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European phylogeography of the epiphytic lichen fungus *Lobaria pulmonaria* and its green algal symbiont

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Abstract

In lichen symbiosis, fungal and algal partners form close associations, often codispersed by vegetative propagules. Due to the particular interdependence, processes such as colonization, dispersal or genetic drift are expected to result in congruent patterns of genetic structure in the symbionts. To study the population structure of an obligate symbiotic system in Europe, we genotyped the fungal and algal symbionts of the epiphytic lichen *Lobaria pulmonaria* at eight and seven microsatellite loci, respectively, and analysed about 4300 *L. pulmonaria* thalli from 142 populations from the species' European distribution range. Based on a centroid approach, which localizes centres of genetic differentiation with a high frequency of geographically restricted alleles, we identified the South Italy–Balkan region as the primary glacial refugial area of the lichen symbiosis. Procrustean rotation analysis and a distance congruence test between the fungal and algal population graphs indicated general concordance between the phylogeographies of the symbionts. The incongruent patterns found in areas of postglacial recolonization may show the presence of an additional refugial area for the fungal symbiont, and the impact that horizontal photobiont transmission and different mutation rates of the symbionts have on their genotypic associations at a continental scale.

Keywords: algal symbiont, biogeography, centres of genetic differentiation, centroid method, comparative phylogeography, *Dictyochloropsis reticulata*, fungal symbiont, geographically restricted alleles, glacial refugia, *Lobaria pulmonaria*, microsatellite, test of congruence

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Introduction

The response of organisms to changing climatic conditions is threefold: they become extinct, adapt or migrate to new sites with suitable environmental conditions. One way to predict the response of organisms to current climate change is to study past migrations during historical periods of climate changes. During multiple glacial cycles, European biota had to deal with extreme environmental fluctuations, and, accordingly, species distributions changed dramatically. Northern

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Europe as well as parts of Central Europe were covered by ice during glacial periods, and most species went extinct in these glaciated regions (Lister 1992; Huntley 1993; Latham & Ricklefs 1993; Svenning 2003), only surviving in southern glacial refugia. Many studies (Bennett *et al.* 1991; Hewitt 1996; Taberlet *et al.* 1998; Petit *et al.* 2002, 2003) confirmed that important glacial refugia were located in the South of Europe, namely on the Iberian and Italian Peninsulas and in the Balkans. These Southern European refugia offered large environmental variation in topography, climatic conditions and habitats, providing ecological refuges during glaciation (Hewitt 1996, 1999). Species survived in southern refugia during both glacial and interglacial periods (Bennett *et al.* 1991; Hewitt 1996; Taberlet *et al.* 1998; Pearson 2006).

The Pleistocene climatic changes caused population contraction, extinction and expansion. These population processes left their footprints in the genetic diversity and structure of organisms and their extant populations (Emerson & Hewitt 2005). Phylogeographic studies make use of these footprints to explore the history of species, with samples taken from potential glacial refugia and from formerly glaciated regions (Widmer & Lexer 2001). Due to genetic drift, long-term isolation of populations in different refugia can lead to genetic differentiation and to private (i.e. unique to a population) or geographically restricted alleles (GRAs) (Provan & Bennett 2008). As the climate warmed, many species that had persisted in glacial refugia experienced massive migrations into newly available territory. New populations were often founded by few individuals only representing a subsample of the genetic diversity found in the source populations (Widmer & Lexer 2001). The recolonization of Central and Northern Europe, for example, should have resulted in a series of founder events, causing changes in allele frequencies and a reduction in local genetic diversity (Ibrahim *et al.* 1996; Provan & Bennett 2008). Genetic differences between refugial populations and newly founded populations persist in the extant populations (Hewitt 1993, 1996; Soltis *et al.* 1997; Bernatchez & Wilson 1998). However, recolonization from different refugia also results in contact zones with the admixture of genetic lineages originating from different glacial refugia, resulting in high local genetic diversity. In contrast to refugial areas, contact zones do not harbour private or GRAs (Provan & Bennett 2008). Genetic diversity arising through mutation in recolonized areas during interglacial periods (e.g. in Central and Northern Europe) is expected to be lost during subsequent glacial periods due to population extinction (Bennett *et al.* 1991; Bernatchez & Wilson 1998).

The phylogeography of European species has intensively been studied (Beheregaray 2008). The glacial histories of many taxa have been described, and comparative phylogeographical studies identified general patterns in the biogeographic history of codistributed species (Bermingham & Moritz 1998; Taberlet *et al.* 1998; Avise 2000; Hewitt 2000; Schönswetter *et al.* 2005; Alvarez *et al.* 2009; Médail & Diadema 2009). In general, recurrent patterns were detected for European taxa, with northern regions generally colonized from Iberic and Balkanic refugia, while the spread of lineages from Italian refugia was often blocked by the Alps (Bilton *et al.* 1998; Taberlet 1998; Taberlet *et al.* 1998; Hewitt 2001). Many comparative phylogeographic studies showed that long-term geographical association of organisms, even when phylogenetically and ecologically distant, leads to phylogeographic congruence (Schneider *et al.* 1998; Riddle *et al.* 2000; Arbogast & Kenagy 2001; Lapointe & Rissler 2005; Victoriano *et al.* 2008; Alvarez *et al.* 2009). This should especially be the case in close species associations such as host/parasites or symbiotic systems (Parker & Spoerke 1998; Funk *et al.* 2000; Thompson *et al.* 2005; Criscione & Blouin 2007). To date, many studies on the spatial genetic structure of associated species examined host–parasite associations (Dybdahl & Lively 1996; Althoff & Thompson 1999; Jerome & Ford 2002; Johnson *et al.* 2002; Mutikainen & Koskela 2002; McCoy *et al.* 2005; Nieberding & Olivieri 2006; Criscione & Blouin 2007; Whiteman *et al.* 2007; Li & Dong 2009). In general, a significantly shared structure between the populations of the host and the pathogen was found in those cases where the distribution and migration of the host imposed a major constraint on the distribution of the pathogen. On the other hand, the absence of congruence in population structure should be indicative of different migration routes of host and pathogen. The degree of congruence between the genetic structure of co-occurring species is thus highly dependent on how strongly the species are ecologically linked to each other, the degree of specificity and the obligate nature of the interaction.

Lichens are ecologically obligate symbiotic associations between a fungus (mycobiont) and at least one photosynthetic organism (photobionts; an alga, a cyanobacterium or both) (Ahmadjian 1993; Lutzoni & Miadlikowska 2009). Lichens are interesting study systems for investigating the combined demographic histories of symbiotic organisms because (i) lichen reproduction and dispersal require the transmission of fungal and photosynthetic partners from one generation to the next as the fungus is completely dependent on its photobiont (Hawksworth & Honegger 1994; Honegger 1998; Lutzoni & Miadlikowska 2009), (ii) because the two lichen partners are often codispersed in vegetative propagules and (iii) because some species are important

indicator species for a variety of topics in ecology and conservation (e.g. ecological continuity, response to air pollution; Conti & Ceccetti 2001).

