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The threatened epiphytic lichen *Lobaria pulmonaria* in the Iberian Peninsula: Genetic diversity and structure across a latitudinal gradient

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ARTICLE INFO

Article history:
Received 23 April 2014
Received in revised form
11 March 2015
Accepted 14 May 2015
Available online 27 May 2015
Corresponding Editor:
Martin Grube

Keywords:
Climatic variables
Geographical gradient
Marginal populations
Population genetic
Spain

ABSTRACT

The current genetic diversity and structure of a species plays a marked role in the species' future response to environmental changes. Identification of the factors that might ensure the long-term viability of populations along its distribution area is therefore important for conserving biodiversity. In this work, infraspecific genetic diversity and structure of the threatened lichen *Lobaria pulmonaria* was investigated along a latitudinal gradient, spanning the Spanish latitudinal range of *L. pulmonaria*. Eighteen populations in Northern, Central, and Southern Spain were analysed using six specific fungal microsatellites of *L. pulmonaria*. Genetic diversity indices were calculated and compared among populations. Genetic differentiation was assessed using AMOVA and Bayesian methods. Additionally, a redundancy analysis was used to estimate the relative importance of environmental factors on the genetic variation among populations. Annual precipitation was the only factor affecting the genetic diversity probably through its influence on population and thallus size of *L. pulmonaria*, and significantly higher levels of genetic diversity were detected in southern populations. Isolation by distance was not significant, being environmental variables most important factors controlling genetic variation in *L. pulmonaria* populations.

Introduction

The genetic structure of species over wide geographical areas results from historical events, life history traits, and ecological factors (Avise 2000). Understanding the patterns and processes associated with geographical variation in population genetic structure across species' geographic ranges is one of the bases to answer questions in ecology, evolution, and conservation biology. Species with broad distributions may perform well within a wide range of environmental conditions (Joshi *et al.* 2001). However, species may become less abundant in marginal situations across species geographic ranges, as adverse conditions depart from the optimum compromising survival and reproduction (Gaston 2003). In this sense, marginal populations are exposed to environmental constraints, limitation or absence of essential resources, or physiological or ecological difficulties that change local population dynamics (Brown 1995; Gaston 2009). Consequently, current population structure is controlled by the interaction of genetic drift, gene flow, and natural selection, which may be strongly influenced by the demography and spatial distribution of populations (Eckert *et al.* 2008). It is expected that marginal and/or

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http://dx.doi.org/10.1016/j.funbio.2015.05.004

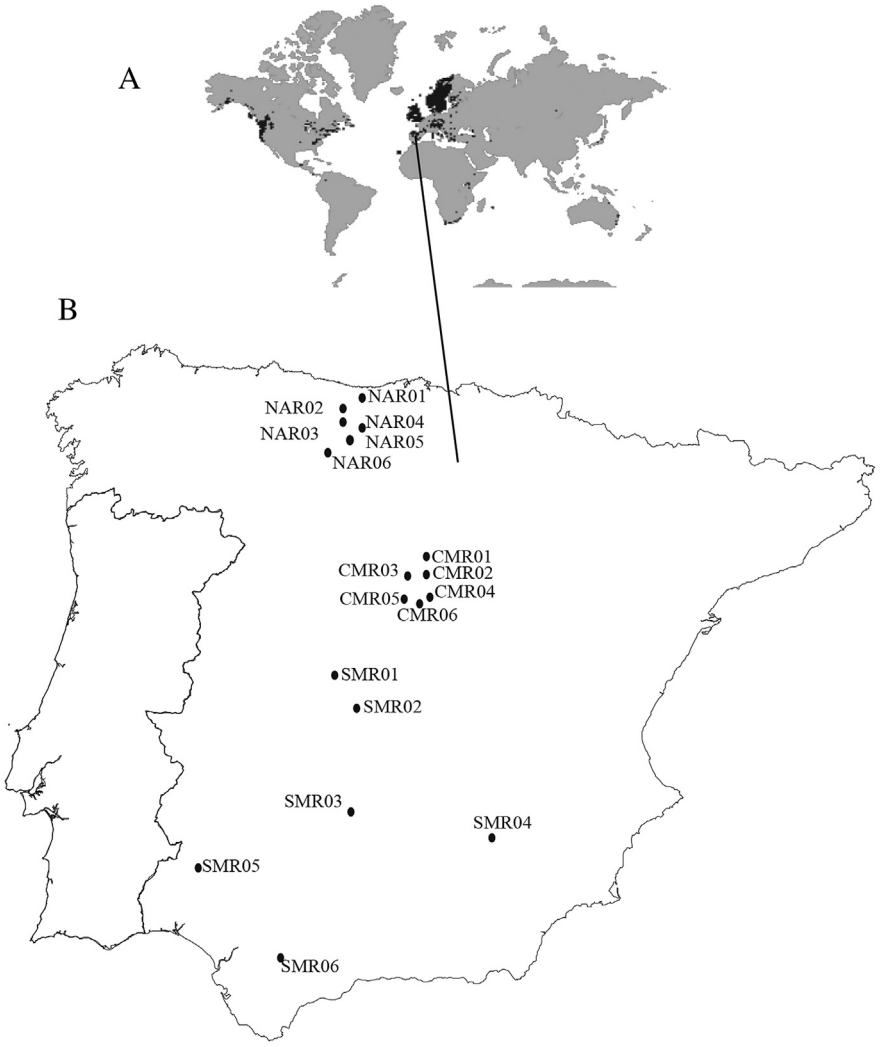

Fig 1 – (A). World distribution map of *Lobaria pulmonaria* (data obtained from GBIF). (B). Geographic locations of the sampled 18 *L. pulmonaria* populations along the Iberian Peninsula.

isolated populations likely exhibit a reduction in genetic diversity and higher genetic differentiation, resulting in negative effects on the evolutionary dynamics of species (Gaston 2003; Eckert *et al.* 2008).

