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Propagule size is not a good predictor for regional population subdivision or fine-scale spatial structure in lichenized fungi

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ABSTRACT

Propagule size has important consequences on the genetic structure of wind-dispersed species, as species with small propagules have higher capability of long-distance dispersal. Here, we studied reproductive modes and compared local and regional population structures in three Macaronesian lichenized fungi differing in propagule size. First, we quantified size distribution of propagules in each species. Second, genotype simulations based on microsatellite data were used to infer the reproductive mode. Third, using spatial analysis and population genetic approaches, we quantified the local and regional scale genetic structures of the fungal species. The three species differed in size distributions of propagules. The majority of populations exhibited clonal reproductive mode. Identical reproductive modes occurred often across species in the same sites, implying a possible relationship between reproductive mode and local site conditions. Contrary to expectation, at the local scale, the species exhibited similar patterns of spatial autocorrelation in genotypes. However, in agreement with the expectation based on propagule size, the species with highest frequency of small vegetative propagules (*L. pulmonaria*) exhibited lowest regional genetic differentiation. Nevertheless, altogether, our results show that propagule size is not a good predictor of population subdivision in lichenized fungi, neither at local nor regional spatial scale.

Introduction

Propagule size is an important factor affecting dispersal capacity in wind-dispersed plants. In plants, species with small seeds (i.e. low seed mass) are more efficient at dispersing over long distances than those with larger seeds, but establishment success is higher in species possessing large seeds (Morse & Schmitt 1985; Greene & Johnson 1993; Gravuer *et al.* 2003; Greene & Quesada 2005; Skarpaas *et al.* 2010; De Ryck *et al.* 2012). Hence, there is a tradeoff in propagule size, where high dispersal capability excludes high establishment success. Moreover, propagule size can have repercussions on population structure. Species with small, widely dispersing propagules would have less genetic differentiation between

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sites, as long as the small propagules are able to establish successfully. No effect of propagule size on population structure is expected if the establishment success of small propagules is low. Species may exhibit a size range in propagules, and effective dispersal rates may depend on the amount of small, lightweight propagules relative to heavier propagules.

The reproductive mode of a species has important consequences on its population subdivision (Hartl & Clark 1997). Lichenized fungi are interesting objects for population genetic studies because they form an obligate symbiosis with photosynthetic green-algae and/or cyanobacteria ('photobionts') and can reproduce either vegetatively with specialized clonal propagules or sexually with meiotic spores produced in apothecia (Büdel & Scheidegger 2008; Werth & Sork 2008; Scheidegger & Werth 2009). Some lichenized fungi have a mixed reproductive system with vegetative and sexual reproduction, as has been reported e.g. for the heterothallic lichenized fungus *Lobaria pulmonaria* (Dal Grande *et al.* 2012; Werth & Scheidegger 2012) and other species (Kroken & Taylor 2001; Högberg *et al.* 2002). Different mating systems are known for the haploid lichenized ascomycetes. For successful sexual reproduction, heterothallic species require a partner with compatible mating type (Scherrer *et al.* 2005; Seymour *et al.* 2005b; Scheidegger & Werth 2009; Singh *et al.* 2012). Heterothallism is thus analogous to obligate outcrossing in higher plants. For lichenized fungi, heterothallism is the most common mating system. However, some species were found to be homothallic, i.e. able to reproduce *via* selfing (Honegger *et al.* 2004; Seymour *et al.* 2005a; Honegger & Zippler 2007).

Here, we investigate the relationship between propagule size and genetic structure as well as reproductive mode in three codistributed species of lichenized fungi in Macaronesia, the Macaronesian endemics *Lobaria immixta* and *L. macaronesica*, and the widespread *L. pulmonaria*. These species can reproduce clonally and sexually but they differ in type and size distributions of clonal propagules.

We use a simulation approach to infer the reproductive mode of populations. Genotype simulations allow determining whether the number of multilocus genotypes (MLG) is consistent with a randomly mating population (Werth & Sork 2008; Werth & Scheidegger 2012). Compared with random mating, clonal reproduction leads to a reduction of the number of MLG in a sample. In fungal species sharing the same photobiont pool such as the species we studied (Dal Grande 2011), recombinant population structures in the same sites across species could either be the result of unknown favourable environmental factors (e.g. beneficial microclimate; high quality of microhabitats in a site) or of local availability of compatible photobionts.

First, we quantified the size distributions of vegetative propagules in each species to assess whether the species were likely to have different capabilities for long-distance dispersal. Second, we investigated the reproductive modes of the studied populations, as we were interested in evaluating whether reproductive modes were similar across species within the same site. To this end, we also evaluated whether sexual thalli occurred spatially aggregated, which would imply that beneficial environmental factors may trigger concerted fruiting across species. Third, we compared patterns of regional genetic differentiation across species. We hypothesized that the genetic differentiation among archipelagos would be lowest in the species with the smallest, putatively most far-dispersing clonal propagules. Fourth, we contrasted the fine-scale spatial autocorrelation in genotypes between species. We predicted that due to its small-sized and putatively far-dispersing clonal propagules, the extent of spatial autocorrelation in genotypes should be largest in the species with the highest fraction of small-sized propagules.

Material and methods

Study species, study area, and sampling

We investigated all species within *Lobaria* sect. *Lobaria* occurring in our study area: *Lobaria immixta* Vain., *Lobaria macaronesica* Cornejo & Scheid., and *Lobaria pulmonaria* (L.) Hoffm. These species are closely related (Cornejo & Scheidegger 2010). *Lobaria immixta* and *L. macaronesica* are sister species, and the widespread *L. pulmonaria* is their closest relative. While *L. pulmonaria* is widespread in Mediterranean to boreal parts of the Northern Hemisphere, with some occurrences in the Southern Hemisphere (Yoshimura 1971), *L. immixta* and *L. macaronesica* are Macaronesian endemics, with only one known occurrence on the Iberian Peninsula in an oceanic site with a high affinity to the Macaronesian flora (Burgaz & Martinez 1999; Cornejo & Scheidegger 2010; Werth *et al.* 2010, 2013b). The reasons for the discrepancy in distribution between the endemics and the widespread species are thus far unknown. It could be that the endemic species have a narrower ecological amplitude; on the Macaronesian islands, the endemics occur almost exclusively in the moistest sites (Laurisilva), while *L. pulmonaria* also occurs in the drier Canary Pine forest. Similar to what has been observed in Macaronesian endemic fungi of the genus *Nephroma*, *L. immixta* and *L. macaronesica* might represent neoendemics that arose after island formation (Sérusiaux *et al.* 2011), and if this was indeed the case, they would not have had equally much time to expand their distribution as *L. pulmonaria*. The three species of *Lobaria* are all associated with the same primary photobiont, the green alga *Dictyochloropsis reticulata* (Dal Grande 2011). They form large, foliose lichens inhabiting trunks and branches of trees in moist forests of the study area where they are associated with the Lobarion community (Rose 1988; Gauslaa 1995; Schumm 2008). According to our field observations from Macaronesia, all three species frequently form fruiting bodies (apothecia), the structures where microscopically small fungal ascospores are produced. The three fungi frequently cooccurred in the Lobarion community on the same host trees (phorophytes), which led to equal spatial sampling across species, a prerequisite to compare fine-scale spatial autocorrelation. The three taxa are recognized morphologically based on their clonal, symbiotic propagules: globose as well as cylindrical, secondarily developed structures (soredia) in *L. pulmonaria*; larger coralloid branched or unbranched structures (isidia) in *L. macaronesica*; and flat marginal protuberances (phyllidia) in *L. immixta* (see Fig 4 in Cornejo & Scheidegger 2010).