On account of the considerable interest of conservation biologists, *Lobaria pulmonaria* (L.) Hoffm., is perhaps the best studied lichen species worldwide, both from an ecological and from a population genetic point of view (Grube & Spribille 2012). The foliose epiphytic lichen *L. pulmonaria* is widely distributed in Europe, Africa, North America and Asia (Yoshimura 1971). Throughout Europe and in parts of North America, it is declining due to habitat loss, air pollution and (potentially) the effects of climate change. This lichen grows on the bark of several tree species primarily in old-growth forests, but also in wooded pastures and chestnut orchards (Rose 1992; Gauslaa 1994). *Lobaria pulmonaria* is a tripartite lichen symbiotic association between the respective fungus, the green alga *Dictyochloropsis reticulata* (Tschermak-Woess) Tschermak-Woess as the primary photobiont and the cyanobacterium *Nostoc* sp. (Jordan 1970) as secondary photobiont. Its primary photobiont *D. reticulata* forms symbiotic associations only with members of two fungal genera, that is, *Lobaria* and *Sticta* (Lobariaceae), and its main fungal host in Europe is *L. pulmonaria* (Dal Grande 2011). Contrary to other photobionts (e.g. *Trebouxia*, Wornik & Grube 2010), *D. reticulata* is completely dependent on the lichen symbiosis because it has never been found in a free-living stage (Dal Grande 2011; Stecher 2011). In Central Europe, *L. pulmonaria* has a generation time of 35 years or longer (Scheidegger & Goward 2002). A recent study (Dal Grande *et al.* 2012) has shown that the mycobiont of *L. pulmonaria* is mostly dispersed together with its algal photobiont through vegetative propagules and thallus fragments (also see Walser *et al.* 2001; Werth *et al.* 2006). Vegetative lichen dispersal implicates a long-term continuity of the symbiotic interaction (Dal Grande *et al.* 2012; Werth & Scheidegger 2012). When the fungus reproduces sexually, fungal sexual spores are distributed without the algal symbiont. These sexual spores have to find a compatible new photobiont to reconstitute the lichen symbiosis.

Understanding the large-scale spatial distribution patterns of interacting species is an important step to infer processes such as local adaptation and co-evolution among symbionts (Anderson *et al.* 2004; Werth & Scheidegger 2012). So far, only one study investigated the genetic structure of lichen mycobionts and photobionts at a continental scale (Fernández-Mendoza *et al.* 2011). These authors reported partially congruent genetic structures between symbionts.

The *L. pulmonaria–D. reticulata* symbiosis shows significant within-population genetic structure due to restricted gene flow and vertical photobiont transmission at the local scale (Dal Grande *et al.* 2012; Werth & Scheidegger 2012). This study aimed at investigating the strength of ecological linkage between lichen symbionts at a continental scale and tested the hypothesis whether the congruent patterns of genetic diversity of the fungal and algal partners found at the small spatial scale also lead to congruent large-scale phylogeographic patterns in the two symbionts. In particular, (i) we reconstructed the spatial distribution of genetic diversity and the common phylogeographic patterns shared between the mycobiont of *L. pulmonaria* and its green algal photobiont at the European continental scale using microsatellite data, (ii) we localized centres of genetic differentiation and inferred and compared the locations of glacial refugia and postglacial colonization routes of the two symbionts in Europe and (iii) we evaluated potential discrepancies in the overall distribution patterns of genetic variation between the symbionts.

For these purposes, we analysed more than 4300 *L. pulmonaria* thalli from 142 populations in 27 countries throughout the species' European distribution, and we genotyped the fungal and the algal symbionts at eight and seven microsatellite loci, respectively.

Materials and methods

Sampling

A total of 4323 *L. pulmonaria* thalli were sampled from 142 populations in 27 countries throughout the species' European distribution range (Appendix S1a,b, Supporting information). We focused sampling on areas that could have served as glacial refugia (56 populations), namely the Iberian and Italian Peninsulas as well as the Balkans (Taberlet *et al.* 1998; Petit *et al.* 2002, 2003; Magri *et al.* 2006; Liepelt *et al.* 2009), while in Central and Northern Europe, we applied a more scattered sampling (86 populations). A population was considered as a patch of trees colonized by *L. pulmonaria*. One to three thalli were randomly taken from an average of 23 nearest neighbour trees (i.e. proceeding from one sampled tree to its nearest yet unsampled neighbour; Walser *et al.* 2004).

Microsatellite analysis

Total lichen DNA was isolated from dried thallus material (35–50 mg) using the DNeasy 96 Plant Kit (Qiagen) according to the manufacturer's protocol. For the fungal symbiont, total DNA was amplified with eight fungus-specific microsatellites (LPu03, LPu09, LPu15, LPu23, LPu24, LPu25, LPu28, MS4; Walser *et al.* 2003; Widmer *et al.* 2010; Dal Grande *et al.* 2012) using fluorescently labelled primers. To obtain consistent

PCR amplification, LPu25 had to be redesigned. For primer sequences, labelling and PCR conditions see Appendix S2 (Supporting information). Fragment lengths were determined on a 3730 DNA Analyser (Applied Biosystems). Genotyping was carried out with GeneMapper 3.7 (Applied Biosystems) using ROX-500 (multiplex 1) or LIZ-500 (multiplex 2) as internal size standards.

For the algal symbiont, total DNA was amplified with seven alga-specific microsatellites (LPh1–LPh7; Dal Grande *et al.* 2010). For primer sequences, labelling, PCR protocols and genotyping see Dal Grande *et al.* (2010).

The total number of alleles and the percentage of different multilocus genotypes per population were calculated for each haploid symbiont separately (Ellstrand & Roose 1987). *Lobaria pulmonaria* and *D. reticulata* are highly clonal organisms (Dal Grande *et al.* 2012), and genetically uniform or depauperate populations primarily reflect within-population clonal dispersal (Dal Grande *et al.* 2012). We therefore performed all analyses on a conservative data set of population samples that was corrected for clonality by removing all recurrent samples with identical microsatellite profiles within population except for one. This data set of unique fungal–algal multilocus pairs for each population was used to account for the potential bias introduced by sampling the same genotypes (and thus genetic individual) several times and to avoid overestimation of allele frequencies due to high local clonality (Xu *et al.* 1999; Arnaud-Haond *et al.* 2007). Clonal bias would also affect the genetic differentiation of populations, the measurement of distribution distances of fungal and algal alleles as well as their respective centres of distribution (Sunnucks *et al.* 1997; Delmotte *et al.* 2002; De Meeûs *et al.* 2006).

Sequencing analysis

We observed pronounced length differences between alleles of the fungal microsatellite locus LPu09. To exclude insertions/deletions or base substitutions to be the cause for this size variation, we amplified and sequenced this locus covering the allele range from 173 to 659 bp using the primers LPu09-1F and LPu09-1R. For primer sequences and sequencing details see Appendix S3 (Supporting information).

Multivariate analyses

To investigate the population genetic structure of the two lichen symbionts of *L. pulmonaria*, we summarized multilocus population allele frequency data by performing principal component analysis (PCA) and spatial principal component analysis (sPCA). Multivariate methods are increasingly used in population genetics (Hanotte *et al.* 2002; Patterson *et al.* 2006; Jombart 2008; Novembre & Stephens 2008; Novembre *et al.* 2008; Jombart *et al.* 2009). Principal component analysis summarizes multivariate genetic information into a few synthetic variables, the PCs, without any assumptions such as Hardy–Weinberg equilibrium or linkage equilibrium (Johnson *et al.* 1969; Smouse *et al.* 1982; Cavalli-Sforza 1996; Jombart *et al.* 2009). Principal components (PCs) can be spatially interpolated and visualized on maps demonstrating how PC values (i.e. genetic variation) for each sampled population vary across geographic space (Cavalli-Sforza *et al.* 1994; Novembre & Stephens 2008; François *et al.* 2010). GENODIVE (Meirmans & van Tienderen 2004) was used to derive PC scores for each population. The scores were calculated from the covariance matrix of population allele frequencies, and a permutation test for the significance of population differentiation represented by the first three PCA-axes scores was performed (999 permutations; randomizing individuals among populations and recalculating PCA; Goudet 1999). As a result of the eigenvalue analysis performed in PCA, axes scores were scaled and centred. Discriminant analysis of principal components (DAPC) was used to define genetic clusters and calculate individual membership probabilities (Jombart 2008; Jombart *et al.* 2010) and populations were majority assigned to one cluster.