Historically, plants and animals have been well studied in terms of a population genetics and phylogeographic framework, describing the genetic relationships among populations at different geographical scales. However, fungi and other cryptogams have been largely overlooked (Grundmann *et al.* 2008). Cryptogamic species, including lichens, usually exhibit a wider geographic distribution than vascular plants (Lücking 2003). The vast majority of lichens are dispersed either sexually by means of ascospores (dispersing only the mycobiont) or asexually *via* vegetative soredia or isidia (both symbionts are dispersed together). Studies of genetic structure and diversity of lichen-forming fungi have lead to different conclusions regarding species and geographical scale (Cassie & Piercey-Normore 2008; Werth 2010; Scheidegger *et al.* 2012). Although several large scale studies have been conducted at an intercontinental scale (Walser *et al.* 2003, 2005; Buschbom 2007; Geml *et al.* 2010; Fernández-Mendoza *et al.* 2011; Dal Grande *et al.* 2012; Fernández-Mendoza & Printzen 2013), very little previous researches have been carried out in European ecosystems, with most of the studies covering relatively small areas in boreal, oceanic, and alpine regions but almost absent from the Mediterranean region (Zoller *et al.* 1999; Lindblom & Ekman 2006, 2007; Lättman *et al.* 2009; Jüriado *et al.* 2011; Hilmo *et al.* 2012; Widmer *et* al. 2012).

The aim of this paper is to understand how the genetic diversity and differentiation of *Lobaria pulmonaria* populations vary throughout a latitudinal gradient in Spain. This gradient is the primary axis of climatic variation and we specifically aimed for addressing the effect of regional environmental factors and assessing genetic diversity on most isolated populations located in the southernmost part.

Lobaria pulmonaria is an emerging model species that is considered to be an indicator of forests of high ecological value (Werth *et* al. 2006a; Scheidegger & Werth 2009). The species is widely distributed in the northern hemisphere, including boreal, temperate, and Mediterranean forests and has few

occurrences in tropical forests. In many European countries the species is considered threatened, due to a marked population decline in the last centuries, primarily from habitat fragmentation and agricultural intensification (Belinchón et al. 2009; Scheidegger & Werth 2009). In the Iberian Peninsula, the species is more common in areas with oceanic influence from the north and west, becoming rarer toward the south where it only appears in mountainous and well-preserved forests with higher humidity values (Burgaz & Martínez 1999). The Iberian Peninsula is characterized by a steep latitudinal climatic gradient, with pronounced higher temperatures and lower precipitation towards the southern areas. On the other hand, the Iberian Peninsula is characterized by a long history of forest fragmentation, degradation, and deforestation, which have been more drastic towards the southern parts (Carrión et al. 2003; Urbieta et al. 2008; González-Martínez et al. 2010). Consequently, the Iberian distribution of this species is discontinuous, with more continuous and suitable stands in the northern area and a few isolated populations in southern Spain (Burgaz & Martínez 1999, Fig 1).

Recently, Scheidegger et al. (2012) and Widmer et al. (2012) developed studies evaluating genetic diversity of *L. pulmonaria* populations throughout the species European distribution. They concluded, using a phylogeographic approach, that the genetic diversity of *L. pulmonaria*, across Europe, is high with a few hotspots in Italy and the Balkans. Here we investigated the genetic diversity and spatial genetic structure of *L. pulmonaria* populations across a latitudinal gradient in Spain. Specifically we studied the importance of climatic *versus* geographic factors on *L. pulmonaria* populations. And secondly, we assessed the implications of these factors on the genetic structure of the isolated populations in southern Spain.

Methods

Sampling

The study site comprised a latitudinal gradient along Spain, covering the Spanish latitudinal range of *Lobaria pulmonaria*. A stratified selection of forest stands encompassed three zones included in two Biogeographical Regions: North (Atlantic Region), Central (Mediterranean Region), and South (Mediterranean Region). Individual samples were collected from six populations in each region (Table 1, Fig 1). Individuals growing in one forest stand were considered a single population. A total of 18 populations were finally considered in the study, including *Fagus sylvatica* (beech), *Quercus pyrenaica* (oak), *Abies pinsapo* (Spanish fir), and *Castanea sativa* (chestnut) forests. In order to avoid spatial clustering the populations were selected in localities at least 5 km apart. Six populations were selected in the North, where *L. pulmonaria* is more common (NAR01-NAR06). In central Spain (Mediterranean region), where *L. pulmonaria* occurs in well-preserved forests we included six populations in the middle of the central range (CMR01-CMR06). In the south, all populations that have been previously reported (SMR01-SMR06) were sampled and included in the study (Table 1, Fig 1). Sixteen to forty individuals were collected per population, with a total sample size of 635 individuals. In order to avoid repeatedly the same genotype, lichen specimens were collected randomly from different trees at least 10 m apart.