Our study area included the Canary Islands, Madeira, and Azores archipelagos with their typical Laurisilva and Canary Island Pine forest vegetation (Table 1). In order to incorporate a large spread of geographic distances while still allowing observation of spatial patterns such as clumped MLGs or clumped apothecial distributions, we sampled thalli along transects through the collecting sites, wherever the local population size was large enough. Balancing the sample sizes of *L. pulmonaria*, *L. macaronesica*, and *L. immixta* across trees and sites proved to be difficult because when thalli were small or otherwise poorly developed, we could not rely on our field identifications of *L. macaronesica* and *L. pulmonaria* (Werth *et al.* 2010; Werth & Scheidegger 2013). Thus, our strategy was to sample 15 thalli per tree from different heights and azimuths in order to maximize the chance of sampling multiple species, and to perform molecular species identifications by RealTime PCR (see below). We attempted to include at least three thalli per species from each tree, and sampled lichens from ten to 15 trees in each site depending on the local population size. In our final analysis, we included the 15 sites with a balanced number of samples of each species to enable a comparison of spatial autocorrelation among species. As we sampled the three fungal species from the same trees, any differences in autocorrelation between species were due to differential spatial genetic structures, and not due to confounding factors such as varying distance class distributions.

Molecular analysis

DNA was extracted using the DNeasy 96 plant kit (Qiagen, Hilden) according to the manufacturer's instructions. Each thallus was visually inspected for the presence of apothecia and parasites, and only thallus parts free of these structures were utilized for DNA extractions. Molecular species identifications involved a RealTime PCR assay, which has been described previously (Werth *et al.* 2010).

For population genetic analyses, samples were genotyped at six fungal microsatellite loci (Walser *et al.* 2003; Widmer *et al.* 2010). We recently developed additional microsatellites for *Lobaria pulmonaria* (Werth *et al.* 2013a), but the new markers did not increase the number of MLGs substantially (15 *vs.* 17 and 30 *vs.* 35 MLGs in two sites), and hence, for the purpose of our analyses, six loci were used. For two loci, we used newly developed primers that worked for all species (sequences below). Another four loci were amplified without modifications of the original protocol. An additional two fungus-specific microsatellites, LPu03 (Walser *et al.* 2003; Widmer *et al.* 2010) and MS4 (Dal Grande *et al.* 2012), were excluded because we were unable to develop primers that worked for all species and the primers did not amplify in *Lobaria macaronesica* and *Lobaria immixta*. Hence, our analyses were based on the same six loci for each species (LPu09, LPu15, LPu23, LPu24, LPu25, and LPu28). Each PCR reaction contained 200 nmol LPu09F, 200 nmol LPu09R-PET, 350 nmol of LPu15F, 350 nmol LPu15R-PET, 200 nmol

Table 1 – Overview of sites included in the population genetic analysis of the lichenized fungi *Lobaria immixta*, *L. macaronesica*, and *L. pulmonaria*, and basic diversity statistics. *N*, sample size; *X* and *Y* coordinate (units: decimal degrees; map datum: WGS84); NTrees, number of trees sampled from; *H*, Nei's unbiased gene diversity; *G*, number of six-locus genotypes; *G/N*, genotype–sample size ratio.

Pop	N	NTrees	X	Y	Area	*Lobaria immixta*				*Lobaria macaronesica*				*Lobaria pulmonaria*			
						N	H	G	G/N	N	H	G	G/N	N	H	G	G/N
FA2	139	21	−28.66011	38.56357	Azores, Faial	56	0.295	13	0.232	71	0.293	13	0.183	12	0.301	6	0.500
FA3	104	23	−28.69877	38.61300	Azores, Faial	58	0.314	7	0.121	27	0.288	10	0.370	19	0.456	7	0.368
MA2	113	11	−25.09038	36.98361	Azores, Santa Maria	38	0.309	12	0.316	55	0.332	12	0.218	20	0.461	11	0.550
SM2	123	28	−25.32551	37.75486	Azores, Sao Miguel	37	0.380	8	0.216	43	0.405	18	0.419	43	0.511	16	0.372
TE2	126	34	−27.20858	38.73381	Azores, Terceira	45	0.305	8	0.178	47	0.314	16	0.340	34	0.483	13	0.382
TE4	72	14	−27.23514	38.69492	Azores, Terceira	27	0.352	6	0.222	21	0.307	10	0.476	24	0.055	3	0.125
SG1	76	22	−17.29624	28.15044	Canary Islands, La Gomera	33	0.281	14	0.424	30	0.413	19	0.633	13	0.615	9	0.692
SG3	87	26	−17.25664	28.13171	Canary Islands, La Gomera	36	0.349	20	0.556	24	0.364	15	0.625	27	0.295	8	0.296
SG4	81	25	−17.21544	28.12161	Canary Islands, La Gomera	27	0.319	16	0.593	39	0.416	33	0.846	15	0.618	14	0.933
SG5	103	24	−17.25648	28.13071	Canary Islands, La Gomera	26	0.240	10	0.385	64	0.388	45	0.703	13	0.571	10	0.769
SH1	88	22	−17.98010	27.76129	Canary Islands, El Hierro	48	0.259	19	0.396	16	0.371	14	0.875	24	0.496	8	0.333
SP1	172	37	−17.83508	28.61152	Canary Islands, La Palma	26	0.269	7	0.269	41	0.344	16	0.390	105	0.489	73	0.695
ST2	135	27	−16.81057	28.32860	Canary Islands, Tenerife	87	0.300	19	0.218	21	0.315	8	0.381	27	0.422	7	0.259
PM13	132	22	−17.13160	32.76352	Madeira	59	0.407	39	0.661	46	0.513	31	0.674	27	0.509	13	0.481
PM16	99	22	−17.14059	32.80709	Madeira	56	0.397	34	0.607	32	0.545	22	0.688	11	0.291	3	0.273
Total	**1650**	**358**				**659**				**577**				**414**			