Only recently PCA was modified to use spatial information to investigate the part of the genetic variation that is or is not spatially structured (sPCA; Jombart *et al.* 2008). In sPCA not only the variance of the PCs, but also the spatial autocorrelation of the samples is optimized (Jombart *et al.* 2008). It has been shown that sPCA is a complement to PCA whenever spatial genetic patterns are investigated (Jombart *et al.* 2008, 2009). We performed sPCA with the R package adegenet (Jombart 2008). We spatially interpolated spatial principal components (sPC) between sampling locations and visualized the first three PC's on maps. Spatial principal component analyses were performed with the function 'spca' on centred population allele frequencies. Missing values were replaced by mean allele frequencies. Barplots of the sPCA eigenvalues were produced. To test for the existence of global and/or local structure, we performed two Monte Carlo multivariate tests (1000 permutations, functions 'global.rtest' and 'local.rtest'; Jombart *et al.* 2008). To perceive the spatial pattern of the genetic variability of the fungal and algal symbiont, we visualized genetic clines in interpolating (function 'interp') each of the first three lagged principal scores on a European map (Borders_MWDB3.cno, http://www.giss.nasa.gov/tools/panoply/overlays/).

Centres of distribution of geographically restricted alleles

To calculate the centres of distribution of all the alleles, the geographic coordinates of samples with a particular allele were extracted. From these coordinates, the central geographic *x*- and *y*-coordinates (centroids) and the corresponding standard deviations were calculated for each allele and locus. To test whether the observed centroid of an allele was geographically significantly different (i.e. GRA) from a centroid expected by chance alone, a bootstrap approach randomly subsampled *n* individuals (where *n* is the number of individuals having a given allele) from the whole data set. This process was re-iterated 5000 times to estimate the null distribution of the centroid position, from which its 95% confidence interval (CI) was inferred. All calculations were carried out in R (R Development Core Team 2011, code available from the authors upon request).

For the centroids falling outside the 95% CI, the standard deviation ellipses were calculated using the Spatial Statistics Tool of ARCMAP 9.3 (www.esri.com/software/arcgis/arcinfo). This was calculated to measure the directional trend of the distribution of GRAs. The distributional ellipses give an average area. If such an ellipse is falling between sampled areas, it may give an indication of gene flow between the two areas. The Directional Distribution option with one standard deviation was selected. Ellipses were rasterized (cell size = 0.02), and the Spatial Analyst Extension (Euclidean Distance and Raster Calculator in action) was used to assign the reciprocal of the distance from the centre of the ellipse to each raster cell within the ellipse of a given centroid. To calculate the relative quantity of GRAs for each location (raster cell), the cell values of superimposed ellipses were added in a final output raster (Raster Calculator option). The resulting raster was smoothed with neighbourhood statistics calculated per cell, that is, the mean value for each cell within a radius of 50 cells was calculated using the Focal Statistics option of the Spatial Analyst Extension. To display the density distribution of centroids of GRAs, the resulting surface was divided into 32 equal classes and visualized in ARCSCENE (www.esri.com/software/arcgis/arcinfo). The relative density of centroids (*z*-values) corresponded to the cell values after calculation of neighbourhood statistics.

Spatial analysis of shared alleles

To compare the overall distribution pattern of fungal and algal alleles, we tested the difference of the distribution of fungal and algal alleles from what one could expect under panmixia given the same spatial sampling with SASHA (Kelly *et al.* 2010). For each symbiont, we calculated the observed distribution of geographic distances between occurrences of each allele in the overall data set and per marker and compared it with the null distribution (i.e. allele distribution under panmixia). To test for significant deviations of the observed mean distances from the expected, we performed 1000 nonparametric permutations of the allele-by-location data set. We then compared the overall and the GRA distribution pattern among the fungal and algal symbiont. To compare the observed mean distributions of fungal and algal GRAs of the South Italy/Balkans region a Mann–Whitney *U*-test was applied.

Test of concordance between fungal and algal spatial genetic variation

To test for congruence between the genetic structure of the fungal and algal symbionts, we performed a Procrustes rotation test (Peres-Neto & Jackson 2001), and a test for distance congruence and similarity of vertex locations of population graphs (Dyer & Nason 2004). To test the degree of congruence between two data sets, for example, between two interacting species, Procrustean superimposition has proven to be as powerful (or even more so) than the Mantel test (Gower 1971; Peres-Neto & Jackson 2001; James *et al.* 2011). Procrustes rotation scales and rotates raw data matrices or their ordination solutions in order to find an optimal superimposition that maximizes their fit, that is, minimizing their sum-of-squared differences (Gower 1971; Peres-Neto & Jackson 2001). To test the Procrustes statistics, that is, the significance of the similarity between two matrices, a correlation-like statistics derived from the symmetric Procrustes sum of squares can be calculated (PROTEST; Jackson 1995). Procrustes rotation test is similar to the Mantel test as it also tests the significance of the correlation between distance matrices. However, the Procrustes test seems to be more powerful than the Mantel test as the analysis is performed on the raw data (or their ordination solutions), rather than derived distance matrices (Peres-Neto & Jackson 2001). To calculate the strength of the correlation between fungal and algal population allele frequencies, we summarized variation in allele frequencies among collection sites (populations) for each symbiont using PCA. These calculations were carried out in R (R Development Core Team 2011) using the R function 'dudi.pca' (ade4 package, Dray & Dufour 2007; adegenet package, Jombart 2008). Allele frequencies were centred before PCA. Procrustes analysis was performed on the fungal and algal PC matrices of the first three axes in R using the 'procruste' and 'procuste.randtest' function in the ade4 package. To get a more scale-independent and symmetric statistic (Procrustes m^2), the configurations of the Procrustean rotation were scaled

to equal dispersions (unit variance). Randomization tests (Monte Carlo test on the sum of the singular values of a procrustean rotation) for the association between matrices were carried out using PROTEST (Jackson 1995) with 9999 iterations.

For a second test of congruence, we used Population Graphs, which focuses on the analysis of marker-based population genetic data within a graph theoretic framework (Dyer & Nason 2004). This model-free approach focuses on quantifying genetic structure by characterizing the topology resulting from genetic interactions between populations. Relationships are in the form of a graphical topology, which captures the genetic covariance relationships between all populations simultaneously rather than in a pairwise fashion (Dyer & Nason 2004). Populations that exchange migrants show conditional covariance and are connected in the network by edges whose length is inversely proportional to the genetic covariance between the populations. In the absence of gene flow, populations are not connected in the network (Dyer *et al.* 2010). Conditional genetic distance (cGD) can be extracted from the population networks and is defined as the length of the shortest path connecting pairs of populations (Dyer *et al.* 2010). Compared with more traditional measures of pairwise genetic distances (such as F_{ST}), cGD is calculated based upon the differences in genetic covariation associated with both direct and indirect gene flow among populations (Dyer *et al.* 2010). As we can test for distance congruence based on direct and indirect gene flow as well as for the similarity of vertex locations, Population Graphs is a robust analysis to test for the congruence between the spatial genetic variation of co-evolving species or of species sampled at the same location. Distance congruence is in fact a measure of similarity in the distance through a graph and is based upon the shortest path algorithm. It may be that edges are connected differently but the relative distances between the graphs are the same for two species compared. Second, one can test for topological similarity in vertex locations within each graph.

Population graphs for the fungal and algal symbiont were performed online (http://dyerlab.bio.vcu.edu/software/popgraph/), while all other graph analyses were carried out in R using GSTUDIO v.0.8 (http://cran.r-project.org/web/packages/gstudio/index.html). Population graphs were visualized using the 'layout.fruchterman.reingold' function. From the population graphs, we calculated the congruence graph (i.e. the graph with an edge set consisting of those edges that the fungal and algal graphs have in common) by using the functions 'get.adjacency' and 'graph.adjacency'. First, we tested for similarity in vertex locations within each graph. We used the function 'closeness' and then performed a Pearson's correlation test with the function 'cor.test'. We tested distance congruence by first calculating cGD (Dyer *et al.* 2010) with the function 'shortest.paths' and then running a Mantel test with 10 000 permutations with the function 'mantel' (R package ecodist, Goslee & Urban 2007) on the cGD matrices.

