MOLECULAR ECOLOGY

Molecular Ecology (2014) **23**, 5164–5178 doi: 10.1111/mec.12928

# Microclimatic differentiation of gene pools in the *Lobaria pulmonaria* symbiosis in a primeval forest landscape

OLGA NADYEINA,*† LYUDMYLA DYMYTROVA,*† ANNA NAUMOVYCH,‡ SERGYI POSTOYALKIN,‡ SILKE WERTH,†§ SARAN CHEENACHAROEN† and CHRISTOPH SCHEIDEGGER†

*Lichenology & Bryology, M. G. Kholodny Institute of Botany, Tereschenkivska str. 2, 01601 Kyiv, Ukraine, †Botany, Kherson State University, 40 Rokiv Zhovtnya str. 27, 73000 Kherson, Ukraine, ‡Biodiversity and Conservation Biology, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zürcherstrasse 111, CH-8903 Birmensdorf, Switzerland, §Faculty of Life and Environmental Sciences, University of Iceland, Sturlugata 7, 101 Reykjavik, Iceland

## Abstract

**Population genetics of the tree-colonizing lichen *Lobaria pulmonaria* were studied in the largest primeval beech forest of Europe, covering 10 000 ha. During an intensive survey of the area, we collected 1522 thallus fragments originating from 483 trees, which were genotyped with eight mycobiont- and 14 photobiont-specific microsatellite markers. The mycobiont and photobiont of *L. pulmonaria* were found to consist of two distinct gene pools, which are co-existing within small areas of 3–180 ha in a homogeneous beech forest. The small-scale distribution pattern of the symbiotic gene pools show habitat partitioning of lineages associated with either floodplains or mountain forests. Using approximate Bayesian computation (ABC), we dated the divergence of the two fungal gene pools of *L. pulmonaria* as the Early Pleistocene. Both fungal gene pools survived the Pleistocene glacial cycles in the Carpathians, although possibly in climatically different refugia. Fungal diversification prior to these cycles and the selection of photobionts with different altitudinal distributions explain the current sympatric, but ecologically differentiated habitat partitioning of *L. pulmonaria*. In addition, the habitat preferences of the mycobiont are determined by other factors and are rather independent of those of the photobiont at the landscape level. The distinct gene pools should be considered evolutionarily significant units and deserve specific conservation priorities in the future, for example gene pool A, which is a Pliocene relict.**

*Keywords*: altitude, approximate Bayesian computation, *Dictyochloropsis reticulata*, epiphytic lichen, mycobiont, population genetics

*Received 18 September 2013; revision received 30 August 2014; accepted 15 September 2014*

## Introduction

*Lobaria pulmonaria* forms a tripartite lichen symbiosis, which consists of a fungal and a primary green-algal partner (myco- and photobiont), whereas its secondary photobionts are symbiotic cyanobacteria. This obligate, stable symbiosis develops a foliose thallus and is a common epiphyte of primeval or old-growth forests in continental Europe. Regionally, the species can also be found in wooded pastures and chestnut orchards (Rose 1976, 1992; Gauslaa 1994; Werth *et al.* 2006; Jüriado *et al.* 2011), where trees frequently persist for centuries. Occupying a wide range in the Holarctic across temperate and boreal zones (Yoshimura 1971), *L. pulmonaria* is threatened in many European countries (Scheidegger & Werth 2009). The species is known to be sensitive to various anthropogenic influences, including air pollution (Rose 1976; Gauslaa 1995), forest management, habitat fragmentation and land-use changes (Werth

Correspondence: Olga Nadyeina, Fax: +41 44 739 22 15; E-mail: nadyeina@gmail.com

*et al.* 2006; Belinchón *et al.* 2009; Otalora *et al.* 2011; Jüriado *et al.* 2012; Scheidegger *et al.* 2012).

Being a flagship species in forest conservation (Scheidegger & Werth 2009), *L. pulmonaria* is widely used as a model to study symbiosis (Dal Grande *et al.* 2012; Werth & Scheidegger 2012), population genetics (Zoller *et al.* 1999; Walser *et al.* 2004; Werth *et al.* 2007; Hilmo *et al.* 2012; Singh *et al.* 2012), phylogeography (Scheidegger *et al.* 2012; Widmer *et al.* 2012), eco-physiology (Gauslaa & Solhaug 2000, 2001; Bidussi *et al.* 2013), secondary metabolism variation (Asplund & Gauslaa 2007; Asplund *et al.* 2009; Asplund 2011) and transplantation and acclimatization to local environments (Scheidegger *et al.* 1995; Walser 2003; Gauslaa & Goward 2012). Recently, four gene pools for the fungal symbiont of *L. pulmonaria* and three for its algal symbiont have been identified in Europe (Scheidegger *et al.* 2012; Widmer *et al.* 2012). The divergence of the *L. pulmonaria* symbionts is said to have occurred during the last glaciations (Widmer *et al.* 2012) or earlier (Scheidegger *et al.* 2012). The evolutionary processes leading to this genetic pattern, that is sympatric or allopatric divergence, have not been addressed so far.

Most studies on *L. pulmonaria* have focused on managed old-growth forests or relatively small fragments of primeval forests, which predominantly consist of forests up to 200 ha. Detailed landscape-level genetic studies are, thus, lacking for large, floristically continuous and undisturbed areas. Our study focused on the genetic pattern of *L. pulmonaria* in the Uholka–Shyrokyi Luh forest massif, which is situated in the Eastern Carpathians in Ukraine. This forest is more than 10 000 ha and is the largest primeval beech forest in Europe (Brändli & Dowhanytsch 2003; Commarmot *et al.* 2013). This remote area has not experienced significant anthropogenic or natural disturbances for centuries (Brändli & Dowhanytsch 2003; Trotsiuk *et al.* 2012) and may thus be considered as a reference ecosystem for studying the distribution, abundance and genetic structure of *L. pulmonaria*. Moreover, the vegetation in the primeval forest of Uholka–Shyrokyi Luh is homogeneous and consists almost purely of common beech (*Fagus sylvatica*) stands (Brändli & Dowhanytsch 2003).

Beech is one of the most frequent host tree species for *L. pulmonaria* in large parts of Europe (Mikryukov 2011; Scheidegger *et al.* 2012). Uholka–Shyrokyi Luh primeval beech forest thus provides an ideal setting to study the population structure of the rare and threatened lichen species with its two symbionts, the mycobiont *L. pulmonaria* and the green-algal photobiont *Dictyochloropsis reticulata*. The aims of this study were (i) to test whether the fungal and algal symbionts form genetically subdivided populations (gene pools) in the continuous forest landscape; (ii) to determine gene pool associations of the *L. pulmonaria* symbionts with environmental variables, including local topography, forest-stand parameters and microclimate (using altitude as a proxy); and (iii) to infer evolutionary processes that lead to differentiation among gene pools and estimate the divergence time between gene pools.

## Materials and methods

### *Study area*

The study area, the Uholka–Shyrokyi Luh Massif of the Carpathian Biosphere Reserve, is situated in the south-western part of Ukraine at 48°18′N and 23°42′E (Fig. 1). The mountain slopes within the two continuous areas Uholka and Shyrokyi Luh are covered by pure beech forests, extending over 10 000 ha, of which 8500 ha (88%) are primeval forests (Brändli & Dowhanytsch 2003). Several tributaries flow into the Tysa River from the massif. This forest is remote from the historical places of extensive logging in the Carpathians and has probably not been disturbed for centuries (Brändli & Dowhanytsch 2003; Commarmot *et al.* 2013). It is a UNESCO World Heritage site (Brändli *et al.* 2008).

The massif ranges in elevation between 400 and 1400 m a.s.l., with the timberline at around 1140 m a.s.l. The slopes in the massif have different aspects and inclinations, ranging from flat areas to steep slopes. The mean inclination is ~50% (Commarmot *et al.* 2013). South-facing and less steep slopes are frequent at lower altitudes in the Uholka area, while the Shyrokyi Luh area is more varied (Commarmot *et al.* 2013). The climate is temperate, with a mean annual temperature of +8 °C and an annual precipitation of 1130 mm [measured at 430 m a.s.l. according to Bursak (1998) and Commarmot *et al.* (2013)]. In the Shyroky Luh area, the annual temperature is slightly lower than in the Uholka area (Brändli & Dowhanytsch 2003).

The beech stands constitute 97% of the total forest cover and are characterized by an uneven-aged, multi-layered structure. The canopy is mostly closed, making the forest floristically monotonous (Bursak 1998; Commarmot *et al.* 2005), with little local differentiation of the epiphytic lichens (Dymytrova *et al.* 2013). The mean tree age on randomly cored beech trees from Uholka is above 200 years, but trees can reach ages up to 550 years (Trotsiuk *et al.* 2012).