To assess the effect of climate and geographic factors on the genetic diversity and structure the following predictors were measured for each forest stand: Pannual = annual precipitation (mm); summer precipitation (mm); Tm = mean annual temperature (°C); P/T humidity index, which is an

Table 1 – Populations of *L. pulmonaria* sampled on the Iberian Peninsula. Population code, geographical distribution range, locality, for each population. Latitude and longitude coordinates used correspond to the system UTM (System of Universal Transverse Mercator coordinates) in WGS 84 datum.

Pop. Code	Locality	Phorophyte	CoorX (UTM)	CoorY (UTM)	Altitude (m)
North–Atlantic Region (NAR)					
NAR01	Cantabria, Saja	Beech	30T395715	4775223	570
NAR02	Cantabria, Laguna	Beech	30T380587	4769914	1205
NAR03	Palencia, Piedrasluengas	Beech	30T380821	4766740	1347
NAR04	Palencia, Brañosera	Oak	30T394960	4754234	1323
NAR05	Palencia, Parapertú	Oak	30T389016	4753124	1225
NAR06	Palencia, Ruesga	Beech	30T373498	4746469	1200
Central-Mediterranean Region (CMR)					
CMR01	Guadalajara, Cantalojas	Oak	30T469196	4565988	1494
CMR02	Segovia, Riaza	Beech	30T465780	4563066	1676
CMR03	Guadalajara, Peñalba de la Sierra	Oak	30T466936	4555757	1300
CMR04	Guadalajara, Cardoso	Oak	30T463465	4551141	1460
CMR05	Madrid, Montejo de la Sierra	Oak	30T458697	4550930	1284
CMR06	Guadalajara, El Cardoso de la Sierra	Oak	30T465710	4547178	795
South-Mediterranean Region (SMR)					
SMR01	Toledo, El Real de San Vicente	Oak	30S352182	4445537	1130
SMR02	Toledo, Estena	Oak	30S361657	4372537	797
SMR03	Ciudad Real, Fuencaliente	Oak	30S390654	4256003	884
SMR04	Albacete, Riopar	Oak	30S545885	4252111	1420
SMR05	Huelva, Aracena	Chestnut	29S179500	4201319	650
SMR06	Cádiz, Grazalema	Fir	30S285154	4071807	1280

Table 2 – Genetic characteristics of six microsatellites loci for all sampled individuals from the 18 populations.

Locus	RAS	Na	He	Ar
Lpu03	187–193	4	0.40 (0.03)	2.82 (0.08)
Lpu09	158–473	22	0.63 (0.03)	6.27 (0.65)
Lpu15	149–211	26	0.73 (0.02)	8.66 (0.54)
Lpu23	294–312	6	0.41 (0.04)	3.11 (0.19)
Lpu24	223–235	3	0.02 (0.01)	1.22 (0.10)
Lpu28	270–332	29	0.70 (0.04)	8.38 (0.89)

RAS: range of allelic size; **Na**: total number of alleles detected; **He**: average allelic diversity, mean (s.d); **Ar**: average of allelic richness by populations, mean (s.d).

estimator of the efficiency of precipitation in relation to the temperature; geographical location (utm); altitude (m a.s.l.). Geographical location and altitude were taken in the field with a GPS (GPSmap 60CSx, Garmin GPS). The climate variables were obtained from CLIMOEST, a climate simulator for the Iberian Peninsula (http://www2.montes.upm.es/Dptos/DptoSilvopascicultura/Edafologia/aplicaciones/Aplicaciones.htm = Programa Estimaciones Climáticas, Sánchez-Palomares *et al.* 1999). We carried out correlation analysis of the mentioned variables, and exclude those with correlation values above 0.7 to avoid problems of multicollinearity (see Martínez *et al.* 2012).

DNA extraction and microsatellite genotyping

Fresh material from *Lobaria pulmonaria* samples was ground in liquid nitrogen. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Six unlinked fungal microsatellites were analysed (Walser *et al.* 2003; Widmer *et al.* 2010). Two multiplex PCRs were performed in a total 10 μl reaction volume using 1 μl of genomic DNA (10–50 ng/μl^{-1}), 2 μl of the Qiagen Multiplex PCR Master Mix, and 200 nmol of each primer. The forward primer was fluorescent-labelled. The first multiplex PCR included the primers Lpu03F-NED, Lpu03R, Lpu09F-HEX, Lpu09R, Lpu15F-FAM, and Lpu15R. The second multiplex PCR included the primers Lpu23F-NED, Lpu23R, Lpu24F-HEX, Lpu24R, Lpu28F-FAM, and Lpu28R (Walser *et al.* 2003; Widmer *et al.* 2010). DNA amplifications were carried out in a Peltier thermal cycler (PTC-100), under the following cycle parameters: initial denaturation at 95 °C for 15 min; 27 cycles of 94 °C for 45 s, 54 °C for 90 s, and 72 °C for 60 s; followed by a final extension at 72 °C for 30 min. Fragment sizes of PCR products were determined on an ABI3100-avant automatic sequencer (Applied Biosystems). Allele assignment was performed using GeneMapper v3.7.