LPu23F-6FAM, 200 nmol LPu23R, 200 nmol LPu24F2-VIC (5′-TGA GGA GTA GAG ATA CAA CGT-3′, this study), 200 nmol LPu24R, 300 nmol LPu25F3-NED (5′-CTA TTC ATT TCT TGT GTT GAG TG-3′, this study), 300 nmol LPu25R3 (5′-CAT GAA ACG GTT TTG GTT GA-3′, Widmer *et al.* 2010), 200 nmol LPu28F, and 200 nmol LPu28R-VIC. Primer sequences not shown above are given in Walser *et al.* (2003). VIC, PET, NED, and 6FAM were the fluorescent labels we used, supplied by Applied Biosystems or Sigma–Aldrich. In addition to primers, each reaction contained 2.5 μL Qiagen multiplex PCR mix (Qiagen, Hilden), 0.5 μL diluted DNA (c. 15–50 ng), and H_2O to a total volume of 5 μL. Cycling conditions were 95 °C for 15 min; 35 cycles of 95 °C for 15 s, 52 °C for 30 s, 72 °C for 1 min; followed by a final elongation step of 60 °C for 30 min. Fragment analyses were run on an automated capillary sequencer (3730xl DNA Analyzer, Applied Biosystems, Rotkreuz), and alleles were typed using an internal size standard (LIZ500). Samples were genotyped using GeneMapper version 3.7 (Applied Biosystems, Rotkreuz).

Data analysis

Propagule size differences

To assess whether the size distributions of vegetative propagules differed between the three species, we measured the size of a total of 150 propagules, sampling 50 from each of three thalli for each species. Thalli were sampled from different localities and reflected the typical morphological range of vegetative propagules in the species. Propagule length (l) and width (w) were measured to calculate surface area as lw. We computed analyses of variance for three responses (length, width, area) across species (predictor). To infer whether there were statistically significant differences, Tukey's Honest Significant Difference (HSD) *post hoc* tests (Crawley 2002) were computed. Calculations were performed in R version 2.14.0 (R Development Core Team 2011) using the functions 'aov' and 'TukeyHSD'.

Reproductive mode of populations

To infer if the microsatellite data were consistent with random mating, we performed a simulation test comparing the observed numbers of MLGs in each population against the expectation under random mating using the method developed in Werth & Scheidegger (2012) and Werth & Sork (2008). Clonality reduces the number of MLG in a population and increases MLG recurrence, i.e. the number of times that an MLG is expected to occur more than once. The simulations were run with 1000 replicates in R, constructing MLGs based on the observed allele frequencies within sites for each species. Then, based on the simulated MLGs, we calculated two probabilities that the observed number of MLGs was smaller than or equal to the expectation under random mating ($P_{R.SMALLER}$ and $P_{R.EQUAL}$).

We compared the inferred reproductive mode from our MLG simulation with the observed frequency of fertile specimens in each species and site. Sites were classified as 'clonal' when the observed percentage of fertile specimens was below 10 %; otherwise, sites were classified as 'sexual'. Four types of responses were calculated separately for each species. In 'clonal matches', the inferred reproductive mode from MLG simulations was 'clonal' and matched the observed site classification ('clonal': <10 % of fertile thalli). In 'sexual matches', the inferred reproductive mode from MLG simulations was 'sexual' and matched the observed site classification ('sexual': >10 % of thalli in a site fertile). In 'clonal mismatches', the inferred reproductive mode from simulations was 'clonal', but the site classification based on observations of fertile thalli was 'sexual'. In 'sexual mismatches', the inferred reproductive mode from MLG simulations was 'sexual' and did not match the classification based on observational data of fertile thalli ('clonal').

We were interested to find out whether the percentages of matches between inferred and observed reproductive strategies could be observed by chance alone. Hence, we devised a permutation procedure as a test. Input data for the test contained a variable giving the inferred reproductive mode from our MLG simulations for each site, and a second variable, 'observed', stating the site classification based on observations of fertile thalli. Then, using our own code in R, we randomly sampled 15 values (=number of sites) with replacement from the variable 'observed' and added these simulated values as a new variable to our data set. We repeated this procedure until we had added 1000 resampled variables to the data set, and the percentages of clonal matches, sexual matches, clonal mismatches, and sexual mismatches were calculated across all sites for each resample. These percentages represented the distribution of values under randomness. From this distribution, we calculated the average and the 95 % confidence interval (CI) according to the formulae $SE = \sigma/\sqrt{N}$ and $CI = \bar{x} \pm 1.96 \cdot SE$. CIs were based on the number of sites. For example, in *Lobaria immixta*, nine of 15 sites had the inferred reproductive mode 'clonal'. Hence, the 95 % CI for 'clonal matches' of this species was based on $N = 9$, and the 95 % CI for sexual matches was based on $N = 6$.

We used the software MLGsim2 (Stenberg *et al.* 2003) to compute the probability that a given MLG occurred repeatedly due to sexual reproduction (P_{SEX}), given observed population allele frequencies. Significance of P_{SEX} was calculated by constructing 1000 random populations of observed size and comparing the simulated distribution of P_{SEX} with observed values (Stenberg *et al.* 2003). From the MLGsim2 outputs, we calculated the percentage of clonal thalli that were unlikely to be derived from sexual reproduction. Analyses were performed separately for each species.

Correlated fertility within trees

To find out if sexual forms were more frequent on individual trees than expected by chance, we simulated the expected percentage of thalli containing apothecia on each tree colonized by a respective fungal species. Our data set contained the species identity, tree number, thallus identity, and a variable indicating whether or not a thallus was apotheciate. To simulate the expected percentage of sexual thalli on each tree for each fungal species under randomness, we randomly sampled the values of the latter variable (apotheciate/not apotheciate) with replacement within each population. This analysis was also carried out separately for each species. Based on 1000 resamples, we calculated the probabilities that the observed proportion of sexual thalli on a given tree was smaller ($P_{F.SMALLER}$) than, equal to ($P_{F.EQUAL}$), or larger than ($P_{F.LARGER}$) the expectation under randomness. For example, if apotheciate thalli of

a given species cooccurred more often on a given tree than expected by chance, $P_{F.LARGER}$ should be high.