### *Sampling and variables*

We sampled a total of 483 trees with *Lobaria pulmonaria* for population genetic analyses during an intensive nonstratified plot-based forest inventory in 2010 (Commarmot *et al.* 2013; Fig. 1). A dense net of 314 plots

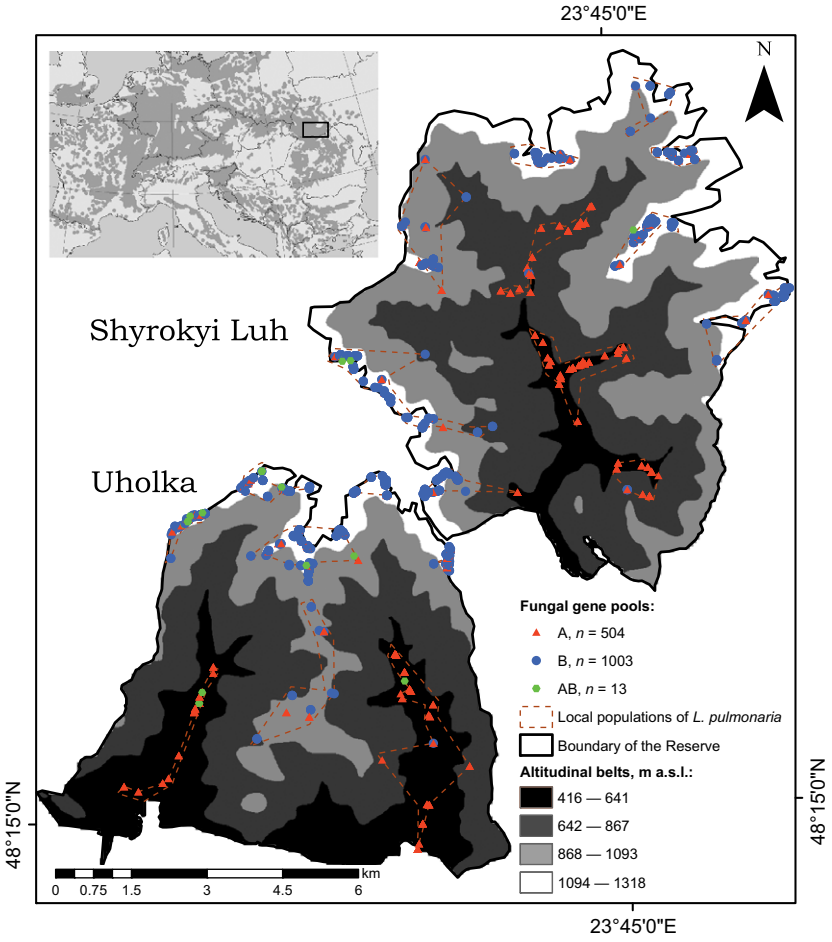


**Fig. 1** Geographic location of the Uholka–Shyrokyi Luh primeval beech forest in Europe and map of the study area. Dark grey shadings show the distribution of *Fagus sylvatica* according to http://www.euforgen.org. The Uholka–Shyrokyi Luh area is shown with altitudinal belts. Trees with *Lobaria pulmonaria* ($n$ = 483) that harbour individuals of the fungal gene pool A are marked in red and those with B in blue. The first-generation hybrids AB are marked in green.

fully covered all altitudinal levels and topographic elements within an inventory perimeter of 10 282 ha. This approach, described in details in Nadyeina *et al.* (2014), allowed an intensive screening of the whole area (covering the plots and the area in between) and thus a near complete sampling of the trees with *L. pulmonaria*. The trees harbouring *L. pulmonaria* are scattered over the forest massif, and we therefore sampled each tree found to host *L. pulmonaria*. The lichen fragments were collected up to a height of 5–6 m on the tree trunk using an avalanche probe (Black Diamond, Reinach, Switzerland) for thalli above 2–3 m, and along the whole trunk on lying trees. We grouped these trees into 19 local populations of *L. pulmonaria* according to their geographic position in homogenous areas and along narrow altitudinal belts (Fig. 1). Each population contained 18–198 specimens from 6 to 58 trees, scattered over 8–343 ha. At least three thallus fragments (considered here as individuals) were sampled per tree, preferably from different sides of the trunk. The height and aspect of the thallus fragments on the trunk were recorded for each sample (Appendix S1, Supporting information).

For each tree with *L. pulmonaria*, the aspect and slope were extracted from a WorldView-2 digital surface model (Commarmot *et al.* 2013), while other variables were measured in the field. This included coordinates, altitude a.s.l., tree characteristics (species, diameter and vitality) and relief (Appendix S1, Supporting information), as these variables have been shown to be of importance for the distribution of *L. pulmonaria*, for example in Belinchón *et al.* (2009), Mikryukov (2011), Jüriado *et al.* (2012) Scheidegger *et al.* (2012) and Nascimbene *et al.* (2013a,b). The distances of the *L. pulmonaria* phorophytes from the nearest permanent water source or stream were calculated in ArcGIS.10 (http://www.esri.com/software/arcgis) and then classified as either close to rivers (<30 m) or far from rivers (>30 m). A positive effect of the proximity to a river on the presence of *L. pulmonaria* has been shown in a Mediterranean beech forest (Belinchón *et al.* 2009).

We used altitude as a proxy for climate-related environmental factors, like air temperature and solar radiation (Barry 1981; Körner 2007), and altitude and distance from rivers were used as proxies for air humidity. As Bennie

*et al.* (2006) show, aspect and slope affect local insolation. The height of the lichen thallus on a trunk is related to the degree of canopy openness, with optimum light availability higher up on the tree trunks under the dense forest canopies in lowland floodplains (Nadyeina *et al.* 2014).

### *Molecular analysis*

Total DNA was isolated from cleaned and lyophilized lobe tips of *L. pulmonaria* using the DNeasy 96 plant kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Eight unlinked, fungal microsatellite loci, LPu03, LPu09, LPu15, LPu23, LPu24, LPu25, LPu28 and MS4 (Walser *et al.* 2003; Widmer *et al.* 2010; Dal Grande *et al.* 2012), and 14 alga-specific microsatellite loci, LPh1–LPh9, LPu16, LPu19, LPu20, LPu26 and LPu27 (Walser *et al.* 2003; Widmer *et al.* 2010; Dal Grande 2011), were analysed (Appendix S2, Supporting information). PCRs were set up according to the methods reported in Dal Grande *et al.* (2012) and performed with a PTC-100 thermal cycler (MJ Research). Fragment lengths of the PCR products were determined on a 3730 DNA Analyzer using LIZ-500 as the internal size standard (both Life Technologies Corporation, Rotkreuz, Switzerland). Genotyping was performed with GENEMAPPER 3.7 (Life Technologies).

### *Statistical analyses*

To infer the genetic pattern in local populations of *L. pulmonaria*, Bayesian analysis of the population structure was run for the fungal and algal data separately using STRUCTURE version 2.3.2 (Pritchard *et al.* 2000). We assigned the most likely number of clusters (*K*), or gene pools, using the method given in Evanno *et al.* (2005) in software program STRUCTURE HARVESTER v0.6.1, as described in Earl & vonHoldt (2012). The rate of change in the log likelihood of the data between subsequent *K* values (delta *K*) was calculated and ranged from $K = 1$ to $K = 15$ for both the fungal and algal data. A map of the study region with fungal gene pools pattern was drawn in ArcGIS.10. Principal coordinate analyses (PCoA) based on seven mycobiont- and 14 photobiont-specific loci were run in GENALEX 6.5 (Peakall & Smouse 2006).

All genetic parameters were calculated for both symbiotic partners separately. Genetic diversity indices, including the average number of alleles per locus ($N_a$), the number of private alleles per population (*P*) and Nei's unbiased gene diversity (*H*), were calculated with GENALEX 6.5 (Peakall & Smouse 2006). Allelic richness (*A*) was corrected for different sample sizes by setting the population size equal to the minimum number of samples (18 lichen individuals) using the rarefaction method implemented in the R package *hierfstat* (Goudet 2013). The same method was used to quantify the allelic richness of the gene pools: a total of 504 and 327 individuals were used for rarefaction for the mycobiont and photobiont, respectively. The number of multilocus genotypes (*G*), the percentage of different multilocus genotypes per population (*M*) and the minimum number of colonization events (*C*), that is the number of alleles at the most variable locus, were calculated using codes originally written by Werth *et al.* (2006) in R (R Development Core Team 2012). These codes were extended by SW to handle individuals with missing data.

Analyses of molecular variance (AMOVA) and population pairwise fixation indices ($F_{ST}$) were calculated on the basis of haplotype frequencies (Excoffier *et al.* 1992) for the two mountain regions, Uholka and Shyrokyi Luh, and the inferred number of gene pools of the mycobiont and photobiont of *L. pulmonaria*, using ARLEQUIN 3.11 (Excoffier *et al.* 2005). The statistical significance of the results was based on 1000 permutations.

To calculate the likelihood of each individual belonging to a particular population, that is to either the Uholka or Shyrokyi Luh, and to one of the gene pools of the mycobiont or photobiont of *L. pulmonaria*, we used the Bayesian algorithm of Rannala & Mountain (1997) in GENECLASS2 (Piry *et al.* 2004). This Monte Carlo method implemented by Paetkau *et al.* (2004) was used to detect the statistical threshold beyond which individuals may be considered as residents or recent migrants. The likelihood ratio of *L_home* (the likelihood of the individual genotype within the population where the individual has been sampled) to *L_max* (the highest likelihood value among all available population samples including the population where the individual was sampled) was used as recommended in Paetkau *et al.* (2004). Individuals were considered as recent migrants when the probability of exclusion reached the significance levels specified below.

One-way analyses of variance (ANOVA) were run with post hoc Tukey–Kramer HSD tests to distinguish between the means of Na, H and A of both symbionts and the two mountain regions or four altitudinal–regional groups. The same analyses were computed to compare the allelic richness of the fungal gene pools occurring in the study region. These were also compared with those of several populations of *L. pulmonaria* in southeastern Europe (Scheidegger *et al.* 2012). Differences between the total allelic richness of the fungal gene pools of *L. pulmonaria* and the algal gene pools from the populations of Uholka–Shyrokyi Luh were estimated for seven fungal and 14 algal loci with Student's *t*-tests. All the above-mentioned statistical analyses were performed with R.

Relationships between the gene pools of both symbionts of *L. pulmonaria* and topographic and forest-stand factors were tested with general linear models (GLM) using a binomial model family in R. The phorophyte tree diameter was log-transformed, aspect was arcsine-transformed, and exposition was expressed as eastness and northness (Roberts 1986). Multicollinearity between environmental factors was assessed on the basis of the variance inflation factor (VIF) with the *car* package (Fox & Weisberg 2011). The cumulative effect of covariables with VIF < 2 was included in the GLM. Nominal factors, relief and distance from rivers were coded as dummy variables with package *dummies* (Brown 2012). The best models were selected on the basis of Akaike's information criterion (AIC, Akaike 1974) using 'backward' selection as implemented in *mass* package (Venables & Ripley 2002).

