtar *et al.* 1994; Beck *et al.* 1998; Friedl & Büdel 2008), and viable photobiont cells are found in faecal pellets of lichenivorous snails (Meier *et al.* 2002; Boch *et al.* 2011). Moreover, horizontal transmission of algae has been shown in asexual (e.g. Nelsen & Gargas 2008, 2009) or nearly asexual (Piercey-Normore 2006; Wornik & Grube 2010) lichen species.

Studies on the mode of transmission of lichen photobionts in natural populations remain scarce. In particular, the genetic composition of a lichen thallus (i.e. its individual mycobiont and photobiont genotypes) and its fine-scale spatial distribution have never been reliably assessed at the within-population scale because of the lack of appropriate genetic markers. Marker resolution becomes in fact critical when studying highly clonal organisms such as lichens, for which multilocus genotypes are the only way to identify genetically distinct individuals (Arnaud-Haond *et al.* 2007).

This work aims to assess the relative contribution of vertical vs. horizontal transmission to the intra-population genetic structure of the mycobiont and of the photobiont of a mainly vegetative lichen species. The model species is the epiphytic lichen *Lobaria pulmonaria*, which is widespread in the northern hemisphere (Yoshimura 1971). Recently, microsatellite markers have been developed for its haploid eukaryotic symbionts (mycobiont: Walser *et al.* 2003; Widmer *et al.* 2010; this study; green algal photobiont: Dal Grande *et al.* 2010). The high mutation rate of microsatellite loci gives them a far greater resolving power than previous, sequence-based studies performed on lichen populations (e.g. Beck *et al.* 2002; Lohtander *et al.* 2003; Printzen & Ekman 2003; Printzen *et al.* 2003; Yahr *et al.* 2004; Lindblom & Ekman 2005, 2007; Selkoe & Toonen 2006; Doering & Piercey-Normore 2009; Lättman *et al.* 2009; Werth & Sork 2010). *Lobaria pulmonaria* is highly selective towards its green algal photobiont, that is, it is associated with the coccoid green alga *Dictyochloropsis reticulata* (Tschermak-Woess) Tschermak-Woess throughout its entire distribution range (Dal Grande 2011).

Earlier studies suggested that the predominant dispersal mode in *L. pulmonaria* is by means of vegetative propagules (Zoller *et al.* 1999; Walser 2004; Wagner *et al.* 2005, 2006; Werth *et al.* 2006a,b, 2007) and showed its mycobiont populations to be highly clonal, suggesting a predominance of vertical transmission of the photobiont. However, the mycobiont of *L. pulmonaria* can undertake sexual reproduction; hence, the photobiont also needs to be transmitted horizontally. While no evidence of free-living photobiont populations has been found to date (Tschermak-Woess 1978; Dal Grande 2011), the presence of zoospores (motile flagellate asexual cells) indicates that the photobiont has the potential to move locally (i.e. on the same tree) once released from the thallus (Richardson 1999; Friedl & Büdel 2008).

The availability of symbionts may impose limits on the distribution of the other partner, particularly in cases where the association is obligate (Andras *et al.* 2011). Werth *et al.* (2007) demonstrated for the mycobiont of *Lobaria pulmonaria* that gene flow is spatially restricted, resulting in spatial aggregation of fungal clones. Based on the notion that spatial processes, such as reproduction followed by dispersal, leave a characteristic spatial signature (Seabloom *et al.* 2005; Wagner & Fortin 2005), analysis of spatial genetic structure may be used to identify the underlying processes. In particular, vertical transmission of the photobiont is expected to result in short-distance spatial aggregation of fungal

and algal clones, while horizontal transmission owing to mycobiont sexual reproduction will decouple photobiont–mycobiont pairs at larger distances (Werth & Sork 2010).

This study addresses the following questions: (i) What is the relative contribution of vertical vs. horizontal transmission of the photobiont to the genetic structure of the lichen populations? (ii) What is the relative contribution of the micro-evolutionary processes of mutation and recombination to the current fungal and algal intra-population genetic diversity? (iii) Are there differences in the within-population spatial genetic structure between mycobiont and photobiont?

To address these questions, we introduce an approach that takes advantage of the microsatellite markers for both the fungal and algal partners. This method allows for the reliable identification of clonal thalli (i.e. thalli with identical multilocus genotypes for the fungus and the alga, respectively). Under the assumption that pairs of thalli with identical multilocus genotypes both for the fungus (MLG_F) and for the alga (MLG_A) within a population result from the vegetative co-dispersal of fungal and algal clones, we can infer within-population evolutionary processes (such as mutation and recombination) by restricting analysis for one symbiont to pairs of thalli with identical MLG in the other symbiont. While statistical inference of (spatial) genetic structure within populations is often limited by a lack of independent replicate populations, we illustrate our approach with a data set of 62 range-wide populations that allows robust statistical analysis.

This research assesses the way photobionts are transmitted in a predominantly asexual taxon and provides insights into the contribution of the micro-evolutionary processes of mutation and recombination to the genetic structure of lichen populations.