Genetic diversity and population structure

We calculated Nei's unbiased gene diversity *H* (Nei 1978), the number of six-locus genotypes *G*, and the genotype per sample-ratio *G/N* in order to provide basic diversity data for each population using our own code in R. Analysis of molecular variance was performed to analyze population genetic structuring (Excoffier *et al.* 1992) in Arlequin version 3.5 (Schneider *et al.* 2000). Populations were nested within archipelagos for analysis of molecular variance. To test whether the degree of population subdivision differed across species, we calculated F_{ST}, the differentiation between subpopulations, according to Weir & Cockerham (1984) with 95 % *CI*s in FSTAT (Goudet 2001). Moreover, to visualize possible differences between archipelagos and islands, we also performed discriminant analysis of principal components (DAPC) (Jombart *et al.* 2010) separately for each species, as implemented in the library 'adegenet' in R (Jombart 2008). For this analysis, we omitted the mainland population due to the low sample size of *Lobaria immixta* and *Lobaria macaronesica*.

Spatial analyses

For the purpose of identifying isolation by distance in each species, we calculated spatial autocorrelation of genotype diversity following Wagner *et al.* (2005) and Werth *et al.* (2006b). Moreover, we tested for a correlation between Roger's genetic distance and Euclidean geographic distance using Mantel tests in R based on 9999 permutations. Roger's genetic distance was calculated between pairs of populations using the package 'adegenet' and function 'dist.genpop' in R (Jombart 2008). Mantel tests were implemented in the R-package 'vegan' (Dixon 2003; Oksanen *et al.* 2010). The *p*-values were adjusted for multiple testing with Holm's method using the function 'p.adjust' in R (Holm 1979). Coordinates were transformed to Universal Transverse Mercator projection using the R-package 'PBS mapping' (Schnute *et al.* 2008).

Results

Propagule size distribution

The vegetative propagules of *Lobaria pulmonaria* were significantly smaller than those of the other two species (Fig 1), but *Lobaria macaronesica* had the longest, and *Lobaria immixta* had the widest vegetative propagules. *Lobaria pulmonaria* had a significantly smaller propagule surface area than the other two species. Propagule surface areas of the endemic species did not differ significantly.

Histograms revealed that the size distributions of propagules differed considerably among species with *L. pulmonaria*

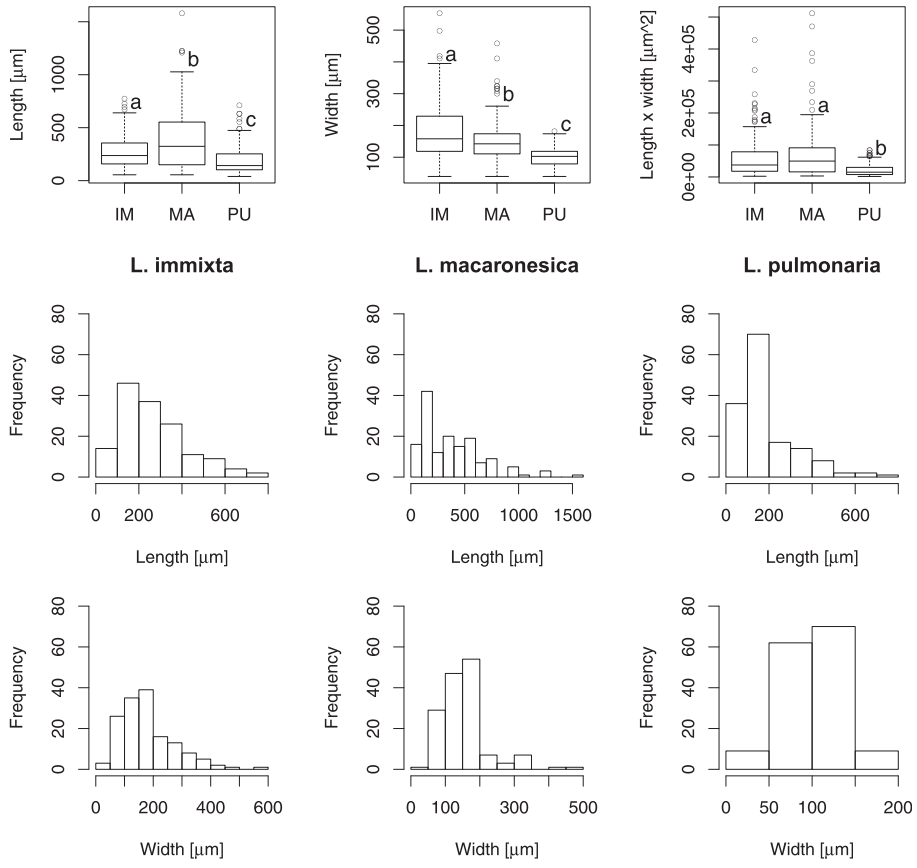

Fig 1 – Distributions of length and width of vegetative propagules in *Lobaria immixta* (IM), *L. macaronesica* (MA), and *L. pulmonaria* (PU). Upper panel: Boxplots. Letters indicate significant differences in mean values based on Tukey's HSD *post hoc* test. Middle panel: Histograms of propagule length. Lower panel: Histograms of propagule width. All measurements were performed in µm.

possessing the highest fraction of short and narrow vegetative propagules (Fig 1). Propagule length distributions in *L. pulmonaria* and *L. immixta* were strongly skewed towards <200 μm long. Twenty-four percent of the propagules of *L. pulmonaria* were shorter than 100 μm, relative to 9.3 % and 10.6 % in *L. immixta* and *L. macaronesica*, respectively. Similarly, 70 % *L. pulmonaria* propagules were shorter than 200 μm, as compared to 40 % and 38 % in *L. immixta* and *L. macaronesica*. These values highlight that *L. pulmonaria* has a higher fraction of small propagules than the other two species.

Reproductive mode of populations

The majority of populations of all three species departed significantly from random mating, implying a high degree of clonal reproduction (Table 3). The percentage of populations in agreement with panmixia differed slightly between species: 40 % (27 %) in *Lobaria immixta*, 33 % (27 %) in *Lobaria macaronesica*, and 20 % (33 %) in *Lobaria pulmonaria*; (values from MLGsim2 in parentheses). Panmixia was observed in the same sites in multiple species in five (four, MLGsim2) cases. Randomly mating populations were spatially aggregated in sites on La Gomera, Canary Islands (Table 3). Results from our MLG simulation method agreed with those of MLGsim2, with a notable exception: For *L. pulmonaria*, in three sites, the inferred reproductive mode from our MLG simulation was clonal, but the percentage of clonal thalli from MLGsim2 was 0 %.

In several sites, inferred reproductive modes from MLG simulations coincided with observed numbers of sexual forms in these sites (Tables 3 and 4; Electronic Supplement Fig S1). Interestingly, we found several mismatches between inferred reproductive mode and frequency of fertile specimens. On the one hand, in all species, a number of sites contained a high percentage of sexual thalli while exhibiting the genetic signature of clonal populations. On the other hand, in a few populations with inferred sexual reproduction, no or few fertile thalli were observed (Table 3).