We tested whether the association between fungal and algal gene pools was random using chi-square ($\chi^2$) tests, randomly replacing specific algal genotypes while keeping the observed fungal gene pool constant. In this way, 1000 resamples of the data were generated. The distribution of randomly constructed associations between the fungal and algal gene pools was then compared with the observed frequencies. We calculated $P_{\text{greater}}$, $P_{\text{equal}}$ and $P_{\text{smaller}}$, the probabilities that the randomized value was larger, equal to or smaller than the observed value. This analysis was performed in *R*.

The lineage diversification of an ancestral population of *L. pulmonaria* into gene pools and the time it took for these gene pools to establish in the Carpathian Mts. were modelled in DIYABC 2.0 (Cornuet *et al.* 2008). Late Pliocene was probably the age of appearance of *L. pulmonaria* as a separate species (Cornejo 2013), and speciation occurred during the Pleistocene in East Asia. Pleistocene glacial cycles (Widmer *et al.* 2012) or pre-Pleistocene (Scheidegger *et al.* 2012) has been suggested as the time frame for the intraspecific diversification into gene pools in the European population of *L. pulmonaria*. Following this rough time frame, we set the genetic diversification time $t_d$ widely, to include the Pleistocene and Pliocene, and the probable time of the invasion of the study area $t_i$ as the Pleistocene (shown as prior distribution in number of generations, Appendix S2, Supporting information). A generation time of 35 years for *L. pulmonaria* (Scheidegger & Goward 2002) was assumed for the approximate Bayesian computation (ABC). The ranges for the genetic diversification time $t_d$ and the time of the invasion of the study area $t_i$ were estimated as number of generations ago × 35 year according to Gibbard and Kolfschoten (2004) geochronology. We have tested several scenarios, assuming different lineage divergence times, that is in the Middle Ages, during the last glacial maximum (LGM), during Early Pleistocene and during Pliocene, respectively. However, the estimation of posterior probability of the most likely scenario is complicated in the case of multiple scenarios. Therefore, we selected two main scenarios at the stage of pre-evaluation of scenarios and prior distributions, which were the closest to the observed data set (results not shown). These two main scenarios of lineage diversification were selected for a DIYABC run (Appendix S2, Supporting information). The first assumed that the divergence occurred due to the bottleneck with a duration $db_d$ at time $t_d$ in the gene pool of pre-A population, which caused a change in population effective size from $NA_{pre}$ to $NA_{db_d}$ and $NB_{db_d}$. The second scenario assumed that the divergence occurred due to the bottleneck with a duration $db_d$ at time $t_d$ in the gene pool of pre-B population, which caused a change in population effective size from $NB_{pre}$ to $NA_{db_d}$ and $NB_{db_d}$. Each scenario included another bottleneck $db_i$ at the time $t_i$, corresponding to the time of invasion of both gene pools of *L. pulmonaria* in the study area, that is founder effects (Appendix S2, Supporting information). Of 3 000 000 data sets were simulated to reveal posterior model checking and relate the observed data set to posterior parameters as implemented in DIYABC (Cornuet *et al.* 2008). The generalized stepwise mutation model (Estoup *et al.* 2002) was used to run posterior model simulations. The significance threshold for all analyses was treated as *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) or $^{ns}$($P > 0.05$).

## Results

### *Genetic diversity of the Lobaria pulmonaria symbiosis*

We analysed 1522 individuals of *Lobaria pulmonaria* and found a total of 129 alleles for the fungal partner and 260 alleles for the algal partner (Appendix S3, Supporting information). All loci, except the fungus-specific LPu24, were polymorphic in our study, as was found by Walser *et al.* (2003) and Werth & Scheidegger (2012). Thus, we excluded this locus from further analyses. The number of alleles per locus for the mycobiont ranged from three (LPu23) to 37 (LPu25), and from four (LPh8) to 60 (LPu26) for the photobiont (Appendix S3, Supporting information). The average number of all alleles ($N_a$) per population for the entire study area was 7.7 for the mycobiont (allelic richness $A = 6.1$) and 8.2 for the photobiont (allelic richness $A = 5.4$; Appendix S4, Supporting information).

### *Gene pools of Lobaria pulmonaria symbionts*

The Bayesian analysis of the genetic population structure revealed two genetically distinct clusters in both

symbionts (Fig. 2, Appendices S5–S7, Supporting information). The highest value of delta *K* as a function of *K* was reached at $K = 2$ for the fungal and algal symbionts (Fig. 2). Further analyses were thus carried out assuming the presence of two distinct gene pools for each symbiont. The definition and abbreviation of the fungal gene pools, A and B, and individuals with mixed or hybrid origin, AB, follow those in Scheidegger *et al.* (2012). The gene pools of the photobiont are abbreviated as $P_1$ and $P_2$. Nearly 66% of all fungal individuals (1005 samples) belonged to gene pool B, whereas individuals of the gene pool A were less common in the study area (504 samples; Figs 1 and 3A, Appendix S4, Supporting information). The individuals of the algal gene pool $P_1$ were more frequent (1149 samples or 75%) than those of $P_2$, which contained 373 individuals (Fig. 3B, Appendix S4, Supporting information).

In each symbiont, considerable genetic differentiation (mycobiont: 32.4% and photobiont: 17.2%) was found between gene pools (Table 1). The genetic differences between the two mountain massifs were small (mycobiont: 0.13% and photobiont: 0.21%; Table 1). The segregation between gene pools is supported by private multilocus genotypes and private alleles in each of the gene pools of both symbionts (Table 2).

The majority of fungal individuals were assigned to one of two gene pools. Only 13 individuals appear to have a hybrid ancestry AB in our analysis (Fig. 1, Appendix S4, Supporting information). These individuals were distributed along high slopes and valleys, but not in the highlands around the timberline. The hybrid individuals were sterile, while fertile individuals were equally distributed between the fungal gene pools A (19 thalli) and B (20 thalli; $\chi^2 = 4.603^{ns}$). A test to identify first-generation migrants (implemented in GENECLASS2) detected one fungal individual with mixed ancestry, but no algal individuals as first-generation migrants (Table 1) were found.

Allelic richness, resampled to the lowest sample size and averaged across loci (Table 2), did not differ significantly between the two fungal gene pools A and B ($t = 0.6^{ns}$) or between the algal gene pools $P_1$ and $P_2$ ($t = 1.3^{ns}$).

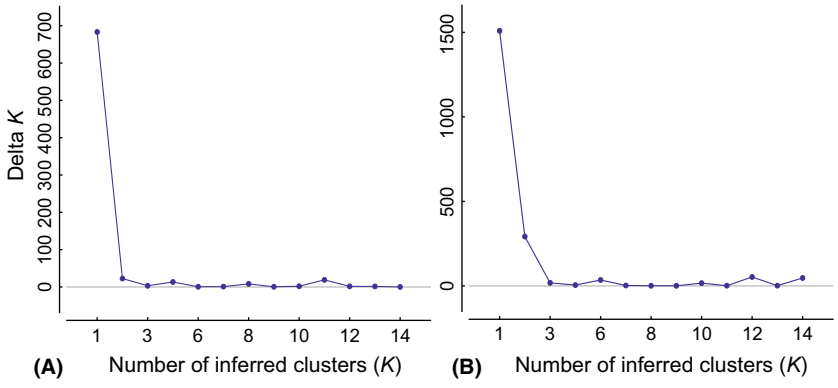


**Fig. 2** Result of STRUCTURE analyses, where delta *K* values (Delta $K$ = mean $((|L''(K)|)/SD(L(K)))$ are plotted for values of *K* from 1 to 15 in STRUCTURE HARVESTER. A, Mycobiont of *Lobaria pulmonaria*. B, Photobiont of *L. pulmonaria*, *Dictyochloropsis reticulata*.

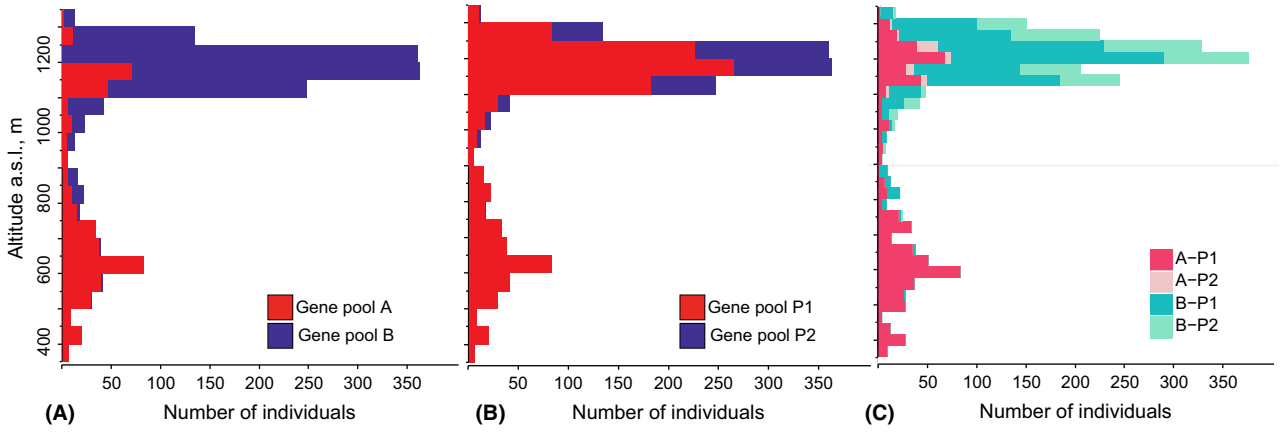


**Fig. 3** Altitudinal distribution of the two fungal gene pools, A and B (A), the two algal gene pools, $P_1$ and $P_2$ (B), and the four associations between them (C). Total number of samples analysed, $n = 1522$.