To assess the significance of correlation between genetic distance matrices of fungal and algal symbionts, we also performed classical Mantel and partial Mantel tests. F_{ST} was used as a measure of population genetic differentiation, as recommended by Meirmans & Hedrick (2011). Population differentiation (F_{ST}) and Mantel tests were calculated in GENODIVE (Meirmans & van Tienderen 2004), while partial Mantel tests (controlling for the geographic distance) were calculated in PASSAGE (ROSENBERG & ANDERSON 2011).

Allelic distribution across European regions

The number of distinct alleles and the number of private alleles (alleles not found in other regions) within regions (or populations) depend on sample size. If sample sizes are different across regions, it is difficult to interpret such numbers. Therefore, we used rarefaction to produce estimates that are comparable between populations with different sample sizes (Hurlbert 1971; Petit *et al.* 1998; Kalinowski 2004, 2005). Populations were first assigned to eight main European regions (Iberian peninsula, Italy, Balkans, South of Alps, North of Alps, Great Britain, Scandinavia and Urals; Appendix S4, Supporting information). To estimate the influence of the sampling intensity on the observed distinct alleles, allelic richness was calculated for random subsamples (138–592) of the same size for each European region. To estimate the genetic relationship between European regions, we then measured the number of distinct alleles private to groups of two regions for random subsamples equal to the smallest sample size across European regions (138 samples for the Great Britain group). For all calculations, we used ADZE (Szpiech *et al.* 2008). Genetic relationship between major European regions was visualized on maps with lines connecting adjacent regions sized according to the mean number of alleles per locus that was private to the respective combination.

Results

The analysis of more than 4300 *L. pulmonaria* samples across Europe with eight fungus- and seven alga-specific microsatellite markers resulted in a total of 314 alleles for the fungus and 191 alleles for the alga. The number of alleles per locus for the fungal microsatellites ranged from four (LPu03, LPu24) to 121 for LPu25, which is a very high allelic variation for a microsatellite locus. There were only < 2.5% of missing data for the

LPu09 marker with no missing alleles for all the other markers in the data set. For the algal microsatellites, the number of alleles ranged from 15 (LPh3) to 36 (LPh1) per locus with no missing data. The proportion of different multilocus genotypes per population ranged from 0.03% to 100% (i.e. every individual sampled within the population had a different multilocus genotype) for the fungus and from 0.08% to 100% for the alga. Sequences of more than 80 fungal LPu09 alleles covering the allele range from 173 to 659 bp revealed no insertions/deletions in the microsatellite or in the flanking region (Appendix S5, Supporting information).

Multivariate analyses

For the fungal symbiont, the first three PCs computed on the covariance matrix of population allele frequencies accounted for 67.27% of the total variance, and all three axes showed significant population differentiation ($P < 0.001$ for all axes). The first PC-axis accounted for 39.22% of the total variance. This axis separated the Northern European from the Southern European populations (together with populations from Great Britain and Spain). The second PC-axis accounted for 16.65% of the total variance and separated Northern and Central European populations from the populations from the Ural Mountains. The third PC-axis represented 11.39% of the total variance and distinguished mainly the populations of Great Britain and Spain from other European populations (Fig. 1A).

For the algal symbiont, the first three PCs accounted for 55.02% of the total variance. The permutation test revealed significant population differentiation for the first ($P < 0.001$) and the second ($P < 0.002$) PC axes, but not for the third PC-axis ($P < 0.583$; 6.25% of total variance explained). The first axis accounted for 38.45% of the total variance and separated populations into two clusters (Fig. 1B). One cluster primarily grouped populations from Southern Italy and the Balkans, while the other cluster grouped the Central and Northern European populations as well as those from the Ural Mountains. The second PC-axis described 10.32% of the total variance and mainly separated populations from Great Britain and Ireland from all other samples.

Spatial principal component analysis revealed for both the fungus and the alga high-positive (fungus: 0.398, 0.149, 0.128; alga: 0.389, 0.073, 0.052) and low-negative eigenvalues (fungus: −0.007, −0.005, −0.004; alga: −0.007, −0.005, −0.005). The global test was significant for the fungus ($max(t) = 0.042$, $P \leq 0.001$) as well as for the alga ($\max(t) = 0.048$, $P \leq 0.01$), while local tests were not significant (fungus: $\max(t) = 0.012$, $P = 0.469$; alga: $\max(t) = 0.010$, $P = 0.949$). Thus, both symbionts showed distinct global spatial genetic structure with populations being differentiated between European regions (Fig. 2).

Centres of distribution of geographically restricted alleles

For the fungal symbiont, 134 centroids of microsatellite alleles (42.7% of all alleles) were significantly geographically restricted ($P < 0.05$; Appendix S6, Supporting information). We localized two hot spots of geographically restricted centroids for the fungal symbiont of *L. pulmonaria* in Europe (Fig. 3 *F*). The most prominent hot spot was located in the Southern Italy–Balkan area and had a maximum centroid density of 26.24. The centroid density statistics calculated for each location describes the relative quantity of GRAs found within

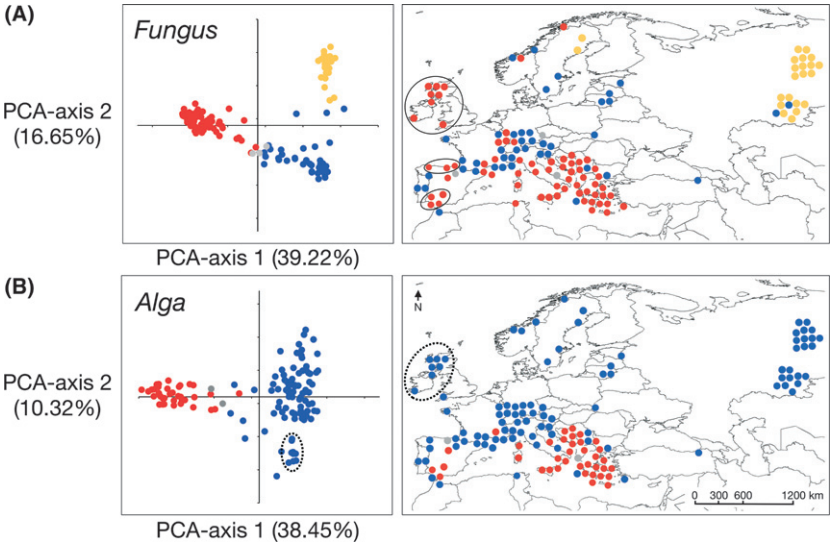

Fig. 1 (A) Principal component analysis (PCA) of the fungal symbiont of *Lobaria pulmonaria* and map of populations coloured according to majority assignment to three genetic clusters. Unassignable populations are coloured in grey. Encircled populations are separated from the other populations along PC-axis 3 (11.39%). (B) PCA of the algal symbiont of *L. pulmonaria* and map of populations coloured according to majority assignment to two genetic clusters. Unassignable populations are coloured in grey. Encircled populations are separated from the other populations along PC-axis 2.