Materials and methods

Sample collection and molecular genetic analysis

The goal of our design was to detect the intra-population genetic structure of the fungal and algal symbionts of *Lobaria pulmonaria* among adjacent trees. This design would not detect either the extent of the overall genetic clustering on the same tree or the extent of gene flow among populations. In total, 2229 thalli of *L. pulmonaria* were sampled from 62 populations across Europe, North America, Asia and Africa (Table S1, Supporting information). The median distance between a population and the nearest neighbour sampled population was 115 km, and all but nine populations were at least 25 km from their nearest neighbour population. For the purpose of our analyses, a population was defined as a stand of trees colonized by *L. pulmonaria*. Across each population, 1–3 thalli were randomly taken from an average of 23 nearest neighbour trees (i.e. proceeding from a sampled tree to its nearest unsampled neighbour tree). The maximum distance among the sampled trees within each population typically was <1500 m except for three populations, with a median maximum distance of 274 m and a minimum of 16 m. Thalli collected on a single tree were separated by about 50 cm and positioned on different sides of the trunk. This sampling design allows for the investigation of microsatellite variation within a population of *L. pulmonaria* (Walser *et al.* 2003; Wagner *et al.* 2005). On average, 31 thalli were collected per population, which has been found to be an appropriate number to resolve within-population mycobiont and photobiont genetic structure (Werth 2010).

Eight fungus-specific (LPu03, LPu09, LPu15, LPu23, LPu24, LPu25, LPu28, Walser *et al.* 2003; Widmer *et al.* 2010; MS4, this study) and seven alga-specific microsatellite markers (LPh1–LPh7; Dal Grande *et al.* 2010) were amplified from total lichen DNA. For primer sequences, including redesigned primers for LPu25, labelling and PCR conditions, see Table S2 (Supporting information). Fragment lengths were determined on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and electropherograms were analysed with GENEMAPPER 3.7 (Applied Biosystems) using LIZ-500 as internal size standard. Multilocus genotypes were defined separately for the fungus (MLG_F, based on eight loci) and for the alga (MLG_A, based on seven loci).

Statistical analyses

Data sets. Recurrent MLGs could either be the result of vegetative reproduction or chance products of sexual reproduction (Arnaud-Haond *et al.* 2007). Therefore, recurrent MLGs were only interpreted as clones if they were unlikely to result from sexual reproduction given the observed allele frequencies in a population. We calculated for each population the probability of observing two sexually produced fungal or algal individuals identical at all eight or seven microsatellite loci, respectively. This method, implemented in the software GENCLONE v2.0 (Arnaud-Haond & Belkhir 2006), is based on the round robin method proposed by Parks & Werth (1993), which allows for each MLG an estimate of the probability of obtaining the observed number of recurrent MLGs in the data set by sexual reproduction under random mating (P_{sex}). The method thus takes into account relative levels of polymorphism (Table S4, Supporting information). We used the P_{sex} to assess the likelihood that identical MLGs were of sexual origin. The significance of P_{sex} was tested at $\alpha = 0.05$ with 1000

simulations. When significant (i.e. P_{sex}<0.05), we considered recurrent MLGs as true clones. Recurrent MLGs with $P_{sex} \geq 0.05$ were excluded from analyses (Arnaud-Haond *et al*. 2007).

To analyse the genetic diversity of the fungal and algal symbionts, each pair of thalli of *Lobaria pulmonaria* was analysed for the number of microsatellite loci at which they differed in the fungal genotype MLG_F (‘*deltaF*’) and in the algal genotype MLG_A (‘*deltaA*’) (see Fig. 1 for a graphic representation). All analyses were restricted to pairwise comparisons of thalli within populations. Three subsets A, B and C of the data were used for analysis as defined in Table 1.

Pairwise comparisons within populations are not independent; hence, statistical tests cannot rely on parametric tests, and true0020replication requires independent data from multiple study sites. To allow for robust statistical estimation, we pooled data over all 62 populations and derived jackknife estimates of SE in R (R Development Core Team 2008) by leaving out one population at a time.

Relative contribution of vertical vs. horizontal photobiont transmission. Pairs of thalli were scored as resulting from co-dependent dispersal of the symbionts (vertical photobiont transmission) if they had identical MLGs of both the fungus and the alga, that is, deltaA = deltaF = 0. We assessed the relative contribution of vertical photobiont transmission to population genetic structure by the proportion of pairs of thalli with identical MLGs for both symbionts among the pairs of thalli in data set B. We derived a jackknife estimate of this proportion by omitting one population at a time.

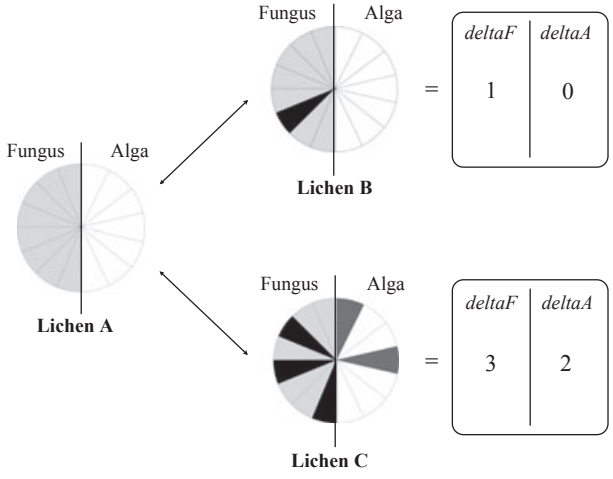

Fig. 1 Graphic representation of the pairwise analysis used in this study to infer the mode of photobiont transmission and to analyse within-population micro-evolutionary processes of the fungal and algal symbionts of *Lobaria pulmonaria*. Each circle represents a single thallus of *L. pulmonaria*. Each slice of the circle represents a microsatellite locus (eight for the fungal symbiont, seven for the algal symbiont). Black (for fungal loci) or dark grey (for algal loci) slices represent differences at the given microsatellite locus between lichen A and lichen B (top) or C (bottom). The number of loci differing between a pair of thalli is defined for each symbiont as *deltaA* (alga) or *deltaF* (fungus).