In *L. immixta*, the percentages of clonal and sexual matches were higher than expected by chance, and the percentage of mismatches was lower than expected (Table 4). In *L. macaronesica* the percentage of clonal matches (i.e. inferred reproductive mode from MLG simulation clonal, observed reproductive mode based on frequency of fertile specimens clonal) was higher than expected by chance, and the percentage of clonal mismatches (i.e. inferred clonal, observed sexual reproductive mode) was lower than expected. The percentage of sexual thalli was significantly correlated between *L. immixta* and *L. macaronesica* (Pearson's correlation coefficient $r = 0.66$, $p = 0.007$), but not between either endemic species and *L. pulmonaria* ($r = 0.08$ and 0.39).

Table 2 – Analysis of molecular variance for the lichenized fungi *Lobaria immixta*, *L. macaronesica*, and *L. pulmonaria*. Populations were nested within three archipelagos. An asterisk indicates statistical significance at $p < 0.05$.

Species	Source	*df*	Perc	Φ-statistic	
L. immixta	Among archipelagos	2	28.2 %	Φ_{CT}	0.281*
	Among populations	12	11.8 %	Φ_{SC}	0.164*
	Within populations	644	60.0 %	Φ_{ST}	0.400*
L. macaronesica	Among archipelagos	2	36.8 %	Φ_{CT}	0.368*
	Among populations	12	6.7 %	Φ_{SC}	0.107*
	Within populations	562	56.4 %	Φ_{ST}	0.436*
L. pulmonaria	Among archipelagos	2	8.3 %	Φ_{CT}	0.083*
	Among populations	12	22.4 %	Φ_{SC}	0.244*
	Within populations	399	69.3 %	Φ_{ST}	0.307*

Correlated fertility within trees

In *Lobaria immixta*, apotheciate thalli cooccurred more often than expected by chance with 25 of 48 trees colonized by multiple thalli ($P_{F.LARGER} > 0.7$; Electronic Supplement, Table S1). Similarly, in *Lobaria macaronesica*, 19 of 40 trees were colonized by multiple thalli and the proportion of sexual thalli was higher than expected by chance. For *Lobaria pulmonaria*, the values were slightly lower: for seven of 30 trees (23.3 %) colonized by multiple specimens, the proportion of fertile thalli was higher than expected by chance.

Genetic diversity and population structure

All populations of the *Lobaria* species were polymorphic (Table 1). Our analysis of the overall genetic structure across species (FSTAT) demonstrated that the two endemic species were similarly structured, with overlapping *CI*s (*Lobaria macaronesica*, $F_{ST} = 0.354$, 95 % $CI = 0.188–0.669$ and *Lobaria immixta*, $F_{ST} = 0.341$, 95 % $CI = 0.199–0.520$). Populations of *Lobaria pulmonaria* showed slightly less structure ($F_{ST} = 0.285$, 95 % $CI = 0.217–0.342$). The *CI* on F_{ST} did not include the F_{ST} estimate of *L. macaronesica*, indicating that *L. pulmonaria* was significantly less structured than the latter species. The F_{ST} value of *L. immixta* was higher than that of *L. pulmonaria*; albeit not a significant difference, the value for *L. immixta* was close to the upper confidence limit of *L. pulmonaria*. The test for differences in regional genetic structure among species based on analysis of molecular variance showed that populations of all species were significantly structured by geographic region, and there was significant structure among populations within geographic regions in all species (Table 2). The rank order of regional genetic differentiation from analysis of molecular variance (Φ_{CT} *L. macaronesica* > *L. immixta* > *L. pulmonaria*) was the same as the order determined by the FSTAT analysis.

DAPC (Fig 3) confirmed the differentiation of populations situated on different archipelagos. Interestingly, the observed pattern was consistent with data simulated under a hierarchical island model (Jombart *et al.* 2010). The Azores archipelago exhibited distinct groups of individuals in *L. immixta* and *L. pulmonaria*, while there was some overlap of the Azores with the Canary Islands in *L. macaronesica* as well as overlap between individuals from Madeira and the Canary Islands in *L. immixta* and *L. pulmonaria*.

Spatial analyses

We found significant isolation by distance in all species at short distances in the variogram analysis (Fig 2). Significant spatial autocorrelation in genotypes extended up to about 50 m in all three species, and the values were fairly similar across species in all distance classes. There was no significant

Table 3 – Simulation of number of MLGs in sites and geographic areas under the assumption of panmixia, based on six-locus microsatellite data of *Lobaria immixta*, *L. macaronesica*, and *L. pulmonaria*. O.MLG, observed number of MLGs; E.MLG, expected number of fungal MLGs based on 1000 simulation replicates; $P_{R.SMALLER}$ and $P_{R.EQUAL}$, the probabilities that the observed number of MLGs is smaller than or equal to the expected number determined from 1000 simulation replicates; R, inferred reproductive mode from MLG analysis (C = clonal, S = sexual); percentages of clonal thalli inferred from MLGsim2 analysis (%Clon), and percentage of thalli bearing apothecia (%Fert). Bold: inferred reproductive mode clonal, but population with ≥10 % of thalli bearing apothecia. Italics: Reproductive mode sexual, but low percentage of thalli with fruiting bodies (<10 %).