**Table 1** Analysis of molecular variances (AMOVA) computed in ARLEQUIN for the mycobiont of *Lobaria pulmonaria* (based on seven fungal loci) and its photobiont *Dictyochloropsis reticulata* (based on 14 algal loci), $n = 1522$. The number of recent migrants $N_m$ and the probability $P$ are shown as the results of analyses in GENECLASS2

| Symbiont | Source of variations | Degrees of freedom | Sum of squares | Variance | Percentage of variation |
|---|---|---|---|---|---|
| Mycobiont | Uholka and Shyrokyi Luh | $F_{ST}$ = 0.0013ns; $N_m$ = 61** | | | |
| | Among | 1 | 6.9 | 0.003 | 0.13 |
| | Within | 3042 | 7056.7 | 2.320 | 99.87 |
| | Gene pools | $F_{ST}$ = 0.324***; $N_m$ = 1** | | | |
| | Among | 1 | 1244.7 | 0.919 | 32.41 |
| | Within | 3016 | 5782.9 | 1.917 | 67.59 |
| Photobiont | Uholka and Shyrokyi Luh | $F_{ST}$ = 0.0021ns; $N_m$ = 67** | | | |
| | Among | 1 | 16.8 | 0.009 | 0.21 |
| | Within | 3042 | 12211.1 | 4.014 | 99.79 |
| | Gene pools | $F_{ST}$ = 0.172***; $N_m$ = 0** | | | |
| | Among | 1 | 881.2 | 0.832 | 17.20 |
| | Within | 2674 | 10703.3 | 4.003 | 82.80 |

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $^{ns}P > 0.05$.

### *Associations between the fungal and algal gene pools*

We found strong associations between the fungal and algal gene pools ($\chi^2 = 133.537$***). The most frequent association was that of the fungal gene pool B and algal gene pool $P_1$ (B–$P_1$: 666 individuals), which was predominantly distributed above 900 m (Table 3, Fig. 3C). The combination of the fungal gene pool A and algal gene pool $P_1$ was less frequent (A–$P_1$: 471 individuals), but it was homogeneously spread over the entire altitudinal gradient (350–1350 m). This combination, A–$P_1$, was the only association found in the lowlands, occurring close to rivers on the lower parts of slopes and along valleys. Combinations of the fungal gene pools B and A with algal gene pool $P_2$ were less frequent (B–$P_2$ and A–$P_2$: 339 and 33 individuals, respectively), and both associations were restricted to areas above 900 m (Table 3, Fig. 3C). The association A–$P_1$ was significantly more abundant than expected under randomness. In contrast, A–$P_2$ was rarer than expected based on the observed frequencies of the fungal and algal gene pools. The observed gene pool frequency of B–$P_1$ was significantly rarer than expected, while that of B–$P_2$ was significantly more frequent (Table 3).

**Table 2** Genetic differentiation between two fungal gene pools of *Lobaria pulmonaria* A and B, and two algal gene pools $P_1$ and $P_2$ of *Dictyochloropsis reticulata*. Specimens with missing data in loci are omitted

| | Gene pool | *n* | $N_a$ | *A* | *C* | *P* | *nP* | *G* | *M* |
|---|---|---|---|---|---|---|---|---|---|
| Mycobiont | A | 487 | 12.3 | 12.3 | 30 | 6.1 | 283 | 303 | 0.60 |
| | B | 969 | 10.1 | 9.3 | 22 | 4.1 | 607 | 379 | 0.37 |
| Photobiont | $P_1$ | 980 | 16.7 | 13.8 | 56 | 7.8 | 816 | 778 | 0.68 |
| | $P_2$ | 327 | 9.9 | 9.9 | 25 | 1.0 | 39 | 221 | 0.59 |

$N_a$, average number of alleles per locus; *A*, mean allelic richness per locus; *C*, number of alleles at the most variable locus; *P*, number of private alleles per locus; *nP*, number of individuals with at least one private allele; *G*, number of multilocus genotypes; *M*, percentage of different multilocus genotypes per population.

### *Ecological specialization of the fungal and algal gene pools*

Our data show that distribution of fungal gene pools depends on altitude above sea level, sample height on the phorophyte trunk and distance from rivers (Table 4). The lowland, floodplain populations of the mycobiont belong predominantly to gene pool A, but gene pool A was also scattered above 1000 m a.s.l. Gene pool B, however, was restricted to higher altitudes, and only a few individuals were found below 1000 m a.s.l. (Figs 1 and 3A). Individuals belonging to gene pool A frequently occurred along rivers, even in the highlands, while individuals of gene pool B grew along high ridges, mostly far away from rivers (Fig. 4A). The thalli of gene pool B were often found lower at the tree trunks, that is below 3 m from the tree base at highlands, while the thalli of both gene pools may grow up to heights of 20–25 m at the trunk (Fig. 4B). The distribution of fungal gene pools was not associated with geographic coordinates, which distinguish the two forest massifs, the topography of the mountain (relief,

**Table 3** Associations between gene pools of mycobiont of *Lobaria pulmonaria* and its photobiont *Dictyochloropsis reticulata*, with the percentages (%) found in two altitudinal zones ($\chi^2$ = 516.2***). Test for randomness of the gene pool associations includes $P_{greater}$, $P_{equal}$ and $P_{smaller}$, which are probabilities that the randomized value is larger, equal to or smaller than the observed value. The expected value based on 1000 resamples of the data is given under exp (this value is the average over 1000 resamples of the data) and the observed number of genotype combinations under Absolute frequency

| Associations | Absolute frequency | $\chi^2$ | $P$ | Highlands, % of total | Lowlands, % of total | Test for randomness of gene pool associations | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Exp | $P_{greater}$ | $P_{equal}$ | $P_{smaller}$ |
| B–$P_1$ | 666 | 102.7 | *** | 39.1 | 4.7 | 757.4 | 1.0 | 0.0 | 0.0 |
| B–$P_2$ | 339 | 104.6 | *** | 21.1 | 1.2 | 247.6 | 0.0 | 0.0 | 1.0 |
| A–$P_1$ | 471 | 294.9 | *** | 11.0 | 19.9 | 380.1 | 0.0 | 0.0 | 1.0 |
| A–$P_2$ | 33 | 2.4 | ns | 1.8 | 0.3 | 123.1 | 1.0 | 0.0 | 0.0 |
| AB–$P_1$ | 12 | 3.0 | ns | 0.4 | 0.4 | | | | |
| AB–$P_2$ | 1 | 0.6 | ns | 0.1 | 0.0 | | | | |
| Total | 1522 | | | 100% | | | | | |

*$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001, $^{ns}P$ > 0.05.

inclination of the slope and aspect), the phorophyte diameter at breast height and aspect of the sample on the tree's trunk.

We also found statistical support for the variation of algal gene pools with altitude and relief (Table 4). The algal gene pool $P_2$ showed clear affinities to highland areas, while gene pool $P_1$ occurred along the entire altitudinal gradient throughout the whole area (Figs 1 and 3B). The individuals belonging to gene pool $P_1$ occupied valleys and mountain slopes, while both gene pools were found along the ridges.

**Table 4** Best-fitting general linear models, which explain the variation in the fungal and algal gene pools of *Lobaria pulmonaria* in terms of the topographic and forest-stand variables listed in Appendix S1 (Supporting information). Data include 1522 thallus fragments collected in the primeval beech forest of Uholka–Shyrokyi Luh

| Variable | Estimate | SE | $z$ | $P$ |
|---|---|---|---|---|
| Mycobiont: Percentage correctly predicted = 20%, McFadden's pseudo $R^2$ = 0.39 | | | | |
| Intercept | −8.18 | 1.43 | −5.72 | *** |
| Altitude | 0.007 | 0.001 | 6.66 | *** |
| Sample height on the host tree | 0.84 | 0.23 | 3.74 | *** |
| Distance from river | −8.07 | 2.73 | 2.96 | ** |
| Altitude × Distance from river | 0.008 | 0.003 | 2.71 | ** |
| Photobiont: Percentage correctly predicted = 77%, McFadden's pseudo $R^2$ = 0.12 | | | | |
| Intercept | −7.57 | 1.23 | −6.15 | *** |
| Altitude | 0.005 | 0.001 | 4.74 | *** |
| Relief: ridge | 0.28 | 0.40 | 0.70 | ns |

*$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001, $^{ns}P$ > 0.05.

### *Lineage diversification in Lobaria pulmonaria*

We found significant support for the hypothesis that the origin of the two gene pools in *L. pulmonaria* derived from an ancestral gene pool pre-A population (Appendices S2 and S8). The most likely scenario was selected based on comparisons using direct and logistic regressions, and principal component (PCo) analysis (Appendix S8, Supporting information). Divergence time ranged from 290 to 790 ka (0.25 and 0.75 quartiles, respectively) with a mean of 374.5 ka, corresponding to the Günz glacial periods during Early Pleistocene (Table 5). The estimated timing for the migration of both gene pools into the Carpathians was found between 65 and 540 ka with mean of 768.5 ka, which corresponds to the Mindel–Riss interglacial during Middle Pleistocene (Table 5).

## Discussion

We found a marked genetic subdivision in populations of *Lobaria pulmonaria* in the floristically homogeneous beech forest of Uholka–Shyrokyi Luh (Fig. 1). This is the first investigation of *L. pulmonaria*, where a nearly complete sample was studied using a dense nonstratified plot network in a region of over 10 000 ha of primeval forest. The populations of each symbiont belonged to two gene pools (Appendix S4, Supporting information). Only the fungal partner of 13 individuals showed properties of both gene pools and thus considered to be the offspring of mating events between parents belonging to the two different gene pools. It is noteworthy that the two fungal gene pools appear to recombine with each other only very rarely (Table 1), even though they live in close proximity in homogeneous

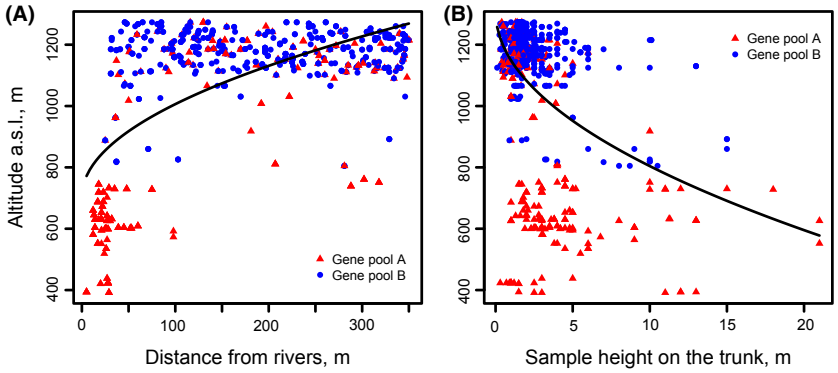


**Fig. 4** Altitudinal distribution of the two fungal gene pools A and B related to distance from rivers (A) and height on the tree trunk (B).

