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RESEARCH ARTICLE

Long-term consequences of disturbances on reproductive strategies of the rare epiphytic lichen *Lobaria pulmonaria*: clonality a gift and a curse

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ABSTRACT

The effect of disturbance on symbiotic organisms such as lichens is particularly severe. In case of heterothallic lichen-forming fungi, disturbances may lead to unbalanced gene frequency and patchy distribution of mating types, thus inhibiting sexual reproduction and imposing clonality. The impact of disturbance on reproductive strategies and genetic diversity of clonal systems has so far received little attention. To infer the effects of disturbances on mating-type allele frequencies and population structure, we selected three populations in the Parc Jurassien Vaudois (Switzerland), which were affected by uneven-aged forestry, intensive logging and fire, respectively. We used microsatellite markers to infer genetic diversity, allelic richness and clonal diversity of the epiphytic lichen *Lobaria pulmonaria* and used *L. pulmonaria*-specific MAT1-1 and MAT1-2 markers to analyse the frequency and distribution of mating types of 889 individuals. Our study shows that stand-replacing disturbances affect the mating-type frequency and distribution, thus compromising the potential for sexual reproduction. The fire-disturbed area had a significantly lower genetic and genotypic diversity and a higher clonality. Furthermore, the majority of compatible mating pairs in this area were beyond the effective vegetative dispersal range of the species. We conclude that stand-replacing disturbances lead to lower chances of sex and symbiont reshuffling and thus have long-lasting negative consequences on the reproductive strategies and adaptive potential of epiphytic lichen symbioses.

Key words: disturbance; lichen-forming fungi; genetic diversity; population structure; mating-type gene frequency; allelic richness

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INTRODUCTION

Lichens are symbiotic associations consisting of a fungal partner (mycobiont) and one or more photosynthetic partners (photobionts) living together to form a coherent structure called thallus (Ahmadjian 1993). Epiphytic lichens are strongly affected by the population dynamics of their carrier trees e.g. via tree logging or stand-replacing forest disturbances such as fire and windthrow. The long-term consequences of perturbations on populations of lichens may have severe and long-lasting consequences on lichen populations as successful recruitment involves complex processes of overcoming dispersal and establishment limitations (Bailey 1976). After disturbance, the remaining individuals may serve as founders for population recovery but the resulting genetic diversity would be a fraction of the genetic diversity of the original population (Wright 1951). Low genetic diversity may lead to low fitness and adaptive potential (Muller 1963; Nei, Maruyama and Chakraborty 1975). This effect however can be counterbalanced by immigration and by recruitment of individuals from propagules and/or thallus fragments. Moreover, recombination during sexual reproduction may also introduce genetic variation in populations.

Reproduction in a lichen-forming fungal species can be exclusively asexual, strictly sexual or alternatively clonal and sexual, where the same individual exhibits both asexual and sexual reproduction. In ascomycetes, almost the entire sexual cycle is regulated by a single regulatory mating-type (MAT) locus (Coppin *et al.*, 1997; Turgeon and Yoder 2000; Honegger *et al.*, 2004), which has two alternative forms or idiomorphs, MAT1-1 and MAT1-2 (Coppin *et al.*, 1997; Turgeon and Yoder 2000). Sexual reproduction occurs only between compatible MAT types. The two MAT idiomorphs can be present in the same haploid individual, thus being self-fertile (homothallic species) (Turgeon and Yoder 2000). Alternatively, each haploid individual may harbor either MAT1-1 or MAT1-2 (heterothallic species), thus being self-incompatible.

For sex to occur in heterothallic fungi, apart from suitable environmental conditions, presence of both mating types in the population is essential (Zoller, Lutzoni and Scheidegger 1999; Skagerberg 2011). The relative proportion and spatial distribution of mating types can have a critical influence on the reproductive mode and sexual potential of the population and also on population structure (Vekemans, Schierup and Christiansen 1998; Dayakar, Narayanan and Gnanamanickam 2000; Douhan, Murray and Dyer 2002; Siah *et al.*, 2010). Ours is the first study analysing the spatial distribution of mating types in populations re-established after disturbance. As the distribution of mating types plays a central role in the life cycle of heterothallic fungi, the knowledge of MAT distribution is important for a proper understanding of the population genetics, adaptive and evolutionary potential of fungi and to devise meaningful conservation strategies.