Site	*Lobaria immixta*							*Lobaria macaronesica*							*Lobaria pulmonaria*						
	O.MLG	E.MLG	$P_{R.SMALLER}$	$P_{R.EQUAL}$	R	%Clon	%Fert	O.MLG	E.MLG	$P_{R.SMALLER}$	$P_{R.EQUAL}$	R	%Clon	%Fert	O.MLG	E.MLG	$P_{R.SMALLER}$	$P_{R.EQUAL}$	R	%Clon	%Fert
FA2	13	21.63	1.000	0.000	C	62.5	0.0	13	37.36	1.000	0.000	C	87.3	1.4	6	8.37	0.909	0.066	S	0.0	0.0
FA3	7	21.54	1.000	0.000	C	98.3	6.9	10	15.62	0.993	0.005	C	66.7	3.7	7	17.48	1.000	0.000	C	78.9	0.0
MA2	12	17.63	0.989	0.008	**C**	55.3	13.2	12	34.53	1.000	0.000	C	69.1	0.0	11	18.07	1.000	0.000	C	45.0	0.0
PM13	39	41.89	0.768	0.101	S	3.4	20.3	31	44.52	1.000	0.000	**C**	34.8	37.0	13	25.87	1.000	0.000	**C**	63.0	29.6
PM16	34	39.93	0.966	0.017	**C**	10.7	28.6	22	31.26	1.000	0.000	**C**	37.5	37.5	3	6.93	0.995	0.004	C	90.9	0.0
SG1	14	16.51	0.795	0.101	S	0.0	3.0	19	26.03	0.999	0.001	**C**	13.3	10.0	9	12.80	0.999	0.001	C	0.0	0.0
SG3	20	22.66	0.799	0.102	S	13.9	2.8	15	18.40	0.922	0.042	S	8.3	12.5	8	15.61	1.000	0.000	C	0.0	3.7
SG4	16	16.18	0.433	0.187	S	11.1	14.8	33	34.22	0.665	0.149	S	5.1	7.7	14	14.93	0.936	0.059	S	13.3	0.0
SG5	10	11.44	0.685	0.145	S	7.7	19.2	45	49.97	0.921	0.037	S	3.1	12.5	10	12.87	1.000	0.000	**C**	0.0	15.4
SH1	19	18.99	0.424	0.146	S	0.0	10.4	14	13.65	0.276	0.305	S	0.0	18.8	8	21.43	1.000	0.000	C	75.0	4.2
SM2	8	23.29	1.000	0.000	C	81.1	0.0	18	37.22	1.000	0.000	C	37.2	2.3	16	40.53	1.000	0.000	C	81.4	4.7
SP1	7	10.18	0.987	0.011	C	61.5	3.8	16	27.63	1.000	0.000	**C**	46.3	14.6	73	94.38	1.000	0.000	**C**	24.8	39.0
ST2	19	28.47	1.000	0.000	**C**	48.3	32.2	8	13.00	0.996	0.004	**C**	38.1	19.0	7	21.63	1.000	0.000	C	88.9	3.7
TE2	8	21.60	1.000	0.000	C	95.6	2.2	16	28.88	1.000	0.000	C	36.2	0.0	13	31.64	1.000	0.000	C	73.5	0.0
TE4	6	15.42	1.000	0.000	C	88.9	3.7	10	13.06	0.926	0.057	S	38.1	14.3	3	3.65	0.565	0.298	S	0.0	0.0

Table 4 – Results from permutation analysis of clonal matches, sexual matches, and mismatches. The table gives observed (Obs) and expected percentage of matches and mismatches (Exp) based on 1000 permutations. Expected values are averages across 1000 resamples, with 95 % CI in parentheses. Significant values (observed values outside 95 % CI) are printed in bold.

Category	*Lobaria immixta*		*L. macaronesica*		*L. pulmonaria*	
	Obs	Exp	Obs	Exp	Obs	Exp
Clonal matches[a]	**66.7**	53.2 (42.6–63.7)	**50**	39.5 (30.1–48.9)	75	79.9 (73.2–86.6)
Sexual matches[b]	**66.7**	46.2 (29.9–62.5)	80	61.5 (42.4–80.7)	0	19.5 (0.0–45.5)
Clonal mismatches[c]	**33.3**	46.8 (36.8–57.4)	**50**	60.5 (51.1–69.9)	25	20.1 (13.4–26.8)
Sexual mixmatches[d]	**33.3**	53.8 (37.5–70.1)	20	38.5 (19.3–57.6)	100	80.5 (54.5–100.0)

a The inferred reproductive mode from resampling of MLGs was clonal, and matched the site classification based on observations of fertile thalli (clonal: <10 % of the thalli fertile).
b Inferred reproductive mode sexual, observed mode sexual.
c Inferred reproductive mode clonal, observed mode sexual.
d Inferred reproductive mode sexual, observed mode clonal.

correlation between genetic and geographic distances (Mantel correlation coefficients: *Lobaria immixta*: $r_M = 0.0193^{ns}$, *Lobaria macaronesica*: $r_M = -0.1225^{ns}$, *Lobaria pulmonaria*: $r_M = -0.0561^{ns}$). Many distances between sites included in the Mantel test were large (tens to hundreds of km) so this result does not conflict with the significant autocorrelation found at short distances (Fig 2).

Discussion

Correlated reproductive modes across species and concerted fertility within trees

The MLG simulation approach (Werth & Scheidegger 2012) gave similar results as the MLGsim2 analysis for all sites except for three with *Lobaria pulmonaria*. The observed discrepancy between our MLG simulation ('clonal') and the MLGsim2 analysis (0 % clonal thalli) is easily explained by the fact that our simulations of MLG did not take into account the possibility of a MLG recurring from panmixia. Nevertheless, in 93 % of the sites, both approaches gave the same results, indicating that the MLG simulation method provides a robust predictor of a population's reproductive mode.

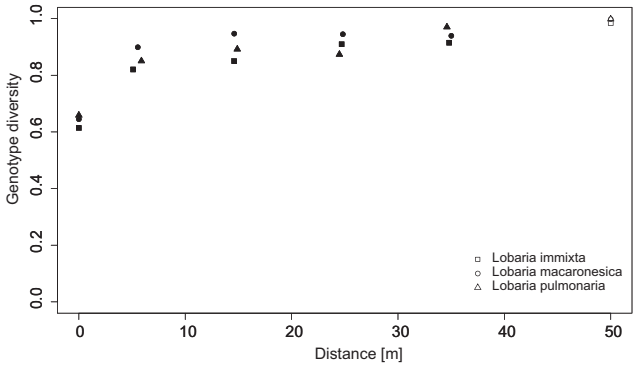

Fig 2 – Spatial autocorrelation in genotype diversity for the lichenized fungi *Lobaria immixta* (■ □), *L. macaronesica* (▲ △), and *L. pulmonaria* (● ○). Significant values of spatial autocorrelation are indicated by filled symbols.

One intriguing result was the mismatch between abundance of fruiting bodies in sampling localities and the localities' reproductive mode as determined by our MLG simulations. These mismatches occurred both with our MLG resampling approach and with MLGsim2. For instance, we found no evidence of a recombinant population structure in some rather abundantly fertile populations in all species. This pattern could be the result of establishment limitation (Werth *et al.* 2006a), caused by low recruitment rates of juveniles from ascospores. We also found, albeit infrequently, the opposite case where populations with few or no sexual thalli were inferred to be sexual. Our determination of the percentage of sexual thalli in each population was made based on samples collected in the field. As we did not sample the entire population, we could have missed sexual thalli, especially when they occurred infrequently.

Demographic effects such as population age could partly explain this mismatch. For example, populations that were fairly young may have been founded by sexual reproduction from areas outside. In this case, the inferred reproductive mode would be sexual, but sexual structures may yet have to develop in the populations. In newly founded populations, individual lichen thalli could have been too young to have produced clonal offspring. Hence, in these cases, the inferred reproductive mode may still reflect the pattern of the initial site colonization.