**Table 5** Posterior timing of divergence between gene pools in mycobiont *Lobaria pulmonaria* estimated using DIYABC (Cornuet *et al.* 2008) for the most likely scenario (see Appendices S7–S9, Supporting information). Geochronological periodization is given according to Gibbard and Kolfschoten (2004)

| | Time (ka) | | | |
|---|---|---|---|---|
| Parameter | Mean | q25 | q75 | Geochronological epoch |
| $t_0$ | 1.5 | 0.6 | 2.4 | Holocene: present, sampling time |
| $t_i + db_i$ | 374.5 | 65.1 | 539.0 | Pleistocene (Middle): Mindel–Riss interglacial |
| $t_d + db_d$ | 768.8 | 289.2 | 792.9 | Pleistocene (Early): Günz glacial |

forest stands, sometimes even on the same tree. This confirms that recombination in the heterothallic, fungal symbiont can occur, although it is relatively rare (Zoller *et al.* 1999; Singh *et al.* 2012). This pattern was also observed by Scheidegger *et al.* (2012). The low genetic exchange between two gene pools could indicate a possible reproductive isolation, but a recent study (Singh *et al.* 2012) found no differentiation of the MAT loci in populations belonging to two distinct gene pools in Europe.

The two fungal gene pools present in the Uholka–Shyrokyi Luh have also been found in southeastern Europe (Scheidegger *et al.* 2012; Widmer *et al.* 2012). Our results, as well as those of Scheidegger *et al.* (2012) and Werth & Scheidegger (2012), show that both symbiotic gene pools, fungal and algal, may co-exist within relatively small areas of 3–180 ha.

Several studies have reported ecological specialization in lichen photobionts (Piercey-Normore 2006; Yahr *et al.* 2006; Werth & Sork 2010; Fernandez-Mendoza *et al.* 2011; Peksa & Škaloud 2011). The first study to present evidence for ecological specialization in lichen-forming fungi was performed by Scheidegger *et al.* (2012), who reported gene pool associations with climate (elevation) in *L. pulmonaria*. With the present data, we have expanded these findings by demonstrating a corresponding pattern of climatic associations in the fungal and the algal gene pools. Moreover, we have shown that these climate and topography-driven gene pool associations occur within the same continuous forest, which indicates an interesting pattern of climate-driven ecological specialization in lichen symbionts.

### *Ecological specialization of the Lobaria pulmonaria symbiosis*

The distributions of fungal and algal gene pools of *L. pulmonaria* within the primeval beech forest of Uholka–Shyrokyi Luh were found to be influenced by ecological and microclimatic factors such as altitude, distance from rivers and sample height on the trunk (Table 4; Figs 3 and 4). Altitude, one of the main factors shaping the distribution of the symbiotic gene pools, can be related to differences in air temperature (Barry 1981; Körner 2007). The altitudinal distribution of *L. pulmonaria* showed a clear bimodal pattern in the primeval landscape we studied (Nadyeina *et al.* 2014). Altitude also explains the diversity and composition of epiphytic lichens (Dymytrova *et al.* 2014) and wood-inhabiting fungi (Ordynets & Nadyeina 2013) in recent studies from this primeval forest. Precipitation and prolonged periods with very humid air, rather than mean temperature, are the main factors promoting high diversity of poikilohydric organisms like fungi, including lichens (Beckett *et al.* 2008). The bimodal distribution of *L. pulmonaria* (Nadyeina *et al.* 2014) and its gene pools (Fig. 3) indicates that habitats are partitioned within the relatively homogeneous beech forest stands.

We have showed recently that *L. pulmonaria* prefers relatively open stands in the forest landscape at higher altitudes and survives in the closed canopy of lowland valley growing high on tree trunks to receive the necessary level of insolation (Nadyeina *et al.* 2014). Our genetic analyses further revealed that different types of

habitats, related to altitude, were colonized by different gene pools. Individuals belonging to the fungal gene pool A preferred lowland valleys and slopes close to mountain springs, where they tended to grow high up on the tree trunks. The individuals of gene pool B preferred highlands, where they grew on lower parts of the tree trunks and were mostly found far away from rivers (Figs 3A and 4). We thus conclude that temperature and levels of air humidity discriminate between the two gene pools. Warmer temperatures with elevated air humidity along rivers tend to favour gene pool A, and colder temperatures with increased precipitation and more frequent fog favour the highland gene pool B. The microclimatic partitioning of the gene pools indicates the existence of two sympatric, but ecologically differentiated evolutionarily significant units (Moritz 1994) in the fungal symbiont of *L. pulmonaria*.

Algal gene pools showed an association with altitude, but not with environmental variables, as in the case of mycobiont (Table 4; Fig. 3B). Gene pool $P_2$ dominated at higher altitudes, while gene pool $P_1$ occurred throughout the entire area. We hypothesize that the strong link to altitude in the algal partner is the result of the codispersal of the photobiont with the mycobiont. Vertical transmission plays a dominant role in *L. pulmonaria* (Dal Grande *et al.* 2012; Werth & Scheidegger 2012), which results in a strong association between the two symbionts, and probably indicates that the mycobiont ecologically buffers its endosymbiotic photobiont.

The availability of compatible photobionts is essential for lichen establishment and development after sexual reproduction (Werth *et al.* 2007; Scheidegger & Werth 2009). Free-living photobionts must be able to survive without the positive effect of ecological buffering provided by the mycobiont. There is a growing body of evidence that lichen photobionts are specialized to particular habitats and ecological conditions (Piercey-Normore 2006; Yahr *et al.* 2006; Werth & Sork 2010; Casano *et al.* 2011; Fernandez-Mendoza *et al.* 2011; del Hoyo *et al.* 2011; Peksa & Škaloud 2011; Werth 2012; del Campo *et al.* 2013; Domaschke *et al.* 2013). Our study is in concordance with those studies and showed a distinct ecological, that is altitudinal, niche differentiation in the photobiont *Dictyochloropsis eticulata* (Table 4, Fig. 3B).

We also demonstrated that the two fungal gene pools associated with genetically different photobionts (Table 3, Fig. 3C). A randomization test of the associations between the fungal and algal gene pools (Table 3) indicated that overall, the gene pools did not associate randomly according to their frequencies, but instead they formed nonrandom associations. Werth & Scheidegger (2012) obtained similar results in their study of *L. pulmonaria* and its photobiont in the Swiss Jura Mountains. In our study area, gene pool B was found to be associated with both algal gene pools, $P_1$ and $P_2$, while the gene pool A preferred only one of the algal gene pools, $P_1$ (Table 3, Fig. 3). The fungal gene pool B is therefore considered unspecific in its association with either one of the two photobiont gene pools.

A broader, infraspecific photobiont spectrum is probably advantageous for the colonization of new habitats, especially with aposymbiotic dispersal units, such as ascospores. The photobiont' ability to associate with either gene pool implies that they can be 'borrowed' from neighbouring thalli of the same species or from other species associated with the same photobiont, that is contribute to the same photobiont-mediated guild (Dal Grande *et al.* 2014). In our study region, *Lobaria amplissima*, which is known to share the same photobiont as *L. pulmonaria* (Dal Grande 2011; Dal Grande *et al.* 2014), was found growing next to it only in a few localities (Dymytrova *et al.* 2013, 2014). *Lobaria amplissima*, however, is a fringe, rather than a core species in this photobiont-mediated guild (Dal Grande *et al.* 2014) and has no symbiotic vegetative diaspores that would spread the photobiont (Rose & Purvis 2009). Prior studies have shown that lichen thalli may host populations of various species of lichen photobionts on their surface as 'epibionts' (Muggia *et al.* 2013). Moreover, lichen photobionts can easily survive the gut passage of invertebrates (Meier *et al.* 2002; Boch *et al.* 2011). Thus, lichenization of a germinating ascospore of *L. pulmonaria* may associate with *Dictyochloropsis reticulata* from an epibiont community or from invertebrate faeces.

### *Phylogeographic signatures of the Lobaria pulmonaria symbiosis*

Differentiation of the gene pools in the *L. pulmonaria* symbiosis associated with ecological/climatic factors and thus raises questions about the timing of the divergence and whether the differentiation of the gene pools occurred sympatrically in the study area or allopatrically in separate refugia. Using approximate Bayesian computation (ABC, Cornuet *et al.* 2008), we were able to show that the gene pool pre-A population was probably the ancestor of both the modern gene pools of *L. pulmonaria*, A and B (Appendices S2 and S8). The ABC results (Table 5) indicate that the fungal gene pool A is a descendant that evolved during the warm and humid climate of the Middle Pliocene (Haywood *et al.* 2000), and the divergence of gene pool B was related to cooling and aridization during the Early Pleistocene (Hewitt 1999). Scheidegger *et al.* (2012) hypothesized that the divergence of the gene pools A and B in *L. pulmonaria* might have occurred prior to the LGM, and our data confirm this suggestion. The current microclimatic

habitat partitioning of the two gene pools across the beech forest supports hypothesis that the two gene pools have evolved in different refugia, possibly under different climatic conditions. Climatic oscillations of the Pliocene and Early Pleistocene have also been identified as drivers of divergence, for example, in an endemic Norwegian peat moss (Stenøien *et al.* 2011), grouse (Drovetski 2003) and *Phlebotomus* sandflies (Esseghir *et al.* 2000).