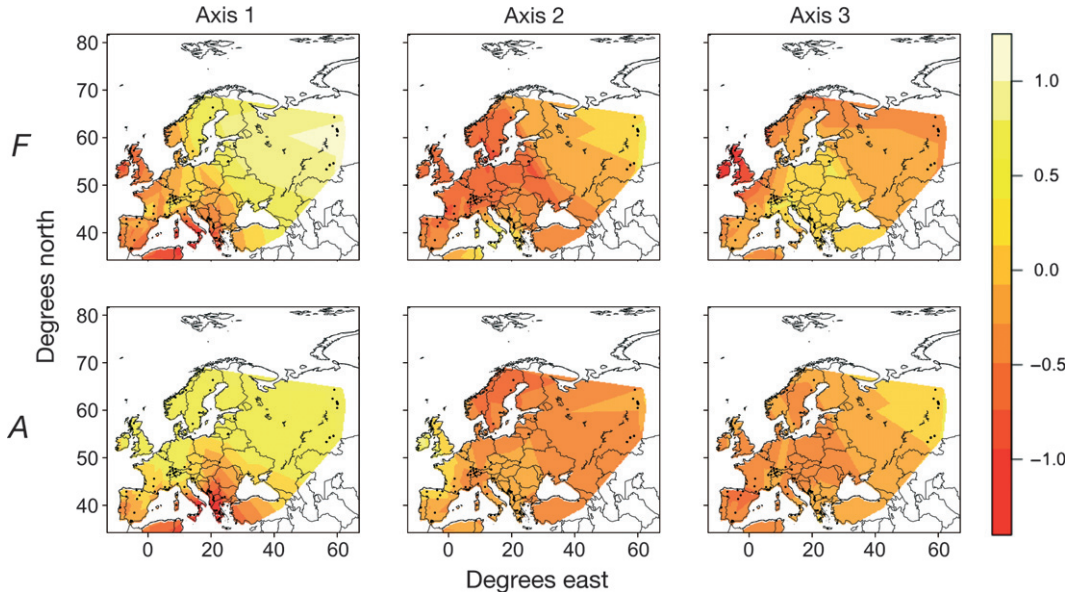

Fig. 2 Interpolation of the first three spatial principal components (sPC) of the fungal (*F*) and algal (*A*) symbiont of *Lobaria pulmonaria*. Sampling locations are marked by black points and colours that correspond to the principal scores can directly be compared between symbionts and different axis.

the specified neighbourhood. In the Southern Italy–Balkan region, we found 28 GRAs from six different microsatellite markers (Fig. 3 *F*). This area of high centroid density extended along a north-western axis towards Northern Italy and France. A second hot spot was found in the Ural Mountains and exhibited a peak of centroid density of 17.61, with 15 GRAs from four different microsatellite loci (Fig. 3 *F*). When recalculating the density distribution of centroids of geographically restricted fungal alleles without locus LPu09, the Ural hot spot was much less pronounced (Appendix S7 F, Supporting information) as the microsatellite locus LPu09 accounted for 80% of the diversity (12 of 15 alleles) in this second hot spot of fungal GRAs.

For the algal symbiont, 79 centroids of microsatellite alleles (41.4% of all alleles) were significantly geographically restricted ($P < 0.05$; Appendix S6, Supporting information). We found only one hot spot of GRAs for the algal symbiont of *L. pulmonaria* in Europe (Fig. 3 *A*). This hot spot was located in the Southern Italy–Balkans area, with a peak of centroid density of 27.42. The cluster of centroids in this hot spot consisted of the centres of distribution of 39 alleles from seven microsatellite loci. It was also characterized by a steep decrease in centroid density away from the hot spot (Fig. 3 *A*).

Spatial analysis of shared alleles

The overall observed mean geographic distance between shared alleles for the fungus was 1868.4 km and was significantly different from the expected mean under the assumption of panmixia (2081.9 km, $P < 0.0001$; Appendix S8a, Supporting information). For the alga, the observed mean distance was 2079.5 km, which was not different from the expected null distribution (2082.3 km, $P = 0.707$; Appendix S8b, Supporting information).

The overall observed mean distance between GRAs for all markers was significantly different from the expected mean for both the fungus and the alga. The mean observed distance of GRAs of all markers of the fungus was significantly smaller (mean observed distance across markers = 1559.62 km; Appendix S9a, Supporting information) than that for the alga (2070.59 km; Appendix S9b, Supporting information). One algal marker was overdistributed with an observed mean that was higher than the expected (LPh6).

When looking at the mean distance of GRAs that centred to the South Italy–Balkans region (Fig. 3), we found strong concordance between the 28 fungal alleles (observed mean: 951.8 km, all alleles significantly different from expected mean; Appendix S10a, Supporting information) and the 39 algal alleles (1034.1 km, all alleles significantly different from expected mean; Appendix S10b, Supporting information). A Mann–Whitney *U*-test confirmed the identical distance distribution of fungal and algal GRAs ($P = 0.3183$).

Test of concordance between fungal and algal spatial genetic variation

Procrustes rotation on the PCA matrices of the two lichen symbionts showed significant correlation of

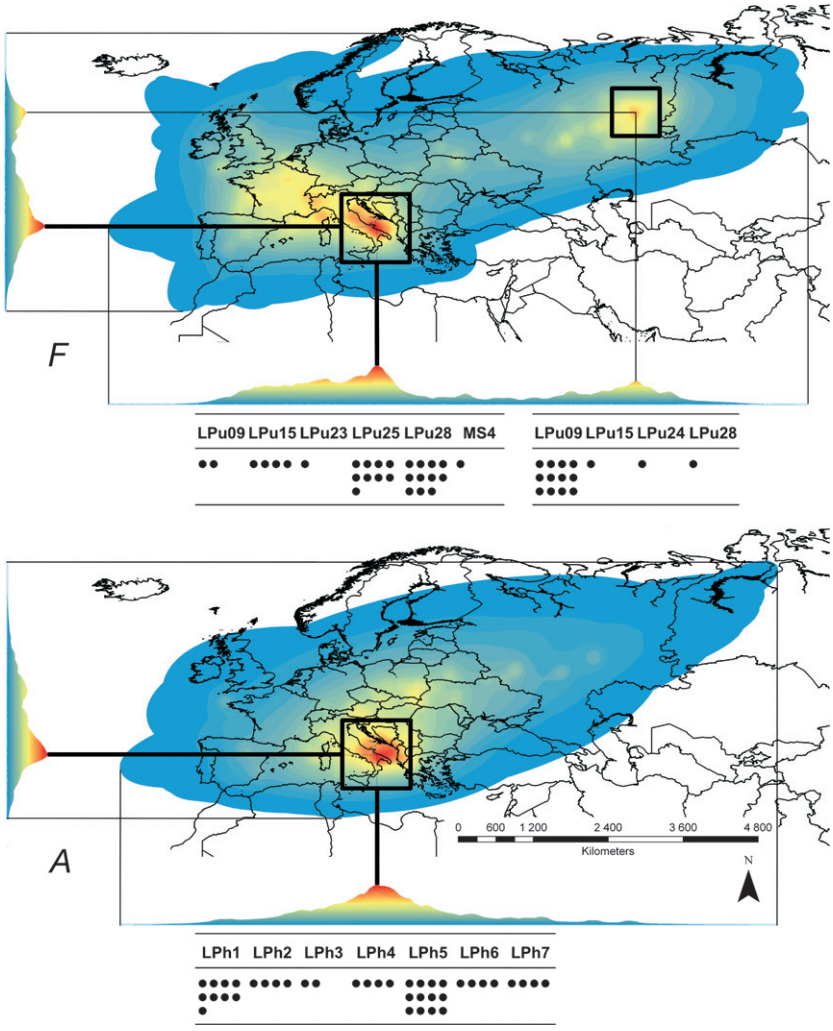

Fig. 3 Density distribution of centroids of geographically restricted alleles for the fungal (*F*) and the algal (*A*) symbiont of *Lobaria pulmonaria*. The latitudinal and longitudinal densities of the centroids in Europe are represented by the height of centroid peaks to the left and at the bottom of the map. Rectangles indicate hot spot regions of centroid density. The bar plot at the bottom gives the number of alleles per locus (fungus: LPu09 to MS4; alga: LPh1 to LPh7) restricted to the region enclosed by the respective rectangles.

population allele frequencies between the fungal and algal partners ($m^2 = 0.727$, $P = 0.001$; Table 1; Appendix S11, Supporting information). When calculating the same statistics without the fungal marker LPu09, there was only a marginal increase in the correlation ($m^2 = 0.730$, $P = 0.001$; Table 1). When analysing the data set at the individual level, a less strong but significant correlation between the lichen symbionts was observed ($m^2 = 0.263$, $P = 0.001$, Table 1). Population graph analysis for the fungal and algal symbiont of *L. pulmonaria* resulted in two graphs having 139 nodes (populations) with 1114 edges for fungal and 1310 edges for algal populations, respectively (Appendix S12a,b, Supporting information). The intersection of the two graph topologies resulted in a congruence graph with 304 edges (Appendix S12c, Supporting information). We tested for similarity of vertex locations within each graph. There was a weak, but significant correlation of 0.25 between the vertex locations of the fungal and algal graphs ($P = 0.0037$). Distance congruence test between the fungal and algal population graph resulted in a significant Mantel correlation ($r = 0.273$, $P \leq 0.001$).