We expect a time of at least one generation between when a population forms the first sexual structures and when sexual reproductive mode can be detected from genetic data. Generation time in *L. pulmonaria* on the continent is about 25 y (Scheidegger & Werth 2009). Slightly shorter generation times have been reported for oceanic boreal rainforest for *L. pulmonaria* (>17 y, Larsson & Gauslaa 2011), but generation time in warmer oceanic areas is likely shorter. Yet, decades may have to pass before the predominating reproductive mode will be reflected in genetic data.

Obviously, the reproductive mode of a site will only manifest itself in a population if the propagules establish successfully. In plants, seed mass correlates with seedling survival probability. If this pattern indeed holds true for lichens, heavier symbiotic propagules would have a better chance to establish than microscopic ascospores, whose survival in

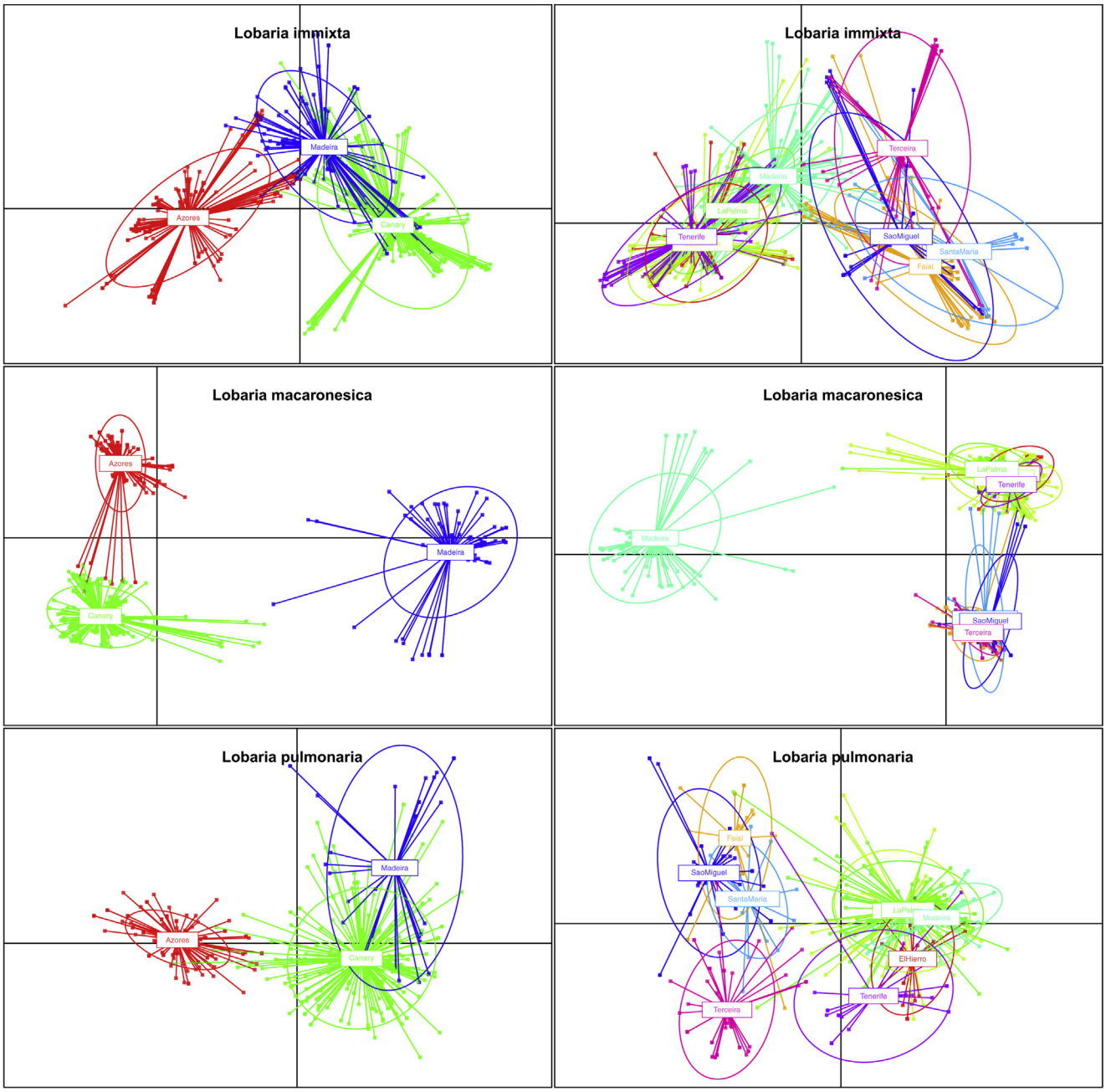

Fig 3 – DAPCs in the lichenized fungi *Lobaria immixta*, *L. macaronesica*, and *L. pulmonaria* in Macaronesia, grouping the data by archipelago. The plots show values from Principal Component Analysis, with individuals as dots and groups (archipelagos/islands) as ellipses.

addition depends on availability of a compatible photobiont. Thus, there may be a longer time lag before a sexual reproductive mode can be detected from genetic data compared to vegetative reproductive mode. In *L. pulmonaria*, transplanted large vegetative propagules had a higher survival rate than small vegetative propagules (Scheidegger 1995). Hence, in some lichens a relationship exists between propagule mass and the survival of juveniles. A well-dispersing species with a high fraction of small-sized propagules such as *L. pulmonaria* may establish less efficiently from these small, far-dispersed propagules than a species with larger propagules. Quantifying establishment of lichens from ascospores, however, is technically difficult due to the microscopic size of ascospores, and potential contaminations by other propagules.

The distribution of reproductive modes across the study area did not appear to be at random: In ten sites (67 %), more than one species was inferred to reproduce predominantly by clonality, in seven of the sites (46.7 %), all three species showed only evidence of clonal reproductive mode and no signal of random mating could be detected, and in five sites (33.3 %), several species were conform with random mating. Sexual reproduction frequently occurred in the

same sites in the two endemic species. Thus, we conclude that the reproductive mode was spatially correlated. Moreover, within sites, a local aggregation of sexual thalli was found, sometimes in more than one species (e.g. sites SH1, SG4, SG3). It could well be that local environmental factors (such as habitat quality) are responsible for this spatially aggregated fruiting pattern.