Currently, individuals of gene pools A and B are sympatric in our study area, but their distribution centres are at different elevations and in different microclimatic niches. Despite the spatial proximity of both gene pools, we found only minimal gene flow between them as the very low number of recent migrants indicates (Table 1). The ecological amplitude of gene pool A is relatively broad, as this gene pool occupies all habitat types across the homogeneous primeval beech forest, including lowland valleys and highland sites near the timberline (Figs 1, 3A and 4A). The more recently diverged gene pool B is restricted to the highlands of the mountains far from rivers, where it co-exists with gene pool A (Figs 1, 3A and 4A).

The two fungal gene pools may have occupied the study area for a similar length of time as the allelic richness of both gene pools, A and B, in Uholka–Shyrokyi Luh was similar. The ABC results showed that both gene pools have co-existed in the Carpathians since the Mid Pleistocene, that is well before the LGM (Table 5). These results support the hypothesis that the Carpathians acted as a refugial area for the fungal gene pools of *L. pulmonaria* (Scheidegger *et al.* 2012; Widmer *et al.* 2012). Moreover, the mean allelic richness of both fungal gene pools of *L. pulmonaria*, A and B, is higher in Uholka–Shyrokyi Luh than in areas in southeastern Europe that were not glaciated during the LGM (Fig. 5). The Carpathian Mountains are considered a glacial refugium for common beech (Hewitt 1999; Magri *et al.* 2006), which is the main host tree for *L. pulmonaria* in Uholka–Shyrokyi Luh (Dymytrova *et al.* 2014). We thus argue that both lineages of *L. pulmonaria* are likely to be related to the phylogeographic history of its phorophyte species, *Fagus sylvatica*. Two fungal gene pools of *L. pulmonaria* may have survived the Pleistocene glaciations in different refugia, including the Carpathian Mountains. Similarly, the Carpathians have served as glacial refugia for crane fly (Ujvárosi *et al.* 2010), bank vole (Wójcik *et al.* 2010), spruce bark beetle (Krascsenitsová *et al.* 2013) and Norway spruce (Gugerli *et al.* 2001).

The clear dominance of the gene pool B individuals in the study area (Figs 1 and 3A) may indicate that the Carpathian populations of *L. pulmonaria* have a biogeographic link with the Dinarides. Scheidegger *et al.* (2012) hypothesized that the distribution of gene pool B is concentrated in the Mediterranean and Dinarides regions. Biogeographic relations to Mediterranean region were found for Ukrainian Carpathian lichen biota (Makarevych 1963) and fauna (Varga 2010).

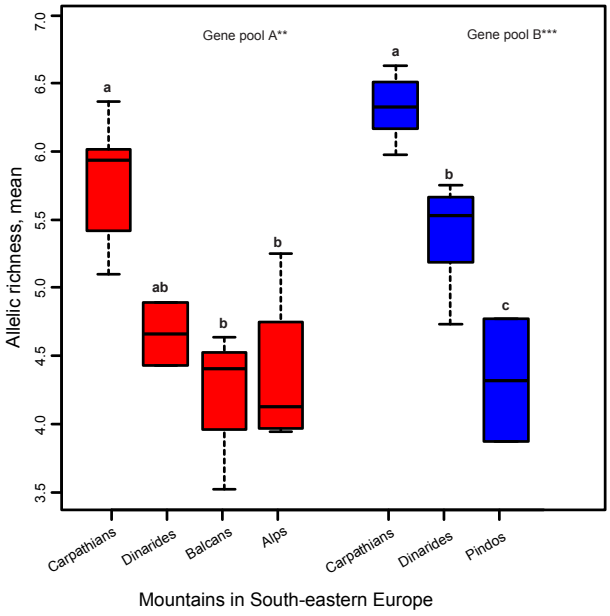


**Fig. 5** Allelic richness in the two fungal gene pools of *Lobaria pulmonaria*, A and B, from the Carpathians (this study) and mountain regions in southeastern Europe (data adjusted from Scheidegger *et al.* 2012). The letters denote differences in the level of means based on Tukey–Kramer HSD *t*-tests. One-way analyses of variance (ANOVA) show differences in allelic richness in the fungal gene pools A ($R^2 = 0.69$, $F = 7.49$**) and B ($R^2 = 0.67$, $F = 16.05$***) among the mountain regions.

## *Implications for conservation*

The two gene pools, that we studied, colonize different ecological niches and have different origins and abundances in our study area. Fragments of a similar genetic pattern might also be found in forest patches and in more open, extensively managed anthropogenic landscapes (Scheidegger *et al.* 2012; Werth & Scheidegger 2012; Widmer *et al.* 2012). We suppose, however, that only large-scale primeval forest landscapes such as Uholka–Shyrokyi Luh have been able to conserve such complex genetic patterns. *Lobaria pulmonaria* is red-listed in Ukraine and in many other European countries (i.e. Türk & Hafellner 1999; Blum *et al.* 2009; Wirth *et al.* 2011). We therefore recommend that Red List assessments, conservation actions and local species recovery plans take into consideration different gene pools of *L. pulmonaria* when developing conservation strategies in future.

The fungal gene pool A is a Pliocene relict that occurs throughout the whole Uholka–Shyrokyi Luh area. It is,

however, three times less frequent than the gene pool B. Gene pool A has a higher selectivity towards its photobiont than gene pool B, but less selectivity for host tree and altitudinal levels (our study and Scheidegger *et al.* 2012). Thus, this gene pool might be more vulnerable in a changing environment than gene pool B, and forest cuts and fragmentation are likely to be the main threats for this gene pool. We therefore suggest that the fungal gene pool A deserves a higher conservation priority in central and southeastern Europe because it has relict status, has higher photobiont selectivity and is less frequent.

On the other hand, the fungal gene pool B is a descendant of the Early Pleistocene' cold and arid climate. It is more frequent in our study area, as well as in southeastern Europe generally (Scheidegger *et al.* 2012), where it is restricted to mountain beech forests, and is currently thus less vulnerable. This gene pool may, however, become more vulnerable in future with gradual global warming (Rosenzweig 2007).

## Acknowledgements

We are grateful to four anonymous reviewers for their constructive comments, and are indebted to Brigitte Commarmot (Birmensdorf, Switzerland), Ruedi Iseli and Beate Hasspacher (Olten, Switzerland) for their support and coordination of this project. We also thank to Vasyl Naumovich (Kherson, Ukraine), Olexander Ordynets (Kharkiv, Ukraine), Volodymyr Savchyn (Lviv, Ukraine) and colleagues from the Carpathian Biosphere Reserve and Ukrainian National Forestry University for assisting with the field work. Prof. Olexander Khodosovtsev (Kherson, Ukraine) kindly organized a preparatory fieldtrip to the Crimean beech forests (Ukraine). We thank Silvia Dingwall (Birmensdorf, Switzerland) for linguistic corrections of the text. Special thanks go to Carolina Cornejo (Birmensdorf, Switzerland) for support in the Molecular Lab. This project was funded by the Swiss State Secretariat for Education, Research and Innovation (SERI). We also appreciated the support of the Genetic Diversity Centre (GDC) at ETHZ (Zurich, Switzerland) and the warm atmosphere they provided.

## References

Akaike H (1974) A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, **19**, 716–723.

Asplund J (2011) Chemical races of *Lobaria pulmonaria* differ in palatability to gastropods. *Lichenologist*, **43**, 491–494.

Asplund J, Gauslaa Y (2007) Content of secondary compounds depends on thallus size in the foliose lichen *Lobaria pulmonaria*. *Lichenologist*, **39**, 273–278.

Asplund J, Solhaug KA, Gauslaa Y (2009) Fungal depsidones—an inducible or constitutive defence against herbivores in the lichen *Lobaria pulmonaria*? *Basic and Applied Ecology*, **10**, 273–278.

Barry RG (1981) *Mountain Weather and Climate*. Elsevier B.V., University of Colorado, Boulder, Colorado, USA.

Beckett RP, Kranner I, Minibayeva FV (2008) Stress physiology and the symbiosis. In: *Lichen Biology*, 2nd edn (ed. Nash TH III), pp. 134–151. Cambridge University Press, Cambridge.

Belinchón R, Martínez I, Otálora MA *et al.* (2009) Fragment quality and matrix affect epiphytic performance in a Mediterranean forest landscape. *American Journal of Botany*, **96**, 1974–1982.

Bennie J, Hill MO, Baxter R, Huntley B (2006) Influence of slope and aspect on long-term vegetation change in British chalk grasslands. *Journal of Ecology*, **94**, 355–368.

Bidussi M, Gauslaa Y, Solhaug KA (2013) Prolonging the hydration and active metabolism from light periods into nights substantially enhances lichen growth. *Planta*, **237**, 1359–1366.

Blum OB, Kondratyuk SY, Khodosovtsev OY (2009) Lichens. In: *Red Book of Ukraine: Plants* (ed. Didukh YP), pp. 728–780. Globalconsulting, Kyiv.

Boch S, Prati D, Werth S, Rüetschi J, Fischer M (2011) Lichen endozoochory by snails. *PloS One*, **6**, e18770.

Brändli U-B, Dowhanytsch J (2003) *Urwälder im Zentrum Europas. Ein Naturführer durch das Karpaten-Biosphärenreservat in der Ukraine Hrsg*. von der Eidgenössischen Forschungsanstalt WSL und Karpaten-Biosphärenreservat, Rachiw, Haupt Verlag, Bern, Stuttgart, Wien.

Brändli U-B, Dowhanytsch J, Commarmot B (2008) *Virgin Forest of Uholka. Nature Guide to the Largest Virgin Beech Forest of Europe A UNESCO World Heritage Site*. Swiss Federal Research Institute WSL, CBR Rakhiv, Birmensdorf.

Brown C (2012) *Create dummy/indicator variables flexibly and efficiently*. An R package 'dummies', version 1.5.6 from June 2012.

Bursak VP (1998) The climate. In: *Biodiversity of the Carpathian Biosphere Reserve* (eds Movchan Ya, Hamor F, Sheliag-Sosonko Yu, Dudka I, Zahorodniuk I), pp. 69–80. Interecocentr, Kiev.

del Campo EM, Catala S, Gimeno J *et al.* (2013) The genetic structure of the cosmopolitan three-partner lichen *Ramalina farinacea* evidences the concerted diversification of symbionts. *Fems Microbiology Ecology*, **83**, 310–323.