Genetic diversity within populations

For each population, the total number of alleles, mean number of alleles per locus, and mean number of private alleles (*Pa*) were calculated in Genalex v. 6 (Peakall & Smouse 2006). Nei's unbiased genetic diversity *H* (Nei 1978), number of multilocus genotypes *G*, and the percent of multilocus genotypes *M* were also calculated, and computed using the codes written by Werth *et al.* (2006a) in R (R Development Core Team), following the same methodology as Werth *et al.* (2006a). Mean within population allelic richness (*Ar*), corrected for sample size using rarefaction, was calculated in Microsatellite analyser MSA (Dieringer & Schlötterer 2003). In order to test if the genetic variation within populations was correlated with the latitudinal gradient and climatic factors, simple linear models were built in R for each of the genetic diversity indexes (*H*,

Table 3 – Standard diversity indices for the studied populations. Pop: population; n: total number of individuals genotyped; Ap: mean number of private alleles from all loci; Nap: number of alleles privates; H: Nei's unbiased gene diversity; G: number of multilocus genotypes; M: percentage of multilocus genotypes per population; Ar: mean within population allelic richness; Tm: annual mean temperature; Pannual: annual precipitation; P/T: humidity index.

pop	n	Ap	NAp	H	G	M	Ar	Altitude	Tm	Pannual	P/T
NAR01	38	0.333	2	0.41	36	0.95	7.02	570	11.4	1433	125.7
NAR02	33	0.167	1	0.63	31	0.94	4.3	1205	8.4	982	116.9
NAR03	37	0.500	3	0.64	34	0.94	4.43	1347	7.6	1122	147.6
NAR04	38	0.333	2	0.49	17	0.45	5.86	1323	7.7	1100	142.8
NAR05	40	0.333	2	0.48	24	0.59	5.13	1225	8.3	985	118.6
NAR06	16	0.000	0	0.49	8	0.5	3.83	1200	8.3	986	118.7
CMR01	38	0.167	1	0.41	16	0.42	3.5	1494	7.4	996	134.5
CMR02	40	0.000	0	0.54	21	0.51	4.57	1676	5.9	1073	181.8
CMR03	24	0.167	1	0.49	16	0.67	3.68	1300	9.2	656	71.3
CMR04	21	0.167	1	0.47	16	0.75	2.22	1460	9.2	656	71.3
CMR05	29	0.000	0	0.56	34	0.9	4.51	1284	9.5	825	86.8
CMR06	40	0.167	1	0.34	20	0.5	4.41	795	12.5	458	36.6
SMR01	38	0.167	1	0.52	24	0.63	4.96	1130	11.2	1178	105.1
SMR02	40	0.000	0	0.4	26	0.65	3.2	797	13.9	669	48.1
SMR03	40	0.000	0	0.59	26	0.66	4.25	884	14.1	693	49.1
SMR04	39	0.167	1	0.45	26	0.67	4.12	1420	11	728	66.1
SMR05	33	1.333	3	0.61	22	0.64	5.20	650	15.6	1315	84.2
SMR06	40	0.500	3	0.45	14	0.36	3.65	1280	12.3	960	78.0

Ar, and *Pa*) using climatic (Tm, Pannual, P/T) and geographic (latitude, altitude) variables as predictive factors.

Population genetic structure and gene flow

Genetic distance matrix for pairwise *Fst* values (Reynolds *et al.* 1983; Slatkin 1995) as well as the number of shared multilocus genotypes between populations were computed in ARLEQUIN v.3.5. Isolation by distance (IBD; Wright 1943) was examined by testing the association between the matrix of natural logarithm of geographic distance and pairwise population differentiation [*Fst*/(1-*Fst*)] using a Mantel test with 999 random permutations among all sampled populations. Analysis of Molecular Variance (AMOVA, Excoffier *et al.* 1992) was applied to assess population differentiation, as implemented in ARLEQUIN. Genetic variation was partitioned into different levels. A two-level AMOVA with populations nested in regions (NAR, CMR, and SMR) was developed. Significance of fixation indices was tested using a nonparametric permutation approach with 1000 permutations, performed by ARLEQUIN v.3.5.

Analyses were performed on the dataset with clones (635 individuals) and on the dataset corrected for clones within populations (411 individuals).

Additionally, a Bayesian clustering method was applied as implemented in the BAPS 5.2 program (Corander *et al.* 2003). This method allows a hierarchical analysis treating the partition among groups of individuals as the parameter of main interest. It treats both allele frequencies (from microsatellite markers), and the number of genetically divergent groups derived from populations as random variables (Corander *et al.* 2003, 2008). We applied BAPS clustering to the entire and corrected dataset using the option clustering of groups of individuals, and spatial clustering of groups using the central coordinates of each population (Corander *et al.* 2003, 2008). We tested ten replicates of $1 \leq K \leq 18$ groups (18 populations). To describe similarities among genetic clusters, we construct a UPGMA diagram in BAPS using Nei's standard distance (Nei 1975).