To study the consequences of disturbance on the population genetics and reproductive strategies, we selected the heterothallic lichen-forming fungus *Lobaria pulmonaria* (Kalwij, Wagner and Scheidegger 2005; Scheidegger and Werth 2009). The generation time of *L. pulmonaria* is 30 years or more for vegetative propagules, and probably longer for ascospores (Scheidegger and Goward 2002). This epiphytic lichen-forming fungus is associated with the clonal green alga *Dictyochloropsis reticulata* and cyanobacteria of the genus *Nostoc* (Cornejo and Scheidegger 2013; Dal Grande *et al.*, 2014b). It is distributed over parts of Europe, Asia, North America and Africa (Yoshimura 1971) and it is considered threatened, particularly in the European lowland regions (Wirth *et al.*, 1996). Studies have shown that both recombination and clonal growth influence population genetic structure of *L. pulmonaria* (Walser *et al.*, 2004; Zoller, Lutzoni and Scheidegger 1999; Dal Grande *et al.*, 2012). The distribution of MAT thalli is crucial for reproduction in heterothallic fungi and might limit the potential of sex in the re-established population. Recently developed markers enable us to determine the mating type of *L. pulmonaria* thalli (Singh *et al.*, 2012). Although an earlier study investigated impact of disturbance on lichen genetic diversity (Werth *et al.*, 2006a), the effect of disturbance on MAT frequency and distribution was not studied.

The objectives of our study are threefold: (i) to investigate the long-term consequences of disturbance on the sexual and adaptive potential of the re-established population, (ii) to understand the process shaping the genetic structure of populations of a symbiotic system re-established after disturbance and (iii) to design effective strategies for conservation of the species aimed at restoring population size as well as ensuring the availability of compatible MAT thalli.

MATERIALS AND METHODS

Sampling

Samples used in this study were collected from a traditionally managed sylvopastoral landscape or '*pasture-woodland landscape*' in the Parc Jurassien Vaudois, Switzerland (Kalwij, Wagner and Scheidegger 2005; Wagner *et al.*, 2006; Werth *et al.*, 2006a, b, 2007; Bolli *et al.*, 2008; Werth and Scheidegger 2012), in the framework of an earlier project. This region includes three areas with different historic disturbance types. The first area (referred to as '*uneven-aged*') is regularly disturbed by uneven-aged forestry, i.e. single stem logging, which is also practiced in the rest our study area in the Parc Jurassien Vaudois. The second area was subjected to intensive logging for charcoal production from 1850 to 1900 (from now on referred to as *logged*). The third area was intensively logged during 1870–1871, followed by windthrow and two weeks of forest fire in the following year (Kalwij, Wagner and Scheidegger 2005) (from now on referred to as *burnt*). A total of 889 samples were included in the study, collected from 41 circular plots, 1 ha each, including areas affected by stand-replacing disturbances (*burnt, logged*) and uneven-aged forestry (*uneven-aged*). From the *logged* sites, 9 plots were chosen and from the *burnt* sites, 12 plots were investigated. Twenty plots were classified as *uneven-aged*. The *burnt* and *logged* sites have few *uneven-aged* sites around them (Fig. 1A). We analysed allelic richness, genetic diversity and MAT frequency of *burnt* and *logged* sites, both at the disturbance level and at the plot level (Tables 1 and 2). The disturbance-level analyses were performed both with and without considering the *uneven-aged* sites in the vicinity of the *logged* and *burnt* sites to assess the influence of stand-replacing disturbance on the distribution of MATs as well as overall MAT representation in an area (Fig. 1B and C). The *uneven-aged* sites were also analysed with and without these plots (Fig. 1A and D).

Molecular analysis

We used data of eight fungal-specific unlinked microsatellite markers (Dal Grande *et al.*, 2010; Werth and Scheidegger 2012).

Lobaria pulmonaria-specific MAT1-1 and MAT1-2 markers (Singh *et al.*, 2012) were used together in a multiplex polymerase chain reaction (PCR) to identify the mating type of the 889 thalli. Multiplex PCR was performed using both MAT1-1 and MAT 1-2 primers in a single PCR reaction. The multiplex PCR reaction consisted of multiple primer pairs specific to different DNA