Correlation between the population genetic distances (F_{ST}) of the fungal and algal symbionts was $r = 0.479$ ($P \leq 0.001$). Without the fungal marker LPu09, the correlation dropped slightly to $r = 0.455$ ($P \leq 0.001$). When the geographic distance matrix was held constant, partial Mantel correlation of the fungal and algal genetic distance matrices was $r = 0.448$ ($P \leq 0.001$) (Table 2).

Allelic distribution across European regions

Mean number of alleles per locus for 138 random subsamples from eight European regions ranged from 9.7 (Urals) to 15.8 (Iberian peninsula) for the fungus and from 10.3 (Great Britain) to 15.6 (Balkans) for the alga with marked standard errors of the mean. For both symbionts, curves of allelic richness did not reach saturation for a standardized sample size of 138, indicating that increased sampling would reveal additional alleles

Table 1 Procrustes rotation test

Principal component analysis	Procrustes rotation	
	m^2	P
Population based		
Full data sets	0.727	0.001
LPu09-locus omitted	0.730	0.001
Individual based		
Full data sets	0.263	0.001
LPu09-locus omitted	0.260	0.001

m^2, Degree of association between two matrices after procrustes rotation (using a rotational-fit algorithm that minimizes the sum-of-the-squared residuals between the two matrices); P, Significance of the correlation (based on a Monte Carlo test based on 1000 permutations on the sum of the singular values of a procustean rotation).

Table 2 Mantel and partial Mantel tests

Genetic distance						
Fungus	Fungus No LPu09	Alga	Geographic distance	Control matrix	r	$P \ll$
F_{ST}	—	F_{ST}	—	—	0.479	0.001
—	F_{ST}	F_{ST}	—	—	0.455	0.001
F_{ST}	—	—	[km]	—	0.416	0.001
—	F_{ST}	—	[km]	—	0.439	0.001
—	—	F_{ST}	[km]	—	0.190	0.001
F_{ST}	—	F_{ST}	—	[km]	0.448	0.001
—	F_{ST}	F_{ST}	—	[km]	0.422	0.001

(Appendix S13, Supporting information). The curves for the alga were flatter and closer to the asymptote than the curves for the fungus (Appendix S13a,b, Supporting information).

The mean number of alleles per locus private to groups of two regions for a standardized sample size of 138 ranged from 0.012 (Balkans-Scandinavia) to 1.067 (Scandinavia-Urals) for the fungus and from 0.005 (Scandinavia-Urals) to 0.740 (Italy–Balkans) for the alga. We observed marked differences in the overall pattern of genetic relationship among European regions between the fungal and the algal symbionts (Fig. 4). For both symbionts, there were pronounced among-marker differences in the number of private alleles, which resulted in high standard errors.

Discussion

The genetic structure of both fungal and algal symbionts of *L. pulmonaria* at the European scale was composed of at least two genetic clusters with the centres of differentiation located in the Southern Italy and Balkans region. Tests of concordance between fungal and algal genetic variation revealed general concordance between the phylogeographies with some incongruent pattern that has been found to be most prominent in the Ural Mountains.

Phylogeographic structure of the fungal and the algal symbiont

When analysing and comparing the spatial pattern of the genetic variation of the fungal and algal symbionts of *L. pulmonaria*, we found high-positive eigenvalues for the fungus and the alga, while negative values were low. For both the fungus and the alga, the first positive sPCA eigenvalues were large compared with other eigenvalues. The global test confirmed that there was indeed one global structure and negligible local structure for both symbionts of *L. pulmonaria*. In other words, we found relevant phylogeographic structure in the fungal and the algal partners of *L. pulmonaria* in Europe with a clear genetic differentiation of populations form the South Italy and Balkans region.

Interestingly, the fungal centroids of GRAs were less clearly clustered than the algal ones, as the area of high fungal centroid density extended towards France

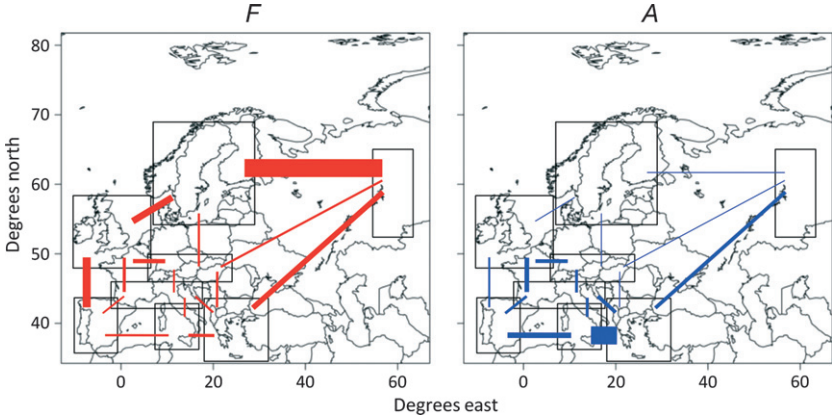

Fig. 4 Private alleles and genetic relationships across eight European regions. Relative sizes of the lines connecting adjacent regions correspond to the mean number of alleles per locus private to the respective region combination (*F*: fungal symbiont; *A*: algal symbiont). Private alleles were calculated considering 138 subsamples from each geographic region.

(Fig. 3). A similar pattern was also found in PC-analysis, where populations located at the northern periphery of the Southern Italy–Balkans region clustered with the Southern Italy–Balkans group in the fungus but not in the alga (Fig. 1). These different patterns can be explained by the fungal reproductive mode, as the fungal symbiont, in contrast to the algal symbiont, also reproduces via sexual spores. These ascospores are more suitable for long-distance dispersal than vegetative propagules, which mainly disperse over smaller distances (Bailey 1976; Galloway & Aptroot 1995; Dal Grande *et al.* 2012). This also explains why the fungal centroids of allele frequencies were geographically more widespread than those of the alga. These results were in accordance with studies on host–parasite population genetics, which found smaller-scale genetic structure in selfing species than in outcrossing species (e.g. trematode parasite of salmon: Criscione *et al.* 2006; anther smut fungus of *Silene*: Vercken *et al.* 2010).

Signatures of postglacial recolonization of Europe from southern refugia

The idea that the main glacial refugia of organisms in Europe were localized in three regions (Iberia, Italy and Balkans) is well-established, although increasing evidence suggests that northern and eastern refugia also existed (Parducci *et al.* 2012).

In *L. pulmonaria*, the distinct phylogeographic structure of fungal and algal symbionts observed at the European scale consists of at least two genetic clusters. The cluster common to both symbionts had a distribution strikingly similar to the above major glacial refugia (Hewitt 2000). In both symbionts, the populations from Southern Italy and the Balkans were genetically different from all other populations along PC-axis 1. This pattern suggested that *L. pulmonaria* probably colonized Europe from at least two main Mediterranean refugia (Italian and Balkan). However, our data support the possibility of additional refugia that likely played a role in the recolonization of Central and Northern Europe. The pattern of private alleles and genetic relationship between regions for both symbionts suggests a relatively close relatedness of the Ural populations with populations in southern refugia as well as with Scandinavian populations (Fig. 4). There is growing evidence that areas west and east of the margin of the Scandinavian ice sheet expanding to the Ural Mountains harboured isolated patches of tree species during the late-glacial and early Holocene and that these populations were important for population expansion and forest development in the early Holocene (Kullman 2008; Binney *et al.* 2009; Väliranta *et al.* 2011; Parducci *et al.* 2012). In addition, many boreal forest species are known to have colonized Fennoscandia from the East (e.g. *Picea abies*, Tollefsrud *et al.* 2008, 2009; boreal forest beetles, Painter *et al.* 2007; brown bears, Taberlet & Bouvet 1994). The phylogeographical patterns found for *L. pulmonaria* indicated complex recolonization dynamics of the European continent, and our results may suggest sequential colonization events. Both symbionts showed distinct gene pools in Southern and North Central Europe (Fig. 1, PCA-axis 1) with only few private alleles shared among the respective regions (Fig. 4). This pattern conforms with a west expansion from additional northeastern refugia and probably less important recolonization of Northern Europe out of the South. However, to resolve European recolonization dynamics in more detail and to draw firm conclusions on the presence of additional glacial refugia of the fungal and/or algal symbionts of *L. pulmonaria* in the East, samples from areas in the West of the Ural Mountains (e.g. Carpathian Mountains) and samples from areas located in the East of the Ural Mountains would be needed.