Casano LM, del Campo EM, Garcia-Breijo FJ *et al.* (2011) Two *Trebouxia* algae with different physiological performances are ever-present in lichen thalli of *Ramalina farinacea*. Coexistence versus competition? *Environmental Microbiology*, **13**, 806–818.

Commarmot B, Bachofen H, Bundziak Y *et al.* (2005) Structures of virgin and managed beech forests in Uholka (Ukraine) and Sihlwald (Switzerland): a comparative study. *Forest Snow and Landscape Research*, **79**, 45–56.

Commarmot B, Brändli U-B, Hamor F, Lavnyy V (2013) *Inventory of the Largest Primeval Beech Forest in Europe—A Swiss-Ukrainian Scientific Adventure*. Swiss Federal Research Institute WSL, L'viv, Ukrainian National Forestry University, Rakhiv, Carpathian Biosphere Reserve, Birmensdorf.

Cornejo C (2013) *An evolutionary view of the taxonomy of the Ascomycetous genus* Lobaria *(Lobariaceae, Peltigerales)*. PhD thesis, University of Bern, Bern, Switzerland.

Cornuet J-M, Santos F, Beaumont MA *et al.* (2008) Inferring population history with DIYABC: a user-friendly approach to approximate Bayesian computation. *Bioinformatics*, **24**, 2713–2719.

Dal Grande F (2011) *Phylogeny and co-phylogeography of a photobiont-mediated guild in the lichen family Lobariaceae*. PhD thesis, University of Bern, Bern, Switzerland.

Dal Grande F, Widmer I, Wagner HH, Scheidegger C (2012) Vertical and horizontal photobiont transmission within populations of a lichen symbiosis. *Molecular Ecology*, **21**, 3159–3172.

Dal Grande F, Beck A, Cornejo C *et al.* (2014) Molecular phylogeny and symbiotic selectivity of the green algal genus *Dictyochloropsis* s.l. (Trebouxiophyceae): a polyphyletic and widespread group forming photobiont-mediated guilds in the lichen family Lobariaceae. *New Phytologist*, **202**, 455–470.

Domaschke S, Vivas M, Sancho LG, Printzen C (2013) Ecophysiology and genetic structure of polar versus temperate populations of the lichen *Cetraria aculeata*. *Oecologia*, **173**, 699–709.

Drovetski SV (2003) Plio-Pleistocene climatic oscilations, Holarctic biogeography and speciation in an avian subfamily. *Journal of Biogeography*, **30**, 1173–1181.

Dymytrova L, Nadyeina O, Naumovych A, Keller C, Scheidegger C (2013) Primeval beech forests of Ukrainian Carpathians are sanctuaries for rare and endangered epiphytic lichens. *Herzogia*, **26**, 73–89.

Dymytrova L, Nadyeina O, Hobi M, Scheidegger C (2014) Topographic and forest-stand variables determining epiphytic lichen diversity in the primeval beech forest in the Ukrainian Carpathians. *Biodiversity and Conservation*, **23**, 1367–1394.

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, **4**, 359–361.

Esseghir S, Ready PD, Ben-Ismail R (2000) Speciation of *Phlebotomus sandflies* of the subgenus *Larroussius* coincided with the late Miocene-Pliocene aridification of the Mediterranean subregion. *Biological Journal of the Linnean Society*, **70**, 189–219.

Estoup A, Jarne P, Cornuet JM (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Molecular Ecology*, **11**, 1591–1604.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611–2620.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47.

Fernandez-Mendoza F, Domaschke S, Garcia MA, Jordan P, Martín MP, Printzen C (2011) Population structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. *Molecular Ecology*, **20**, 1208–1232.

Fox J, Weisberg S (2011) *An {R} Companion to Applied Regression*, 2nd edn. An R package 'car', version 1.2-16 from August 2012. Sage, Thousand Oaks, California.

Gauslaa Y (1994) *Lobaria pulmonaria*, an indicator of species-rich forests of long ecological continuity. *Blyttia*, **52**, 119–128.

Gauslaa Y (1995) The *Lobarion*, an epiphytic community of ancient forests threatened by acid rain. *Lichenologist*, **27**, 59–76.

Gauslaa Y, Goward T (2012) Relative growth rates of two epiphytic lichens, *Lobaria pulmonaria* and *Hypogymnia occidentalis*, transplanted within and outside of *Populus* dripzones. *Botany-Botanique*, **90**, 954–965.

Gauslaa Y, Solhaug KA (2000) High-light-intensity damage to the foliose lichen *Lobaria pulmonaria* within a natural forest: the applicability of chlorophyll fluorescence methods. *Lichenologist*, **32**, 271–289.

Gauslaa Y, Solhaug KA (2001) Fungal melanins as a sun screen for symbiotic green algae in the lichen *Lobaria pulmonaria*. *Oecologia*, **126**, 462–471.

Gibbard P, Kolfschoten TV (2004) The Pleistocene and Holocene epochs. In: *A Geologic Time Scale* (eds Gradstein FM, Org JG, Smith AG), pp. 441–452. Cambridge University Press, Cambridge

Goudet J (2013) *Estimation and tests of hierarchical F-statistics*. An R package 'hierfstat', version 0.04-10 from April 2013.

Gugerli F, Sperisen C, Büchler U *et al.* (2001) Haplotype variation in a mitochondrial tandem repeat of Norway spruce (*Picea abies*) populations suggests a serious founder effect during postglacial re-colonization of the western Alps. *Molecular Ecology*, **10**, 1255–1263.

Haywood AM, Valdes PJ, Sellwood BW (2000) Global scale palaeoclimate reconstruction of the middle Pliocene climate using the UKMO GCM: initial results. *Global and Planetary Change*, **25**, 239–256.

Hewitt GM (1999) Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society*, **68**, 87–112.

Hilmo O, Lundemo S, Holien H, Stengrundet K, Stenøien HK (2012) Genetic structure in a fragmented Northern Hemisphere rainforest: large effective sizes and high connectivity among populations of the epiphytic lichen *Lobaria pulmonaria*. *Molecular Ecology*, **21**, 3250–3265.

del Hoyo A, Álvarez R, del Campo EM, Gasulla F, Barreno E, Casano LM (2011) Oxidative stress induces distinct physiological responses in the two *Trebouxia* phycobionts of the lichen *Ramalina farinacea*. *Annals of Botany*, **107**, 109–118.

Jüriado I, Liira J, Csencsics D *et al.* (2011) Dispersal ecology of the endangered woodland lichen *Lobaria pulmonaria* in managed hemiboreal forest landscape. *Biodiversity and Conservation*, **20**, 1803–1819.

Jüriado I, Karu L, Liira J (2012) Habitat conditions and host tree properties affect the occurrence, abundance and fertility of the endangered lichen *Lobaria pulmonaria* in wooded meadows of Estonia. *Lichenologist*, **44**, 263–276.

Körner C (2007) The use of 'altitude' in ecological research. *Trends in Ecology and Evolution*, **22**, 569–574.

Krascsenitsová E, Kozánek M, Ferenčík J, Roller L, Stauffer C, Bertheau C (2013) Impact of the Carpathians on the genetic structure of the spruce bark beetle *Ips typographus*. *Journal of Pest Science*, **86**, 669–676.

Magri D, Vendramin GG, Comps B *et al.* (2006) A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences. *New Phytologist*, **171**, 199–221.

Makarevych MF (1963) *Analysis of the Lichen Flora of Ukrainian Carpathians*. Academy of Sciences of USSR, Kyiv.

Meier FA, Scherrer S, Honegger R (2002) Faecal pellets of lichenivorous mites contain viable cells of the lichen-forming ascomycete *Xanthoria parietina* and its green algal photobiont, *Trebouxia arboricola*. *Biological Journal of the Linnean Society*, **76**, 259–268.

Mikryukov VS (2011) *Population ecology of the epiphytic lichen* Lobaria pulmonaria *(L.) Hoffm. in Ural and Sibiria*. PhD Thesis, Yekatirinburg University of Plant and Animal, Yekatirinburg, Russia.

Moritz C (1994) Defining 'evolutionarily significant units' for conservation. *Trends in Ecology and Evolution*, **9**, 373–374.

Muggia L, Vancurova L, Škaloud P, Peksa O, Wedin M, Grube M (2013) The symbiotic playground of lichen thalli—a highly flexible photobiont association in rock-inhabiting lichens. *Fems Microbiology Ecology*, **85**, 313–323.

Nadyeina O, Dymytrova L, Naumovych A, Postoyalkin S, Scheidegger C (2014) Distribution and dispersal ecology of *Lobaria pulmonaria* in the largest primeval beech forest of Europe. *Biodiversity and Conservation*, **23**, 1–22.

Nascimbene J, Nimis P, Ravera S (2013a) Evaluating the conservation status of epiphytic lichens of Italy: a red list. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*, **147**, 898–904.

Nascimbene J, Thor G, Nimis PL (2013b) Effects of forest management on epiphytic lichens in temperate deciduous forests of Europe—a review. *Forest Ecology and Management*, **298**, 27–38.

Ordynets O, Nadyeina O (2013) Diversity and ecology of wood-rotting fungi of Uholka-Shyrokyi Luh massif of the Carpathian Biosphere Reserve (Transcarpathian region, Ukraine). In: *Primeval and Ancient Beech Forests of Europe: Problems of Protection and Sustainable Use* (ed. Hamor F), pp. 256–261. Rakhiv, Ukraine.

Otalora MG, Martinez I, Belinchon R *et al*. (2011) Remnants fragments preserve genetic diversity of the old forest lichen *Lobaria pulmonaria* in a fragmented Mediterranean mountain forest. *Biodiversity and Conservation*, **20**, 1239–1254.

Paetkau D, Slade R, Burden M, Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology*, **13**, 55–65.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.