Finally, to evaluate the effect of environmental factors in driving genetic structure, full, and partial redundancy

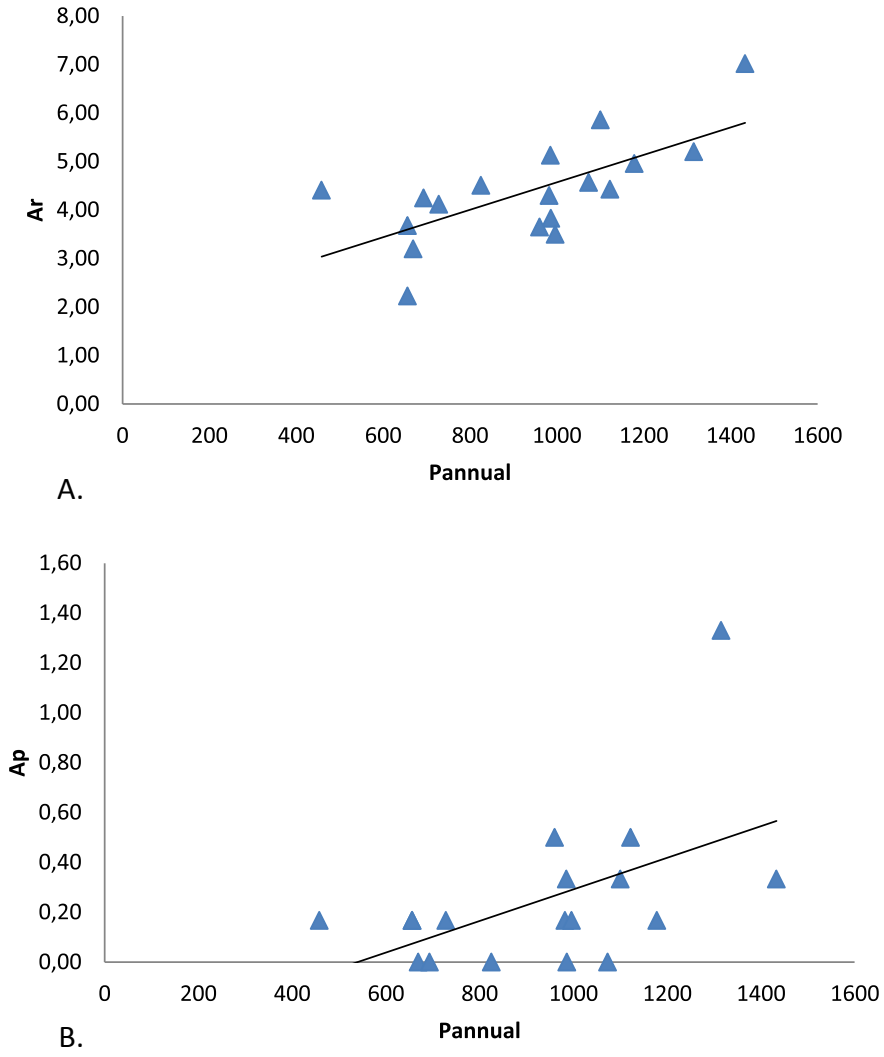

Fig 2 – Relationship between annual precipitation and genetic diversity estimators (Ar: mean within population allelic richness, Ap mean number of private alleles) in the 18 *L. pulmonaria* populations.

analyses (RDA, Legendre & Fortin 2010) were conducted with the vegan package in R. The allele frequencies were used as dependent matrix, geographic, and environmental factors were used as two distinct explanatory matrices. Three different models were constructed in order to determine how much of the genetic variation is uniquely explained by environmental factors, how much is uniquely explained by geographic variables, and how much is due to the combined effect of the two. The first model RDA1 included all variables (environmental and geographic) as explanatory; the second model RDA2 is a partial model in which geography explains genetic data conditioned by environmental variables; the third model RDA3 is a partial model in which environmental factors explain genetic data conditioned by geographic data.

Results

Genetic diversity within populations

Ninety alleles were detected in the 635 sampled individuals, with the number of alleles per locus ranging from three to twenty-nine (Table 2). Allelic diversity ranged from 0.02 for locus LPu24 to 0.734 for locus LPu15. LPu24 locus was variable in only three populations (NAR02, NAR03, and SMR04). Thirteen of the 18 populations examined exhibited at least one private allele, and six possessed more than one private allele (Table 3). The populations with the highest number of private alleles were NAR03, SMR05, and SMR06 (Table 3). A total of 329 different multilocus genotypes were detected across the 18 populations. The genetic diversity within populations based on estimates of multilocus genotypes was slightly variable (Table 3). Genetic diversity (H) ranged from 0.34 to 0.64, the number of multilocus genotypes (G) range was 8–36, and the allelic richness (Ar) varied from 2.2 to 7 (Table 3). The latitude gradient is not correlated to values of genetic diversity (H $r^2 = 0.0013$; $p > 0.11$; G $r^2 = 0.19$ $p > 0.1$; Ar $r^2 = 0.098$ $p > 0.1$; Ap $r^2 = 0.069$). However allelic richness (Ar) and mean number of private alleles (Ap) are significant and positively correlated with annual precipitation (Ar $r^2 = 0.46$ $p < 0.01$; Ap $r^2 = 0.26 < 0.05$) (Fig 2).