Populations in glacial refugia often exhibit a high frequency of private or GRAs, while areas colonized after the ice ages typically show common, widespread alleles (Hewitt 1996). Allelic richness is a good indicator of glacial refugia, but high allelic richness can also be found in hybrid zones (Widmer & Lexer 2001). Generally, when comparing two regions of high allelic richness, it is not known whether the same or different alleles account for high allelic richness in the two regions. For that reason, we used a method that identified regions, where GRAs have high density. The high number of GRAs (43% of the fungal and 41% of the algal alleles) found in the Southern Italy–Balkans region in the present study was well compatible with this area acting as a glacial refugium for both symbionts of *L. pulmonaria*. If a species has undergone some range expansion, one would expect to see more diversity close to the source of the expansion, with a loss of alleles (and heterozygosity) along the expansion routes (Excoffier *et al.* 2009). Thus, the high diversity of GRAs found in this region, with a loss of alleles towards Central and Northern Europe (Fig. 3) supports the hypothesis that the Southern Italy–Balkans region was a source of postglacial range expansion in *L. pulmonaria*.

Our findings are also in agreement with previous reports on the location of glacial refugia of several tree species. For the widespread European beech (*Fagus sylvatica*), glacial refugia were, amongst others, in Southern Italy and the Balkans (Comps *et al.* 2001; Magri *et al.* 2006), and, for deciduous oak species (*Quercus* spp.), the primary refugia were localized, based on fossil pollen evidence, in the southern parts of the Iberian and Italian Peninsulas as well as in the Balkans (Brewer *et al.* 2002). Furthermore, Petit *et al.* (2002) gave

evidence of gene flow between refugial oak populations from the South of Italy and the West of the Balkan Peninsula during interglacial periods. Additionally, the European silver fir (*Abies alba* Mill.) had long-term glacial refugia in South Italy and in the Southern Balkans (Terhürne-Berson *et al.* 2004; Liepelt *et al.* 2009). In agreement with the location of the refugia of single tree species, a multispecies study examining chloroplast DNA variation of 22 widespread European trees and shrubs (Petit *et al.* 2003) located the genetically most unique stands in Southern and Central Italy, Corsica and the Balkans. It is thus evident that Southern Italy and the Balkans were important glacial refugia for many European tree species. Our data demonstrated that the major glacial refugium of the symbionts of *L. pulmonaria* was located in the same region. However, as *L. pulmonaria* can grow on the bark of several tree species, the glacial history of this lichen species cannot be directly linked and explained by the glacial history of any single European tree species.

Differences in phylogeographic patterns between the fungal and the algal symbionts of Lobaria pulmonaria in Europe

Given the symbiotic relationship between the fungal and algal symbionts of *L. pulmonaria*, we expected the genetic and phylogeographic structure of these two species to be similar. All comparisons and test of congruence showed high, but not complete similarity between the genetic structures of the two symbionts. The predominant population genetic pattern was represented by a high similarity in the distribution of GRAs for the symbionts mainly in the South Italy–Balkans region. Not only the centres of distribution closely matched, but they also exhibited identical distribution of GRA mean distances. As refugial areas permit long-term historical continuity of symbiotic associations, they will substantially affect co-evolutionary history: symbionts could accumulate many alleles that are restricted or private in refugia.

While there was high genetic similarity in the southern refugia of the symbionts, there were discrepancies in their genetic structure in recently recolonized areas of Central and Northern Europe. For the fungus, we found genetic evidence of an eastern refugium that was not present in the algal symbiont (see PCA, Fig. 1). This cluster located in the Ural Mountains corresponds to the second hot spot of fungal GRAs (Fig. 1A), for which the microsatellite locus LPu09 accounted for 80% of the diversity (12 of 15 alleles). This was in marked contrast to the Southern European refugium where a large number of alleles from several microsatellite loci contributed to the centroid hot spot. LPu09 alleles restricted to the Ural Mountains were three to four times longer than the alleles of the same locus found in other regions of Europe, and neither insertions nor deletions were observed in the microsatellite repeats or in the flanking region. Tests of concordance between fungal and algal spatial genetic variation without locus LPu09 showed that this fungal microsatellite locus only had a minor overall effect on the congruence of the genetic patterns of the fungus and the alga (Tables 1 and 2). One possible explanation is that the second hot spot in the Ural Mountains is of a more recent origin and derived from a radiation-like diversification at locus LPu09 in this region (Weber 1990; Wierdl *et al.* 1997; Primmer *et al.* 1998; Zhu *et al.* 2000). Another explanation is the presence of an eastern fungal refugium. This idea was supported by the high number of private alleles in the Scandinavia-Urals region (1.067), potentially indicative of a postglacial recolonization route. The existence of a glacial refugium in the Ural Mountains is supported by the genetic structure of the Siberian fir (*Abies sibirica* Ledeb.; Semerikova & Semerikov 2007). However, chemotype data of *L. pulmonaria* samples (unpublished data) suggested that this region was also influenced by colonization from the Altai Mountains and from Far East populations. In the Urals, we found an admixture between two lichen chemotypes, one of which was common in the European part, while the other was common in the Far Eastern part of the distribution range of *L. pulmonaria* (data not shown). This pattern suggests a tantalizing scenario in which the Southern European refugium might have acted as a source for the Ural populations. A second event of colonization took then place from the Far East populations of *L. pulmonaria* to North-Eastern Europe. As stated earlier, further research including areas located further East from the Ural Mountains would be required to draw firm conclusions on the presence of an additional glacial refugium in the East.

Differences in phylogeographic patterns among symbionts can originate from different dispersal rates and distances of the symbionts, as it has been found for fungus-gardening ants and their two microbial mutualists (Mikheyev *et al.* 2008) and for a plant–insect mutualism (Anderson *et al.* 2004). In addition, McCoy *et al.* (2005) showed that gene flow and population genetic congruence of seabirds and their tick ectoparasites are scale-dependent. Different mutation rates between the host/parasites or symbionts may result in different genetic structures. It has been proposed that in lichens, the mutation rate of the fungus might be higher than that of the alga (Law & Lewis 1983). For lichen symbiosis between the fungi *Omphalina* and their green algae *Coccomyxa*, it has been shown that photobionts have a lower rate of genetic change than the fungal host, because