As the sexual life cycle is considered to be the main factor responsible for the independent dispersal of the symbionts (horizontal photobiont transmission), creating new genotypic combinations of fungi and algae, we further analysed pairs of thalli with different MLGs for the fungus and/or the alga. We modelled the contribution of somatic mutation and recombination to the observed differences at the microsatellite loci as follows (see Fig. 2):

1 Empirical null model of recombination (Fig. 2, top). Recombination may result in pairs of thalli with any number of differing loci. We derived an empirical null model of the distribution of the expected number of loci difference (*deltaA* or *deltaF*) based on the observed allele frequencies within each population. We permuted repeat lengths for each microsatellite marker among the thalli sampled from the same population (data set A), separately for the alga and for the fungus. We thus simulated thalli with new MLGs based on the observed allele frequencies within each population under the assumption of random mating within populations, taking into account observed levels of marker polymorphism and clonality in each population. We repeated the simulation 100 times and evaluated for each run the frequency distribution of the number of loci differing between each pair of simulated thalli from the same population (*deltaA*, *deltaF*). We pooled the simulated frequencies across the 62 populations for each simulation run and then averaged over all 100 simulation runs. All calculations were performed in R (R Development Core Team 2008). Simulated probabilities for obtaining identical MLGs by recombination were 3.4×10^{-4} for *deltaF* = 0 and 6.4×10^{-4} for *deltaA* = 0 (not shown in Fig. 2).
2 Negative exponential distribution model accounting for somatic mutation (Fig. 2, centre). Mutations are assumed to occur independently for each locus and for each symbiont. Hence, over many generations and in an otherwise only vegetatively reproducing population, mutation will first lead to difference in a single locus, a subsequent mutation to difference in one additional locus and so on, following a negative exponential model defined by parameter lambda.
3 Model fitting to the fungal and algal data (Fig. 2, bottom). To assess to what degree the observed non-clonal pattern in a symbiont was the result of mutation vs. recombination, the parameter lambda of an

Table 1 Data sets used in this study

Data set	Definition	Number of pairs	Analysis
A	All pairs from within the same population (same for both symbionts)	All: 36 218 pairs from 62 populations	Quantification of intra-population genetic diversity and spatial genetic structure.
B	Fungus: all pairs of data set A with *deltaA* = 0 Alga: all pairs of data set A with *deltaF* = 0	Fungus: 2977 pairs from 62 populations Alga: 3285 pairs from 62 populations	Restriction for each symbiont to pairs with identical MLG in the other symbiont to partial out larger-scale evolutionary processes when assessing the relative contribution of vertical transmission to population genetic structure.
C	Data set B without recurrent MLGs within the same population	Fungus: 269 pairs from 38 populations Alga: 215 pairs from 50 populations	Exclusion of recurrent MLGs within populations to identify signals of within-population mutation and recombination and to assess the relative contribution of horizontal transmission. This avoids potential underestimation of horizontal transmission in genetically uniform or depauperate populations.

exponential function was fitted to data set C using the function 'nls' in R (R Development Core Team 2008) and accounting for the null model of recombination. This resulted in estimates for lambda of 0.55 for the fungus and 0.77 for the alga. We then performed a linear regression of the frequency distribution of the number of differing loci as a function of the exponential model (representing mutation) with the fitted parameter lambda and the null model of recombination, with no intercept, and assessed model fit, statistical significance of regression coefficients, and the relative contribution of the exponential model and the empirical null model of recombination to the frequency of pairs per number of differing loci. For each level of *deltaA* or *deltaF*, we assessed the proportional contribution by each component model to the fitted value (e.g. if 100 pairs were predicted, 37 may be predicted by the exponential model and 63 by the empirical null model of recombination). We multiplied these proportions by the observed frequency of each level of *deltaA* or *deltaF* in data set C to estimate the ratio of mutation vs. recombination among the non-clonal component. Jackknife mean and SE of this ratio were determined by leaving out one population at a time.

Assuming an average microsatellite mutation rate of 10^{-3}, the expected probability of observing at least one mutation in the alga (with seven independent loci) is 0.0068, and the expected probability of observing at least one mutation in the fungus (with eight independent loci) is 0.0077. The expected probability of mutation occurring in both symbionts independently at the same time is $(6.8 \times 10^{-3}) \times (7.7 \times 10^{-3}) = 5.2 \times 10^{-5}$. The probability that both symbionts show a somatic mutation was thus expected to be less than 1% of the probability for somatic mutation in either symbiont and considered negligible.

Spatial genetic structure. To assess spatial genetic structure within populations, we determined for data set A the probability of sampling a pair of thalli from the same population belonging to one of the following categories as a function of their distance in space: (i) clonal thalli (*deltaF* = *deltaA* = 0), (ii) fungal clones associated with different algal MLGs (*deltaF* = 0 and *deltaA* > 0), (iii) algal clones associated with different fungal MLGs (*deltaF* > 0 and *deltaA* = 0) and (iv) different fungal MLGs associated with different algal MLGs (*deltaF* > 0 and *deltaA* > 0). The first distance class contained pairs of thalli sampled from the same tree. Distance class boundaries were defined on a logarithmic scale, with the last distance class containing all pairwise comparisons at distances >500 m.