Peksa O, Škaloud P (2011) Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae). *Molecular Ecology*, **20**, 3936–3948.

Piercey-Normore MD (2006) The lichen-forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*. *New Phytologist*, **169**, 331–344.

Piry S, Alapetite A, Cornuet J-M, Paetkau D, Baudouin L, Estoup A (2004) GENECLASS2: a software for genetic assignment and first-generation migrant detection. *Journal of Heredity*, **95**, 536–539.

Pritchard J, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

R Development Core Team (2012) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences, USA*, **94**, 9197–9201.

Roberts DW (1986) Ordination on the basis of fuzzy set theory. *Vegetatio*, **66**, 123–131.

Rose F (1976) Lichenological indicators of age and environmental continuity in woodlands. In: *Lichenology: Progress and Problems* (eds Brown DH, Hawksworth DL, Bailey RH), pp. 279–307. Academic Press, London.

Rose F (1992) Temperate forest management: its effects on bryophyte and lichen floras and habitats. In: *Bryophytes and Lichens in a Changing Environment* (eds Bates JW, Farmer A), pp. 211–233. Clarendon Press, Oxford.

Rose F, Purvis OW (2009) *Lobaria*. In: *The Lichens of Freat Britain and Ireland* (eds Smith CW, Aptroot A, Coppins BJ, Fletcher A, Gilbert OL, James PW, Wolseley PA), pp. 560–562. British Lichen Society, London.

Rosenzweig C (2007) Global warming is changing the world. *Science*, **316**, 188–190.

Scheidegger C, Goward T (2002) Monitoring lichens for conservation: red lists and conservation action plans. In: *Monitoring with Lichens—Monitoring Lichens* (eds Nimis PL, Scheidegger C, Wolseley P), pp. 163–181. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Scheidegger C, Werth S (2009) Conservation strategies for lichens: insights from population biology. *Fungal Biology Reviews*, **23**, 55–66.

Scheidegger C, Frey B, Zoller S (1995) Transplantation of symbiotic propagules and thallus fragments: methods for the conservation of threatened epiphytic lichen populations. *Mitteilungen der Eidgenössischen Forschungsanstalt für Wald, Schnee und Landschaft*, **70**, 41–62.

Scheidegger C, Bilovitz PO, Werth S, Widmer I, Mayrhofer H (2012) Hitchhiking with forests: population genetics of the epiphytic lichen *Lobaria pulmonaria* in primeval and managed forests in southeastern Europe. *Ecology and Evolution*, **2**, 2223–2240.

Singh G, Dal Grande F, Cornejo C, Schmitt I, Scheidegger C (2012) Genetic basis of self-incompatibility in the lichen-forming fungus *Lobaria pulmonaria* and skewed frequency distribution of mating-type idiomorphs: implications for conservation. *PLoS ONE*, **7**, e51402. doi: 10.1371/journal.pone.0051402.

Stenøien H, Shaw A, Stengrundet K, Flatberg K (2011) The narrow endemic Norwegian peat moss *Sphagnum troendelagicum* originated before the last glacial maximum. *Heredity*, **106**, 370–382.

Trotsiuk V, Hobi ML, Commarmot B (2012) Age structure and disturbance dynamics of the relic virgin beech forest Uholka (Ukrainian Carpathians). *Forest Ecology and Management*, **265**, 181–190.

Türk R, Hafellner J (1999) Rote Liste gefährdeter Flechten (Lichenes) Österreichs. 2. Fassung. In: *Rote Listen gefährdeter Pflanzen Österreichs. 2. Auflage*. Grüne Reihe des Bundesministeriums für Umwelt, Jugend und Familie, Band 10 (ed. Niklfeld H), pp. 187–228. Austria Medien Service, Graz.

Ujvárosi L, Bálint M, Schmitt T, Mészáros N, Ujvárosi T, Popescu O (2010) Divergence and speciation in the Carpathians area: patterns of morphological and genetic diversity of the crane fly *Pedicia occulta* (Diptera: Pediciidae). *Journal of the North American Benthological Society*, **29**, 1075–1088.

Varga Z (2010) Extra-Mediterranean refugia, post-glacial vegetation history and area dynamics in Eastern Central Europe. In: *Relict Species: Phylogeography and Conservation Biology* (eds Habel JC, Assmann T), pp. 57–87. Springer, Heidelberg.

Venables WN, Ripley BD (2002) *Modern Applied Statistics with S*. Springer, New York, NY.

Walser JC (2003) *Populations genetic processes and ecological adaptation in a lichenized fungus assessed by microsatellite analysis and transplantation experiments*. PhD thesis, University of Bern, Bern, Switzerland.

Walser JC, Sperisen C, Soliva M, Scheidegger C (2003) Fungus-specific microsatellite primers of lichens: application for the assessment of genetic variation on different spatial scales in *Lobaria pulmonaria*. *Fungal Genetics and Biology*, **40**, 72–82.

Walser JC, Gugerli F, Holderegger R, Kuonen D, Scheidegger C (2004) Recombination and clonal propagation in different populations of the lichen *Lobaria pulmonaria*. *Heredity*, **93**, 322–329.

Werth S (2012) Fungal–algal interactions in *Ramalina menziesii* and its associated epiphytic lichen community. *Lichenologist*, **44**, 543–560.

Werth S, Scheidegger C (2012) Congruent genetic structure in the lichen-forming fungus *Lobaria pulmonaria* and its green-algal photobiont. *Molecular Plant-Microbe Interactions*, **25**, 220–230.

Werth S, Sork VL (2010) Identity and genetic structure of the photobiont of the epiphytic lichen *Ramalina menziesii* on three oak species in southern California. *American Journal of Botany*, **97**, 821–830.

Werth S, Wagner HH, Holderegger R, Kalwij JM, Scheidegger C (2006) Effect of disturbances on the genetic diversity of an old-forest associated lichen. *Molecular Ecology*, **15**, 911–921.

Werth S, Gugerli F, Holderegger R, Wagner HH, Csencsics D, Scheidegger C (2007) Landscape-level gene flow in *Lobaria pulmonaria*, an epiphytic lichen. *Molecular Ecology*, **16**, 2807–2815.

Widmer I, Grande FD, Cornejo C, Scheidegger C (2010) Highly variable microsatellite markers for the fungal and algal symbionts of the lichen *Lobaria pulmonaria* and challenges in developing biont-specific molecular markers for fungal associations. *Fungal Biology*, **114**, 538–544.

Widmer I, Dal Grande F, Excoffier L *et al.* (2012) European phylogeography of the epiphytic lichen fungus *Lobaria pulmonaria* and its green algal symbiont. *Molecular Ecology*, **21**, 5827–5844.

Wirth V, Hauck M, von Brackel W *et al.* (2011) Rote Liste und Artenverzeichnis der Flechten und flechtenbewohnenden Pilze Deutschlands. *Naturschutz und Biologische*, **70**, 7–122.

Wójcik JM, Kawałko A, Marková S, Searle JB, Kotlík P (2010) Phylogeographic signatures of northward post-glacial colonization from high-latitude refugia: a case study of bank voles using museum specimens. *Journal of Zoology*, **281**, 249–262.

Yahr R, Vilgalys R, DePriest PT (2006) Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. *New Phytologist*, **171**, 847–860.

Yoshimura I (1971) The genus *Lobaria* of Eastern Asia. *Journal Hattori Botanici Laboratory*, **34**, 231–364.

Zoller S, Lutzoni F, Scheidegger C (1999) Genetic variation within and among populations of the threatened lichen *Lobaria pulmonaria* in Switzerland and implications for its conservation. *Molecular Ecology*, **8**, 2049–2059.

This work was carried out in the framework of the project 'Primeval forest structure and biodiversity: population genetic research of *Lobaria pulmonaria* in the primeval beech forest area of Uholka–Shyrokyi Luh'. O.N. is interested in the evolutionary and ecological aspects of the population genetics of lichen symbionts and was responsible for sampling, laboratory work, statistical analyses and the text of the manuscript. L.D. is interested in lichen ecology and the effects of environmental factors on lichen diversity and contributed to sampling. A.N. and S.P. are interested in lichen floristics and also contributed to sampling. S.W. investigates population genetics and genomics of lichen-forming fungi and higher plants and also interested in local adaptations to the environment and in ecological specialization and contributed to the analyses and writing of this study. S.C. is interested in the symbiotic aspects of lichen association and phylogeography of the green-algal photobiont of *L. pulmonaria* and contributed to the laboratory work and draft of the manuscript. C.S.'s scientific focus is on biodiversity assessment and population genetics as the background for conservation and he contributed to the design of the study, sampling, data analysis and the text of the manuscript.

## Data accessibility

Microsatellite genotypes, alleles and environmental data, including location: Dryad doi:10.5061/dryad.7vs2r.

## Supporting information

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

**Appendix S1** List of variables used for trees with *L. pulmonaria* ($n$ = 483) and the specimens collected ($n$ = 1522).

**Appendix S2** Scenarios tested for lineage diversification with DIYABC (A, B; Cornuet *et al.* 2008) and prior historic parameters preselected and estimated (C).

**Appendix S3** The polymorphism of symbiont-specific microsatellite markers applied in the study ($n$ = 1522; abbreviations of genetic parameters used here and in other analyses, as described in the Materials and methods).

**Appendix S4** Genetic parameters of 19 local populations of *L. pulmonaria* symbionts in the primeval beech forest of Uholka–Shyrokyi Luh ($n$ = 1522).

**Appendix S5** Genetic differentiation in the populations of the mycobiont of *L. pulmonaria* from the study region.

**Appendix S6** Genetic differentiation in the populations of the photobiont of *L. pulmonaria*, *D. reticulata*, from the study region.

**Appendix S7** Bar plots of STRUCTURE analysis, where probabilities of membership to either of the gene pools are given on the $y$-axis (0–1) and samples on the $x$-axis.

**Appendix S8** Evaluation of the most likely scenario for the genetic divergence in the mycobiont of *L. pulmonaria*.