Population genetic structure and gene flow

The Mantel test identified weak but significant isolation by distance over the entire dataset (635 individuals, $P < 0.05$) while in the dataset corrected for clones within populations (411 individuals) the test was not significant (Fig 3). AMOVA results indicated that 78.26 % (635 dataset) and 86.6 % (411 dataset) of the variation was within populations, followed by a significant variation among populations within regions in both datasets (Table 4).

The BAPS cluster analysis of the entire and corrected by clones datasets indicated that 12 (K) and 4 (K) respectively were the most likely partition for each data. The partition with $K = 12$ had a log marginal likelihood −5326.27 ($p = 0.997$) and the partition with $K = 4$ had a log marginal likelihood −3882.5627 ($p = 0.876$). We describe the partition obtained from the corrected dataset. The partition revealed moderate structure by region, where single cluster was mainly shared by populations from the same region with exception of cluster 1, which was found once in each one of the three regions (NAR01, CMR06, SMR02). In the northern region, BAPS identified two clusters for the six sampled populations being cluster 2 the most common one, present in five northern populations. This cluster was also found to be present in southern

Table 4 – Analysis of molecular variance (AMOVA) (with the region as cofactor) on the dataset with clones (635 individuals) and on the dataset corrected for clones within populations (411 individuals).

Source of variation	Sum of squares	Variance	%	*P*
635 individuals				
Among regions	44.59	0.32	1.70	<0.001
Among populations	222.60	0.38	20.01	<0.001
Within populations	920.32	1.49	78.26	<0.001
Total	1187.53			
411 individuals				
Among regions	19.82	0.01	1.01	<0.005
Among populations	113.42	0.26	13.11	<0.001
Within populations	642.36	1.63	85.66	<0.001
Total	775.61			

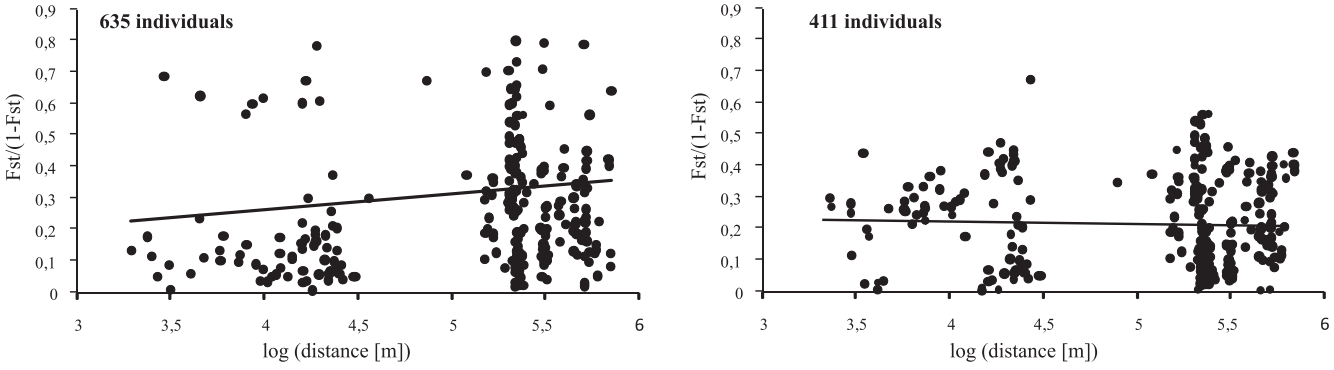

Fig 3 – Mantel test for matrix correlation between genetic distance (*Fst*/(1 − *Fst*)) and geographic distance (Log (distance). A weak but positive correlation is shown (r = 0.21 P = 0.041) when including clones (635 individuals). Not correlation when excluding clones (411 individuals, r = −0.030).