Results

After exclusion of recurrent MLGs that were not assessed as true clones (P_{sex} values >0.05, 209 thalli) had incomplete genotype assessment (55 thalli) or missing spatial coordinates (five thalli), the data set consisted of 1960 thalli. We found 1051 MLGs for the haploid fungus and 1025 MLGs for the haploid alga, with a total of 1256 MLGs based on all 15 markers from both symbionts (Table S1, Supporting information: numbers of different MLGs per population; Table S4, Supporting information: allele frequency distribution per population at eight fungal and seven algal loci). Multiple fungal or algal genotypes within the same thallus were not found in any of the populations.

Our analyses were based on 36 218 pairwise comparisons within populations pooled over 62

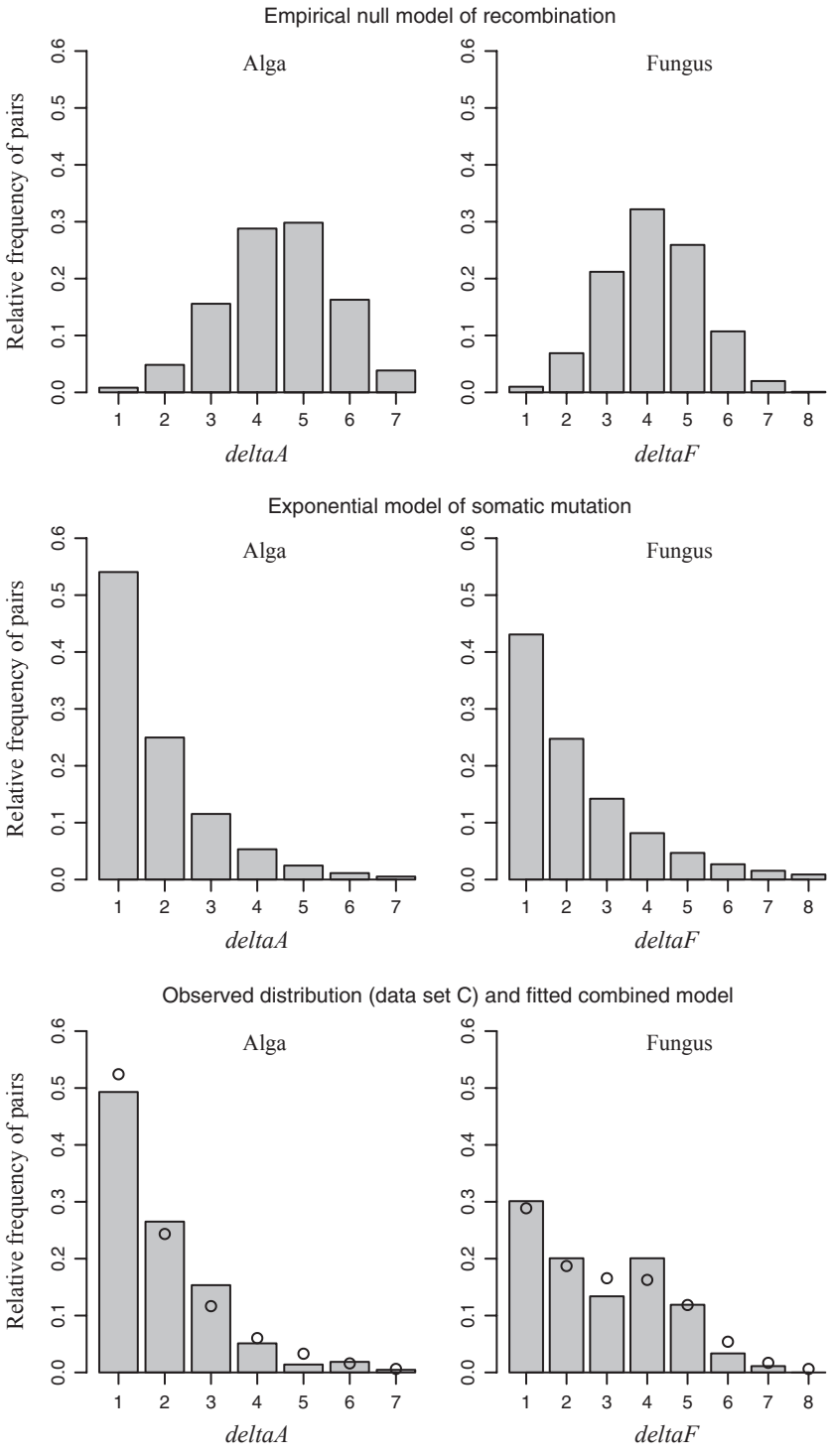

Fig. 2 Models used to analyse the contribution of mutation and recombination to within-population genetic structure of lichen symbionts in *Lobaria pulmonaria* Each barplot shows the relative frequency of pairs of thalli differing in *deltaA* (alga, left) or *deltaF* (fungus, right) loci, as expected for each symbiont under the empirical null model of recombination (top) or an exponential model of somatic mutation (centre). The bottom barplots show the observed relative frequency of pairs in data set C. Empty circles indicate for each level of *deltaA* or *deltaF* the relative frequency predicted by the fitted model combining the empirical null model of recombination and the exponential model of mutation.

populations (Table 1). The relative frequency distribution of the number of loci differences (deltaA, deltaF) calculated over all pairs within populations (data set A) was similar for both symbionts, with the highest frequency of thalli differing by four loci each for the fungus and for the alga (Fig. 3, top). In both symbionts, we found a high frequency of identical pairs of thalli (2977 pairs with deltaA = 0; 3285 pairs with deltaF = 0; Fig. 3, top).