Formation of fruiting bodies in fungi depends on environmental signals such as temperature, humidity, and nutrients (Fultz 1988; Kües 2000). In heterothallic fungi such as *L. pulmonaria* (Singh *et al.* 2012), sexual reproduction depends on the local availability of a compatible mating type (Zoller *et al.* 1999; Honegger *et al.* 2004; Scherrer *et al.* 2005; Seymour *et al.* 2005a; Honegger & Zippler 2007). Hence, concerted sexual reproduction at the tree level could be due to the local presence of compatible mating types. In heterothallic fungi, matings occur at short distances; for instance, in epiphytic species mostly between specimens growing on the same tree (Scheidegger & Werth 2009). Once both compatible mating types are present on a tree, multiple thalli of a species could get fertilized and develop apothecia. This process is expected to take some time; newly founded populations may not yet have been colonized by the two compatible mating types. Indeed, a skewed frequency distribution of the two mating types has been reported for some populations of *L. pulmonaria* (Singh *et al.* 2012). Sexual thalli occurred quite frequently in some of the studied sites, but the percentages of fertile thalli varied drastically between sites. This could be due to some factors involved in the formation of fruiting bodies, at least in some of the studied sites. For instance, in sites TE2 and FA2, only few sexual forms were observed in all species.

All species showed evidence of clonality in the majority of sites. In a number of additional sites, multiple species possessed a recombinant population structure. One possible explanation for this pattern is that specific environmental conditions allowing the establishment of thalli from ascospores occur only in certain sites (Werth *et al.* 2006a). Factors responsible for the successful establishment of thalli from ascospores are still poorly understood in lichenized fungi, including the species studied here (Scheidegger & Werth 2009).

One factor that might lead to similar reproductive modes in sites across fungal species is the local availability of compatible algae. The three fungi we compared here share the same green-algal photobiont species (Dal Grande 2011). In sites where compatible free-living algae are available, multiple fungal species could be able to utilize this common photobiont pool, leading to panmictic population structures. On the contrary, in sites where no free-living *Dictyochloropsis reticulata* is available, all species may have to resort to clonal reproductive mode owing to photobiont limitation (Werth *et al.* 2007), until photobionts become available through vegetative propagules. So far, all attempts to prove the occurrence of free-living populations of *D. reticulata* have been to no avail (Dal Grande 2011; Stecher 2011). Photobionts of lichens may survive the gut passage of mites and snails (Meier *et al.* 2002; Boch *et al.* 2011). At least in boreal environments, *L. pulmonaria* is heavily grazed by snails (Asplund & Gauslaa 2008; Asplund *et al.* 2010a, b; Vatne *et al.* 2010) and thus photobionts could in principle be recruited from invertebrate feces, even if *D. reticulata* does not form free-living populations.

Reproductive mode is an important factor determining population structure. We found evidence of recombination at moderate frequencies in all three *Lobaria* species. Recombination has previously been reported for *Letharia gracilis* (Kroken & Taylor 2001), *Letharia vulpina* (Arnerup *et al.* 2004), *Ramalina menziesii* (Werth & Sork 2008; Werth & Sork 2010), and *Parmotrema tinctorum* (Mansournia *et al.* 2011). Our study species have a mixed reproductive mode, with the predominance of the clonal component in some and of recombination in others populations, a pattern previously reported for *L. pulmonaria* (Dal Grande *et al.* 2012; Werth & Scheidegger 2012). *Letharia* '*lupina*', depending on the study area, had either a recombinant population structure (Kroken & Taylor 2001) or a lack of recombination (Arnerup *et al.* 2004), indicating that other lichens exhibit a mixed reproductive mode.

Population subdivision and spatial autocorrelation

To our knowledge, this is the first study to quantify the relationship between propagule size and fine-scale spatial autocorrelation or regional population subdivision in fungi. Moreover, this study is the first to make a detailed comparison of population genetic structures across multiple fungi collected from the same sites and tree substrates. As expected on the basis of extensive geographic isolation, all species showed significant genetic differentiation among archipelagos. These results are consistent with those of others revealing significant differentiation among geographic regions (e.g. continents) in lichenized fungi (Palice & Printzen 2004; Walser *et al.* 2005; Buschbom 2007; Werth 2010; Fernández-Mendoza *et al.* 2011). The pattern of our data displayed in DAPCs was consistent with that of a hierarchical island model (see Fig 4 in Jombart *et al.* 2010). This model was expected in an island system, where individual islands are proximate enough to allow for some gene exchange, but where gene flow among archipelagos is rare or absent.

Lobaria pulmonaria had the lowest genetic structuring among archipelagos, consistent with our prediction that the species with the highest frequency of small propagules (length and width) should show the lowest degree of regional differentiation. Hence, there was a relationship between genetic structure and propagule size. The species with large phyllidia, *Lobaria immixta*, exhibited a similar amount of genetic differentiation among regions as *Lobaria macaronesica*, the species with large coralloid isidia (FSTAT and analysis of molecular variance results); both of these species also produce smaller propagules in low frequency. Overall, propagule size was not a good predictor of regional population subdivision: The two endemic species that had quite different propagules (largest in *L. macaronesica*) did not differ in genetic structures.

All species showed similar fine-scale genetic variation, with similar spatial extents of autocorrelation. Hence, the type and size distribution of vegetative propagules size did not appear to correspond to the genetic structure of the three species, nor were there major differences in effective population size between species, which could also potentially influence spatial autocorrelation. We would have expected the largest extent of spatial autocorrelation in genotypes in *L. pulmonaria*, the species with the highest proportion of small-sized vegetative propagules, but significant spatial

autocorrelation extended to similar distances in all species. Hence, propagules size was not a good predictor of fine-scale spatial autocorrelation. We found no substantial differences in the fine-scale spatial genetic structures among species, which could be due to the fact that propagule size distributions do overlap between species, all species exhibited variation in propagule size, and all species contained a fraction of propagules with small size.

Spatial correlation in genotype diversity was largely congruent in the three species – hence, the spatial extent of clonality was similar across species, a finding which we did not anticipate owing to the differences in the size distributions of vegetative propagules. Similar genetic structures within populations could result from a shared forest stand history (Werth *et al.* 2006b), leading to a similar population history across species. We found relatively high genotype diversities for all species at small distances, indicating multiple colonization events per each tree, similar to the genetic pattern in small-scale patch dynamics of temperate, humid primeval forests (Scheidegger *et al.* 2012).

In species with small and/or isolated populations, high genetic differentiation is expected as allele frequencies of small populations shift by genetic drift (Barrett & Kohn 1991; Hartl & Clark 1997). The studied populations were not small, but they were geographically isolated from one another on different islands and archipelagos. Hence, we expected substantial genetic differentiation between populations situated on different archipelagos in all species—which was indeed found.

Interestingly, the species differed with respect to population subdivision at the regional scale. The endemics exhibited similarly strong genetic structuring among archipelagos, while the widespread *L. pulmonaria* showed less differentiation at the same spatial scale. We are not aware of other studies that have contrasted the genetic structures of endemic species with that of widespread congeners. Hence, it remains to be seen if the pattern found in our data extends to other species and taxonomic groups.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2013.10.009.

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