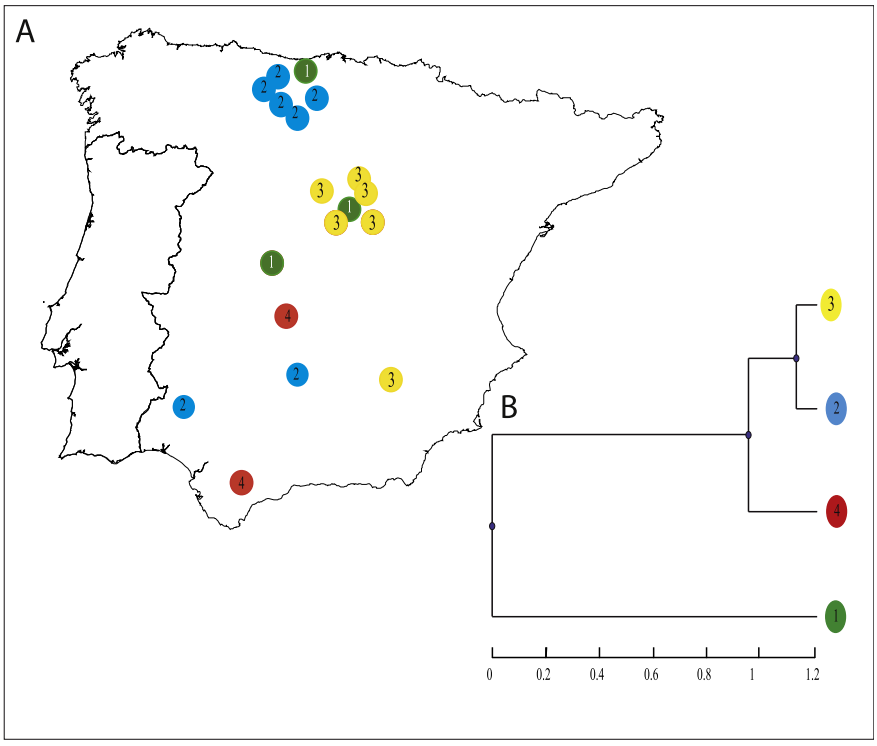

Fig 4 – BAPS Results. (A). Coloured circles are the graphical representation showing genetic clusters resulting from BAPS analysis. Populations indicated by the same colour represent similar genetic groups. (B). UPGMA on Nei'sD a pairwise distance between BAPS-identified genetic clusters. The numbers represent cluster populations.

Table 5 – Explained variance by each RDA model.

	Variance	Probability
Total explainable (RDA1)	43.6	<0.01
Pure environment (RDA2)	26.4	<0.05
Pure geography (RDA3)	7.6	<0.1
Joint climate/geography (RDA1-RDA2-RDA3)	9.6	

populations (SMR03 and SMR05). In the central region only two clusters were discriminated by BAPS, differentiating CMR06 (cluster 1) from the other five populations grouped into cluster 3. In the southern region, all four clusters were identified, being cluster 4 restricted to southern populations (SMR01, SMR06; Fig 4A). The UPGMA on Nei'sD pairwise distance between the clusters indicates that cluster 3 is the most divergent group, in spite of it is found in the three regions (Fig 4B).

Results from partial RDA conditioned by geographic factors showed a significant association between allele frequencies and environmental factors, being altitude the most important factor ($F = 2.36$; $p = 0.015$). Partitioning of total variance analysis (comparing the complete model with a partial model conditioned by environmental factors and a partial model conditioned by geographic factors) found that environmental variables explained 26.4 % of the total explainable genetic variance while geographic factors explained 7.8 % after removing the variance explained by environmental variables. Finally the percentage of the genetic variance in which environment and geography (join effect) cannot be separated due to collinearity is 9.6 % (Table 5).

Discussion

Genetic diversity within populations

Lobaria pulmonaria populations along a latitudinal gradient in Spain exhibited relatively high levels of genetic diversity (H) in most of the populations sampled (mean $H = 0.497$; s.d. = 0.08), with very similar values to those reported from Central and Southeastern Europe (Walser *et al.* 2005; Werth *et al.* 2006a; Scheidegger *et al.* 2012). In fact, Scheidegger *et al.* (2012) found almost identical mean values of genetic diversity and allelic richness between their southeastern European populations and our Spanish populations (H = 0.498 *versus* 0.497, and Ar = 5.09 *versus* 5.08), although we did not include in our study the same Iberian populations and the locus LPu25. The latitudinal gradient across Spain implies a steep climatic gradient: from the wetter north to drier south where there is a reduction in forest cover and quality due to the historic intensive use of these forests (Carrión *et al.* 2003; Urbieta *et al.* 2008; González-Martínez *et al.* 2010). However, Spanish mountains generate a rain-shadow effect across the gradient, implying high precipitation levels in the south comparable to the northern localities (Costa *et al.* 2001).

Thus, although we a priori expected a negative relationship between genetic diversity and latitude linked to the lower

number of populations that are highly isolated we did not find this pattern. However, we have found relationship between annual precipitation and genetic diversity, particularly on allelic richness and number of private alleles.

Population genetic theory predicts that fragmentation into isolated, discrete subpopulations composed of relatively few individuals, as is the case of the southernmost Spanish populations (Martínez *et al.* 2012 and personal observations), contributes to a depletion of overall genetic diversity (Ellstrand & Elam 1993). However, our results showed that annual precipitation exerted some influence on genetic diversity probably through the influence of precipitation on the population size. In that sense, Merinero *et al.* (2014) in a study where they analyze the environmental factors that drive the distribution and abundance of *Lobarina scrobiculata* in the Iberian Peninsula, they found that highest populations where located in the more rainy areas. Furthermore, Martínez *et al.* (2012) found that thallus size of *L. pulmonaria* was positively correlated with humidity whereas sexual reproduction was related to a higher thallus size. Following these previous results, *L. pulmonaria* populations located in more rainy areas will have bigger individuals and a higher probability to reproduce sexually and this could imply a higher genetic diversity. However, Singh *et al.* (2012) recently showed that *L. pulmonaria* is a heterothallic species, needing a compatible partner to reproduce sexually. So, if the frequency of mating type-idiomorphs is rather skewed in the studied populations, the probability to find apothecia is very small. More studies are necessary in order to elucidate the relationship between thallus size, presence of apothecia and genetic diversity.