Vertical transmission of the photobiont

Two thousand two hundred and ninety-four pairs had identical MLGs for both the alga and the fungus

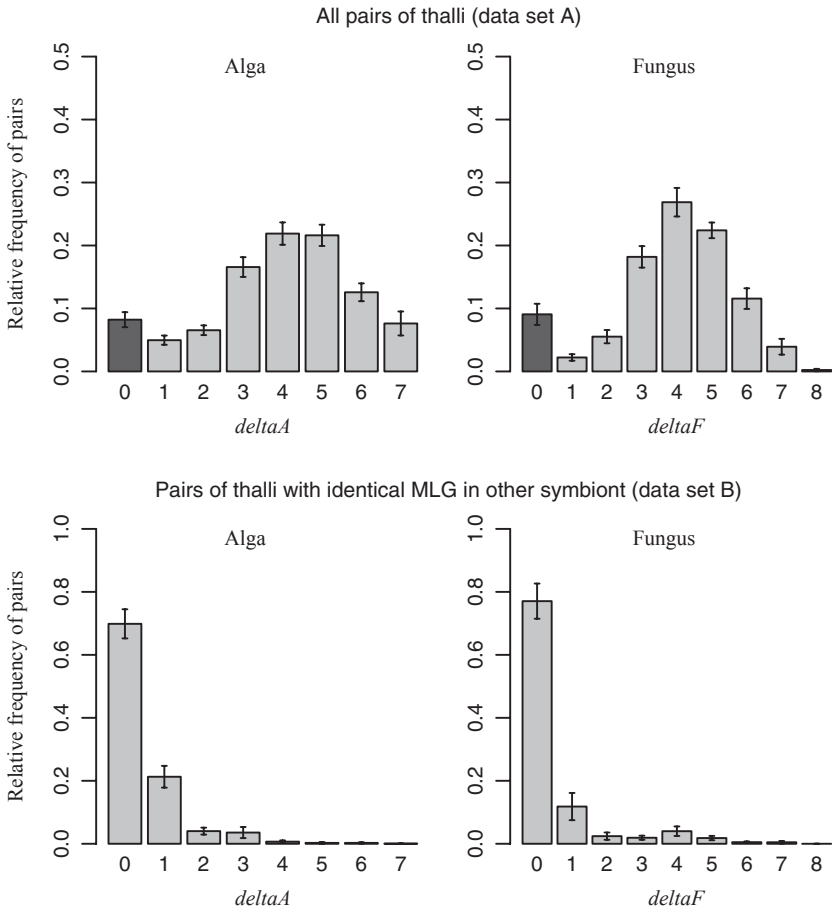

Fig. 3 Barplot of the relative frequency (±jackknife SE) of pairs of thalli (within populations pooled over all populations) differing by 0–7 loci for the alga (*deltaA*) and 0–8 loci for the fungus (*deltaF*) for all pairwise comparisons within each population and pooled over all populations (data set A, top) and for pairwise comparisons in one symbiont constrained to identical pairs of multilocus genotypes for the other symbiont (data set B, bottom).

(deltaA = deltaF = 0). When the analysis was restricted per symbiont to those pairs of thalli displaying an identical MLG in the other symbiont (data set B), both the alga and the fungus displayed a high degree of clonality, with deltaA = 0 and deltaF = 0 as the predominant classes (Fig. 3, bottom). The proportion of clonal comparisons was higher for the fungus (77.06% ± 5.59%) than for the alga (69.85% ± 4.63%).

Identifying micro-evolutionary processes of mutation and recombination

After the exclusion of recurrent genotypes within each population and constraining by clonality in the other symbiont (deltaF = 0 for algal MLGs and vice versa, data set C), we found 215 algal and 269 fungal pairs of MLGs that differed from each other in at least one locus (Table 1, Fig. 2, bottom). For both symbionts, the largest proportion of these pairs differed at only one locus (deltaA or deltaF = 1). The alga showed a strongly skewed distribution of the number of loci differences as expected under a negative exponential model resulting from mutation (Fig. 2, bottom left). In the fungus, the distribution was bimodal, suggesting the presence of an additional process (Fig. 2, bottom right).

In fungal sexual reproduction, each ascoma (i.e. reproductive structure of the fungus) may form either meiotic fungal spores with the same MLGs or spores with different MLGs. Both spore types may form new associations with either the same or a different algal MLG. The empirical null models of recombination based on the observed allele frequencies estimated that under random mating within each population, 0.033% of fungal recombinations and 0.065% of algal recombinations would result in the same MLG as the mother thallus. The secondary peak in the distribution of deltaF given deltaA = 0 was proportional to the frequency distribution expected from the empirical null model of recombination (Fig. 2, bottom right). The combination of an exponential model representing mutation and the empirical null model of recombination explained the distribution of the number of loci differences for each pair of fungi with identical algae well, explaining a total of 96.5% of the variation for the fungus, whereas for the alga, the exponential model alone explained 98.5% of the variation (Table S3, Supporting information).