the latter may have a very narrow ecological niche and thus be more sensitive to abiotic environmental variation and more exposed to competition from other organisms (Zoller & Lutzoni 2003; Otálora *et al.* 2010). This holds true especially for lichens of old-growth forest communities where symbionts are cospecialized due to vertical asexual transmission of the photobiont (Otálora *et al.* 2010), as it is the case in the *L. pulmonaria–D. reticulata* symbiosis. Additionally, if the number of genera and species is used as a proxy for genetic change, lichens seem to conform to a trend of reduction in genetic change in the algal symbiont (Zoller & Lutzoni 2003). This was in concordance with our data that showed higher overall genetic diversity (more microsatellite alleles) in the fungal data set than in the algal data set. Thus, different mutation rates might be an important cause for the observed discrepancies between the genetic structures of the two symbionts. In an earlier study, we showed that the predominant dispersal mode of *L. pulmonaria* is by symbiotic vegetative propagules, shaping the within-population spatial genetic structure up to distances of 20 m (Dal Grande *et al.* 2012). While in both symbionts somatic mutations generated genetic diversity, only the fungus showed evidence for recombination. There is thus evidence for horizontal (independent) photobiont transmission. Horizontal transmission due to mycobiont sexual reproduction will decouple mycobiont-photobiont pairs and will generate discrepancies between the symbionts' genetic structures at larger spatial scales (Werth & Sork 2010). In addition, the specificity of the symbiosis should be taken into consideration. As for many lichen symbioses, the photobiont of *L. pulmonaria* can be shared among several fungal species of the genera *Lobaria* and *Sticta* (Dal Grande 2011). This gives the photobiont a wider niche breadth resulting in broader ecological tolerance. Intriguingly, *D. reticulata* the algal partner of *L. pulmonaria* is also found in two suboceanic lichen species, namely *Lobaria amplissima* and *Lobaria virens* (Dal Grande 2011), whose distributional areas reach the marginal parts of the boreal zone. This could explain the presence of a Great Britain-Ireland PCA cluster in the alga (Fig. 1B), being a region in Europe where these lichen species are rather abundant. As it has been found for other lichens and obligate associations (Rikkinen *et al.* 2002; Yahr *et al.* 2004, 2006; Werth & Sork 2010; Fernández-Mendoza *et al.* 2011; James *et al.* 2011; Fedrowitz *et al.* 2012), the redistribution of photobionts under warmer climatic conditions could indeed rely on the horizontal transmission of locally adapted photobionts, or on the presence of photobiont-mediated guilds formed by fungal hosts with different ecological preferences and adaptive potentials, but horizontally sharing a common photobiont (Rikkinen *et al.* 2002; Peksa & Skaloud 2011).

Caveats of the present analysis

Sampling is crucial in phylogeographic analysis, as sampling intensity influences the number of distinct alleles to be found within geographical regions. In our study, the position of allele centroids might be skewed towards regions of denser sampling and the geographic locations of the selected set of sampling sites determine the null distribution of the centroid. However, the centroid method applied in this study has shown to be robust in identifying global patterns of differentiation on a continental scale even though this method is more powerful at identifying alleles near to the boundary of the distribution than alleles of identical frequencies close to the centroid of a species range. Thus, unless the source of differentiation is located in the centre of the species distribution, this method is effective in identifying centres of genetic differentiation and to test the hypothesis of range expansion from the very centres. In addition, small areas (e.g. cryptic northern refugia) where populations underwent genetic differentiation could have been overlooked due to sparse or nonexhaustive sampling. It is also essential to include all possible refuge areas (Provan & Bennett 2008). In the present study, some regions might not have been sufficiently covered, for example, the Carpathian Mountains, where we only had access to a single population. We thus cannot completely exclude the existence of additional glacial refugia, for example in the Carpathians. Nevertheless, for Central and Northern Europe, where our sampling was denser, we found no evidence for additional glacial refugia.

For both symbionts of *L. pulmonaria*, it has been shown that sample sizes of 20 individuals allows one to describe most of the allelic diversity at the population level, while within landscapes 300–400 samples and about 25–30 populations are sufficient (Werth 2010). The main goal of this study was to reconstruct the common phylogeographic patterns shared between the symbionts of *L. pulmonaria* and to localize centres of genetic differentiation on large spatial (and temporal) scales. For these types of analyses, not all alleles in a region have to be sampled. In fact, the Iberian Peninsula had among the highest richness of distinct fungal alleles in the present study. Nevertheless, only few of these alleles were found to be geographically restricted to the Iberian Peninsula: most alleles were common and also occurred in Central and North-Western European populations.

Conclusions

This study contributes to the understanding of processes that lead to differences in phylogeographical

pattern among ecologically linked taxa in general and among lichen symbionts in particular. We show that both symbionts kept clear footprints of postglacial recolonization from major southern refugia localized in Italy and the Balkans. It is plausible that the genetic differences between symbionts found in different regions are the result of different mutation rates of the symbionts as well as of horizontal photobiont transmission and algal sharing between fungal species with different ecology in areas of recent expansion. Our results suggest the presence of an additional eastern refugium for *L. pulmonaria*, but this hypothesis needs to be validated by increased sample density in East Eurasia and Asia.

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This work was part of I.W. and F.D.G.'s PhD research on the evolutionary history and phylogeography of a lichen symbiosis. I.W. is molecular ecologist interested in landscape genetics and its application to the conservation and management of biodiversity in urban areas. F.D.G. is a postdoctoral research fellow interested in the evolutionary and population genetic impacts of climate change on lichen fungal and algal symbionts. L.E. is a population geneticist interested among other things in the evolution of populations in a spatially explicit context. R.H. is interested in landscape genetics and its application to conservation management. C.K. is biologist interested in lichen systematics and conservation. V.S.M. is interested in various aspects of plant and fungal ecological genetics, including biogeography and population genetics. C.S.'s research interests cover the biodiversity evaluation, population genetics and conservation biology of lichens and plants.

Data accessibility

Microsatellite data: DRYAD entry doi: 10.5061/dryad.rj04r.

Final DNA sequence assembly of fungal microsatellite locus LPu09: uploaded as online supporting information (Appendix S5, Supporting information).

Supporting information

Additional Supporting Information may be found in the online version of this article.

Appendix S1 (a) Map of sampling locations of *Lobaria pulmonaria*. (b) Information on sampled populations of *Lobaria pulmonaria*.

Appendix S2 Microsatellite analysis: (a) primer sequences (Walser *et al.* 2003, 2004; Widmer *et al.* 2010; Dal Grande *et al.* 2012; this study), labelling, primer concentrations and (b, c) PCR conditions for genetic analysis of *Lobaria pulmonaria*.

Appendix S3 Sequencing of microsatellite locus LPu09: (a) primer sequences and (b, c) PCR conditions and sequencing details for genetic analysis of *Lobaria pulmonaria*.

Appendix S4 Eight European regions in which populations of *Lobaria pulmonaria* were sampled (1: Iberian peninsula, 2: Italy, 3: Balkans, 4: South of the Alps, 5: North of the Alps, 6: Great Britain, 7: Scandinavia, 8: Urals).

Appendix S5 Sequences of fungal LPu09 alleles covering the allele range from 173 to 659 bp with emphasis on long, geographically restricted alleles occurring in the Ural Mountains.

Appendix S6 Centroid analysis of *Lobaria pulmonaria*: information on geographically restricted alleles in the fungal (a) and the algal (b) symbiont.

Appendix S7 Density distribution of centroids of geographically restricted alleles for the fungal (*F*; without locus LPu09) and the algal (*A*) symbionts of *Lobaria pulmonaria*.

Appendix S8 Spatial distribution analysis of shared alleles for the fungal (a) and algal (b) symbiont of *Lobaria pulmonaria*.

Appendix S9 Overall distance distribution of geographically restricted alleles for each fungal (a) and algal (b) microsatellite marker.

Appendix S10 Mean geographic distance between single fungal (a) and algal (b) geographically restricted alleles with centroids in the South Italy–Balkans region.

Appendix S11 Plot of the Procrustes analysis for populations of *L. pulmonaria*. Loadings for genetic variables (1: algal microsatellites, 2: fungal microsatellites), eigenvalues scree plot, scores of samples for the two data sets, and projection of these two sets after rotation (arrows link the algal multilocus genotype score to the fungal multilocus genotype score for the same populations) are shown.

Appendix S12 Population graphs with 139 nodes (populations) and 1114 edges for the fungal (a) and 1310 edges for the algal (b) symbiont of *L. pulmonaria* and its intersection resulting in a congruence graph (c).

Appendix S13 Mean number of distinct fungal (a) and algal (b) alleles per locus as a function of standardized sample size for eight major European regions.

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