Moreover, although the latitudinal gradient comprises a decline in habitat availability and a reduction in numbers and size of populations (Martínez *et al.* 2006), surprisingly our results did not support a latitudinal gradient effect on the levels of genetic diversity of the populations. Highly fragmented populations in more marginal areas are generally expected to possess reduced levels of within-population genetic diversity (Kark *et al.* 2008). In this context, we expected that continuously distributed populations (*i.e.* in regions to the north, Fig 1), should exhibit increased genetic diversity compared to geographically isolated populations, as is the case in *L. pulmonaria* southern Spanish populations (Fig 1). The results suggested us that the long term human-induced modifications on *L. pulmonaria* habitats (more severe in southern Spain) have not resulted in a relative decay of the genetic diversity in isolated populations compared with highly connected and well preserved populations in the north. These results contradict previous findings that showed how recent forest fragmentation and intensive management reduce the genetic diversity of *L. pulmonaria* in different woodland habitats in Europe (Jüriado *et al.* 2011; Otálora *et al.* 2011). Nevertheless, our result is partially in agreement with a recent study in Scandinavian fragmented forests where high levels of genetic diversity were retained (Hilmo *et al.* 2012). Other studies have also found that genetic diversity of *L. pulmonaria* populations in Europe did not decrease with latitude (Widmer *et al.* 2012; Scheidegger *et al.* 2012), suggesting that other factors could be mitigating the effects of isolation in marginal areas. In our study, isolated population in the south can be maintaining relatively stable and quite large populations because of the high annual precipitation values and then, relatively high values of genetic diversity.

Population genetic structure and gene flow

When including clones the Mantel test showed a weak significant effect of isolation by distance which is consistent with previous studies of *Lobaria pulmonaria* at regional scales (Walser *et al.* 2005). However, when the clones were excluded the Mantel Test suggested that dispersal is not local nor regional restricted. The AMOVA analysis revealed a low proportion (1.70 % and 1.01 %) of genetic variation attributed to regional differences. *Lobaria pulmonaria* populations in Spain have similar levels of diversity to those detected at intercontinental and local scales reported in previous studies (Walser *et al.* 2005; Werth *et al.* 2006a; 2007; Jüriado *et al.* 2011; Otálora *et al.* 2011; Scheidegger *et al.* 2012). The results from several studies of *L. pulmonaria* genetic structure at the local scale have suggested that dispersal in this species is rather effective, but not spatially unrestricted (Scheidegger & Werth 2009). Bayesian clustering method (BAPS) detected low divergence with four gene pools showing a moderate pattern of regional structure especially in north and central regions. Additionally, this analysis also showed a high level of similarity among distant populations (Fig 4). Organisms dispersed by spores are capable of long distance dispersal, and past establishment of founder populations could cause random gene distribution (Muñoz *et al.* 2004; Grundmann *et al.* 2008). Dispersal limitations at small spatial scales (distance of up few kilometers) have been shown in different lichen-forming fungi species (Walser 2004; Cassie & Piercey-Normore 2008), being in some cases virtually absent at large distance (Walser *et al.* 2005). However, other studies have reported a wide and efficient dispersal in both small and large spatial scales (Printzen *et al.* 2003; Muñoz *et al.* 2004; Werth *et al.* 2006b; 2007; Buschbom 2007; Lättman *et al.* 2009; Geml *et al.* 2010; Otálora *et al.* 2010; Fernández-Mendoza *et al.* 2011).

Historical factors and present day processes acting at different spatial and temporal scales may explain the differentiation patterns of populations. The factors that determine the genetic structure of populations at local scales are related to dispersal capabilities depending on the type of propagules (Freeland 2005), reproduction and recruitment, as well as drift and selection, which are influenced by habitat availability and quality. Here we have found, after removing the geographic distance effect, a significant partial correlation between environmental factors and allele frequencies. This implies a role of environmental heterogeneity in the genetic divergence of populations. Several studies have found that neutral population genetic differentiation is explained by environmental variation rather than by simple geographic distances in plant species (Gram & Sork 2001; Bockelmann *et al.* 2003; Temunovi *et al.* 2012) and animal species (Pilot *et al.* 2006; McGaughran *et al* 2014). Otálora *et al.* (2011) also found this relationship in *L. pulmonaria* in a study developed at local scale (Otálora *et al.* 2011). More recently, Nadyeina *et al.* (2014) showed ecological differentiation of gene pools in *L. pulmonaria* in a study developed in a primeval forest of Ukraine. They found that the distribution of gene pools depended on altitude, distance from rivers and height

in the trunk where the thallus grows. These variables were correlated to climate-related environmental factors such as air temperature and humidity, and solar radiation. The fact that environmental factors affect neutral genetic structure indicates that natural selection may interact with neutral processes of gene flow and genetic drift (McGaughran *et al.* 2014). Future tests with candidate genes could clarify the possible role of natural selection in shaping the genetic structure of *L. pulmonaria*.

Acknowledgements

We thank Tatiana Flores, for help with fieldwork. We also thank J. Más for technical support in the molecular Lab at URJC. This research was supported by the Dirección General del Medio Natural, Comunidad Autónoma de Castilla-La Mancha (projects POII09-0286-4849; GU002806; SC 0001/07), Ministerio de Educación y Ciencia (project CGL2007-66066- C04-04/BOS), Ministerio de Ciencia e Innovación of Spain (CGL2010-22049/BOS), and Ministerio de Economía y Competitividad (EPIDIVERSITY CGL2013-47010-P). The Comunidad Autónoma de Castilla-La Mancha, Madrid and the City Council of Montejo allowed the study of *Lobaria pulmonaria* populations in protected areas.

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