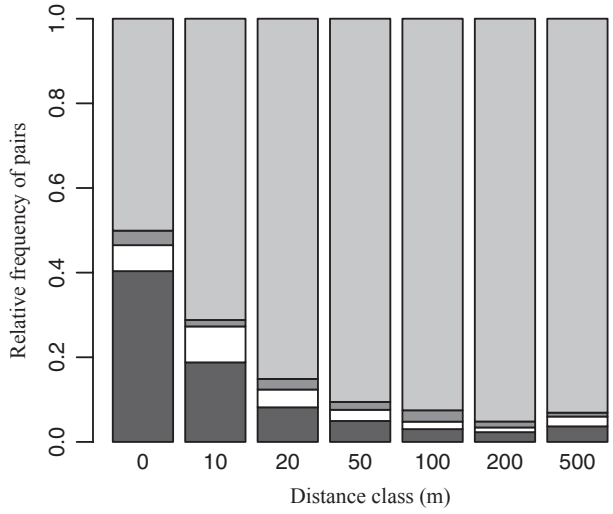

Fig. 4 Spatial distribution of the following categories of thalli: vegetative propagules (deltaF = deltaA = 0; black bars, '00'), fungal clones associated with different algal multilocus genotypes (deltaF = 0 and deltaA > 0; white bars, '01'), algal clones associated with different fungal multilocus genotypes (deltaF > 0 and deltaA = 0; dark grey, '10') and different fungal multilocus genotypes associated with different algal multilocus genotypes (deltaF > 0 and deltaA > 0; light grey, '11'). The first distance class contained pairs of thalli sampled from the same tree. The last distance class contained all sample comparisons at distances >500 m.

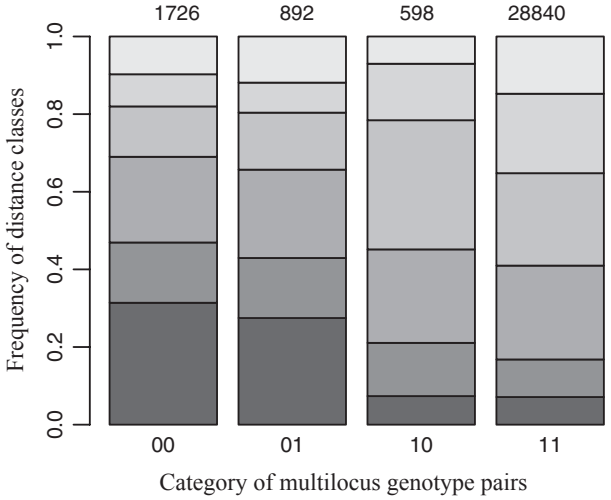

Fig. 5 Relative frequency of distance classes for each group of multilocus genotype pairs defined in Fig. 2. Distance classes: 0–10 m (first bar from bottom; darkest bars); 10–20 m (second bar from bottom); 20–50 m (third bar from bottom); 50–100 m (fourth bar from bottom); 100–200 m (fifth bar from bottom); >500 m (last bar from bottom; faintest bars). The first distance class of pairs of thalli sampled from the same tree was not included in the analysis. The total number of multilocus genotype pairs per group is given on the top of the corresponding bar.

Taking into account the above-mentioned estimate of 77.06% (±5.59%) clonality in the fungus, the fitted models resulted in an overall estimate of 15.21% (±1.49%) of pairwise comparisons of fungal MLG being affected by mutation and 7.73% (±1.49%) being affected by recombination. For the alga with 69.85% clonality (±4.63%), mutation thus accounted for 30.15%.

Spatial genetic structure

Clonality depended strongly on distance (Fig. 4). There was a marked decrease in the frequency of pairs of thalli with identical fungal and algal MLGs (vertically transmitted photobionts, deltaF = deltaA = 0) within the first 20 m, compensated by an increase in the frequency of pairs that differed both in the alga and in the fungus (deltaF > 0 and deltaA > 0).

The relative frequency of distance classes for each type of pairs showed significant differences between the two symbionts (Fig. 5). For the alga, pairs with differences at 1 or more loci (deltaA > 0, deltaF = 0) decreased in number over short distances, similarly to the distribution of clonal thalli. Fungal MLG pairs differing at least at one locus (deltaF > 0, deltaA = 0) showed a different spatial pattern similar to that of pairs differing in both symbionts (*deltaF* > 0, *deltaA* > 0).

Discussion

Prevalence of vertical transmission of the photobiont

Based on the previous evidence that the fungus reproduces mainly clonally (Walser 2004; Wagner *et al.* 2005; Werth *et al.* 2006b, 2007), we expected the photobiont of *Lobaria pulmonaria* to disperse primarily vertically within vegetative propagules. Vegetative reproduction will re-create the MLG of the mother thallus unless there is mutation in at least one symbiont. This should result in a dominating component of pairs of thalli displaying identical MLGs for both symbionts. Indeed, when the analysis was restricted per symbiont to those pairs of thalli displaying an identical MLG in the other symbiont (data set B), we found that the predominant class of MLG comparisons was composed of pairs of thalli having identical MLGs for both symbionts. Considering that the probability of creating identical MLGs through sexual reproduction was small enough to be negligible (as tested here with P_{sex} for each MLG and further supported by the empirical null model of recombination), we interpreted recurring MLGs as clones, that is, resulting from vegetative reproduction. Thus, based on microsatellite fingerprinting of both lichen symbionts, we demonstrated that the photobiont of *L. pulmonaria* is mostly vertically transmitted.

Vegetative dispersal with symbiotic propagules ensures the continuity of a successful combination of MLGs of the two partners across generations (Margulis 1993; Yahr *et al.* 2004, 2006; Reeve & Hölldobler 2007; Zilber-Rosenberg & Rosenberg 2008). It has been suggested that vegetative dispersal in lichens has the advantage of producing large numbers of locally adapted propagules that can readily exploit and colonize the local environment (Ott 1987b; Sanders & Lücking 2002; Walser 2004). A predominance of vertical transmission of photobionts has also been confirmed in other multicellular symbiotic systems for which genetic uniformity is favoured by selection for cooperative traits (Gilbert *et al.* 2009). For instance, in some symbiotic systems, such as maternally inherited endosymbionts (Saffo 1992; Moran & Baumann 1994; Huigens *et al.* 2000), grass endophytes (Clay 1988, 1990; Saikkonen *et al.* 2002), corals (Coates & Jackson 1987) or sea anemones (Geller & Walton 2001), strong population structure and shared phylogenetic history of symbionts are expected because of the vertical transmission of symbionts (Brem & Leuchtmann 2003).

Contribution of mutation and recombination to within-population genetic structure of lichen symbionts

The high variability of the microsatellite markers used in this study, combined with the approach constraining the analyses of one symbiont to pairs of individuals with genetically identical symbiotic partners and the availability of data from 62 replicate populations, provided robust evidence for patterns of mutation and recombination in *Lobaria pulmonaria* symbionts. The alga showed a clear signal of mutation as indicated by the exponential distribution of the number of loci differences (Fig. 2, bottom left). Considering that no statistically significant signal of recombination was found, our results indicate that the photobiont *Dictyochloropsis reticulata* may reproduce strictly asexually. In lichen photobionts, the production of zoospores (motile, flagellate spores) is considered a means to escape from the thallus, close to which they can form colonies (Slocum *et al.* 1980; Scheidegger 1985). The occurrence of free-living colonies is known for the green algal genus *Trebouxia* (Tschermak-Woess 1978; Bubrick *et al.* 1985; Mukhtar *et al.* 1994), and their zoospores are known to frequently undergo sexual fusion in fresh cultures (Ahmadjian 1959). Despite extensive investigation, the production of zoospores was never observed in the photobiont of *L. pulmonaria* (Skaloud 2008).

It is remarkable that the alga, with one marker less than the fungus, exhibited a comparable level of genetic diversity within populations to the fungus (Fig. 3, top). With no evidence for recombination and having shown that the alga is mostly co-transmitted vertically with the mycobiont, this may be the result of faster mutation rates in the algal microsatellites combined with a greater number of generations in the photobiont. An alternative explanation involves the introduction of new alleles into the populations through the horizontal transmission (symbiont capture) from other photobiont populations found in lichen species associated with *D. reticulata* (genera *Lobaria* and *Sticta*; Dal Grande 2011). The evidence of mutation obtained in our study concurs with the hypothesis that mutation is the key process creating genetic diversity in clonal organisms (Higgs & Woodcock 1995; Tomiuk *et al.* 1998; Butlin 2002; Vogler *et al.* 2006; Ally *et al.* 2008; Mock *et al.* 2008).

The sporadic presence of fruiting bodies in *L. pulmonaria* indicates that the mycobiont can undertake sexual reproduction by forming ascospores. Sexual reproduction involves the process of relichenization, that is, the formation of a new thallus once fungal spores found a suitable alga (horizontal transmission). Our results show that recombination significantly contributes to the fungal genetic structure (7.73% ± 1.49% of pairwise comparisons of fungal MLG; Table S3, Supporting information). Hence, despite the predominance of vertical transmission, horizontal transmission plays a non-negligible role in shaping the genetic composition of the lichen population.

Our approach, however, does not allow us to distinguish the effect of horizontal transmission of the photobiont related to fungal sexual reproduction from the process of horizontal movement of photobiont from nearby vegetative propagules, which may affect the interpretation of our results through reshuffling of the genetic composition of lichen thalli independently from fungal sexual reproduction. Previous studies have shown that, even where both partners are co-dispersed in specialized propagules, de-differentiation (separation of algal and fungal partners) allows vertically transmitted algae to be replaced by others available in the environment or even to be captured from other nearby lichen species (Friedl 1987; Ott *et al.* 1995; Ohmura *et al.* 2006; Wornik & Grube 2010; Dal Grande 2011). The frequency of such algal substitutions in nature is unknown (Piercey-Normore & DePriest 2001), but this strategy may provide a mechanism for optimizing symbiotic composition in a local environment (Friedl 1987; Ott 1987b; Ohmura *et al.* 2006; Yahr *et al.* 2006).

The way horizontal algal movement and relichenization take place remains elusive, and these processes deserve further attention. They may well be key evolutionary processes in lichen communities, allowing the formation of photobiont-mediated guilds among unrelated lichen-forming fungi (Beck *et al.* 2002; Rikkinen *et al.* 2002; Rikkinen 2003). In other symbioses, evidence

for horizontal symbiont transmission has been reported, for instance in certain corals and their symbiotic dinoflagellates (Rowan 1998; Brown *et al.* 2000; Loh *et al.* 2001), in insects and their endosymbiotic bacteria (Huigens *et al.* 2000; Sirviö & Pamilo 2010), or in fungus-gardening ants or termites and fungal cultivars (Aanen *et al.* 2002; Mikheyev *et al.* 2007). Our results showed that the lichen symbiosis is formed by a strictly asexual partner (alga) and by a fungal partner that conserved the sexual pathway together with the formation of asexual diaspores co-dispersing both partners. Sexual propagules are considered important for long-distance dispersal of the mycobiont (Walser 2004; Seymour *et al.* 2005; Cassie & Piercey-Normore 2008; Scheidegger & Werth 2009) and increase the number of genotypes in local populations, thus potentially enhancing adaptation (Maynard Smith 1986; Samadi *et al.* 1999; Rice & Chippindale 2001; Blaha *et al.* 2006; Foucaud *et al.* 2006).

The symbiotic relationship is obligatory for the fungal partner in *L. pulmonaria* to complete its life cycle (Ott 1987b; Ingold & Hudson 1993; Honegger 2001). Yet, little is known about how often and under what conditions sexual reproduction and relichenization occur in natural habitats. So far, no corresponding estimates from molecular data were available (Honegger 2001; Dobson 2003). Our study suggests that independent dispersal of the symbionts does occur in natural populations of *L. pulmonaria* and that it has a considerable impact on the genetic diversity of lichen populations.

Spatial genetic structure

We analysed the spatial genetic structure of lichen populations to infer about dispersal processes related to horizontal and vertical transmission of the photobiont. While statistical analysis of spatial patterns is often limited by the lack of replicate study areas, the availability of comparable spatial genetic data from 62 replicate populations allowed robust statistical analysis based on jackknife estimates.

Here, we showed that the fungal and algal clonal components had a large impact on the small-scale spatial genetic structure of the lichen association, and the signal of clonality markedly decreased within a distance of about 20 m (Fig. 4). Our results indicate that vegetative propagules play a dominant role to disperse genetically identical symbionts of *Lobaria pulmonaria* over short spatial distances within populations. They are thus a means of rapid lichen spread at the local scale (Hawksworth & Hill 1984; Heinken 1999; Dettki *et al.* 2000; Sillett *et al.* 2000). The restricted dispersal can be explained by these propagules' larger size compared to fungal ascospores, because the larger the propagule, the shorter the distance they can be carried by wind, water or animals (Heinken 1999; Walser 2004; Werth *et al.* 2006b; Scheidegger & Werth 2009).

The differences in the reproductive modes between the two symbionts of *L. pulmonaria* described above were clearly reflected in their spatial genetic structure. We expected that the symbionts mainly spread within the vegetative propagules of the lichen and thus would present similar spatial structures. The alga, which only showed a signal of mutation, confirmed this assumption by displaying almost an identical spatial pattern as the clones (with $deltaA = deltaF = 0$; Fig. 5). The fungus, which showed signals of both mutation and recombination, exhibited a different spatial genetic structure suggesting dispersal over larger distances.

Caveats to the analysis

As a caveat, we recall some key assumptions to our analysis. First, we assume that long-distance migration leads to the introduction of new genotypes, that is, it is unlikely that the same MLG would originate independently in two populations and migrate from one to the other, thus reflecting larger-scale processes within our data set B. Based on observed allele frequencies within populations, we estimated the probability of independent origin of clones as <0.1% each for the fungus and the alga. The probability of independent origin in different populations and subsequent immigration is expected to be much lower still. Second, we assume that each mutation leads to a new allele, such as expected under an infinite allele mutation model. Multiple identical but independent mutations within the same population, as might be expected under a stepwise mutation model, would lead to underestimation of the relative contribution of mutation because of the exclusion of recurrent MLGs in data set C. Third, we assumed independent mutation in both symbionts at the same time to be negligible, and we estimated its probability as <1% of the probability of mutation in either symbiont. Concurrent mutation in both symbionts would reduce the relative size of data set B but should not otherwise bias results.

Conclusions

This study presents a novel approach to analyse relatively recent, within-population micro-evolutionary processes from the population genetic structure of the lichen *Lobaria pulmonaria*. We provided robust evidence for the predominance of vertical transmission of the photobiont at the intra-population level in a mainly vegetative species.

We inferred the different processes shaping the genetic structure of the symbionts, highlighting that,

even in a species with rare sexual reproduction such as *L. pulmonaria*, fungal recombination is a process shaping the genetic structure between the two lichen symbionts. The possibility of sexual reproduction is important to population genetics. Considering the low germination rate in some lichen species, it may seem unlikely that their ascospores would ever develop into a lichen thallus. However, even if only a few of the thousands of ascospores produced in one ascoma find the proper photobiont to reconstitute the symbiosis, as long as the new thallus multiplies and disperses through vegetative propagules, this may suffice to alter lichen population genetic structure (Honegger & Zippler 2007).

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Data accessibility

Microsatellite data and sample coordinates: DRYAD entry doi: 10.5061/dryad.4jf42ct4.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Information on sampled populations of *Lobaria pulmonaria*.

Table S2 Microsatellite analysis: (a) primer sequences (Walser *et al.* 2003; Walser 2004; this study; Dal Grande *et al.* 2010), labeling, primer concentrations and (b, c) PCR conditions for genetic analyses of *Lobaria pulmonaria*.

Table S3 Model fitting and residuals of observed frequencies of deltaF vs. fitted frequencies (combined exponential and binomial fitting).

Table S4 Allele frequency distribution at eight fungal (LPu03, LPu09, LPu15, LPu23, LPu24, LPu25, LPu28, Walser *et al.* 2003; Widmer *et al.* 2010; MS4, this study) and seven algal (LPh1–LPh7; Dal Grande *et al.* 2010) loci per population. Each line is one repeat length (allele) and each number represents the absolute frequency of that allele in the particular population.

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