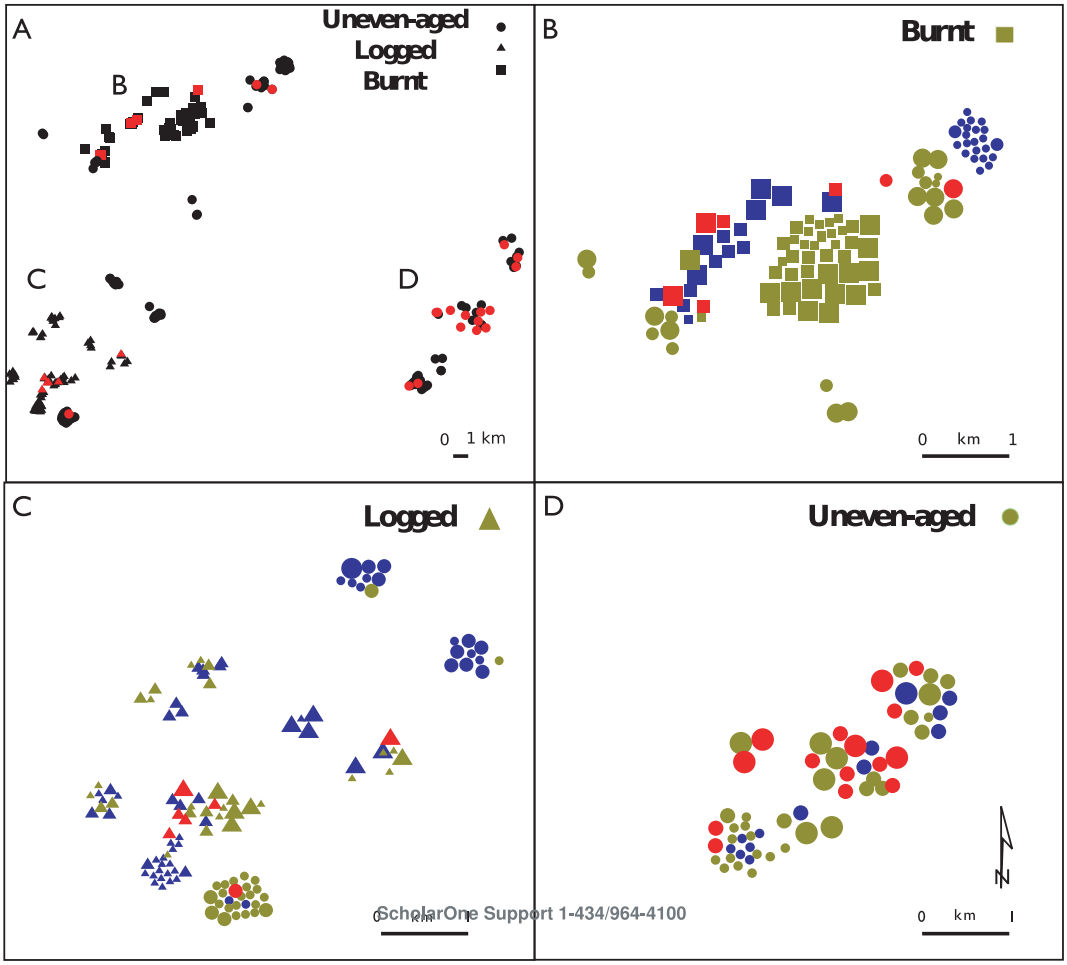

Figure 1. Spatial distribution of MAT alleles in (A) study area, showing all three regions analysed for the MAT distribution of *L. pulmonaria*, (B) *burnt* sites (squares), (C) *logged* sites (triangle) and (D) *uneven-aged* sites (circle) of Parc Jurassien Vaudois. Each colored shape represents a tree, with smallest circles representing one thallus, larger circles representing < = 5 thalli and largest circles representing > 5 thalli. Black symbols represent trees hosting *L. pulmonaria*. Blue-colored shapes represent MAT1-1 thalli, brown symbols represent MAT1-2 thalli and trees having thalli of both mating types are represented by red symbols.

Table 1. MAT analyses in the Parc Jurassien Vaudois region in relation to the disturbance history. See footnotes below for the definition of terms.

Fungus	N	MLGs	#loci	Clonality (%)	R	PR	Ia	rBarD	uH	MAT1-1:MAT1-2 (%)	chi	P-value
Overall	887	139	8	94.14	–	–	–	–	0.468 +/− 0.12	41.71:58.29	**24.36**	<0.0001
Fire	270	21	8	92.22	6.20 +/− 1.55	1.15 +/− 0.42	2.23	0.35	0.45 +/− 0.11	44.4 : 55.6	3.33	0.0679
Fire*	430	38	8	91.16	–	–	–	–	–	34.4 : 65.6	41.75	<0.0001
Logged	188	46	8	75.53	8.22 +/− 2.70	2.57 +/− 1.15	1.17	0.17	0.49 +/− 0.1	62.2 : 37.8	10.94	0.0009
Logged*	260	59	8	77.31	–	–	–	–	–	62.3 : 37.7	15.75	<0.0001
Uneven-aged	429	88	8	79.48	9.09 +/− 2.67	2.76 +/− 1.02	−0.45	−0.08	0.51 +/− 0.11	31.2 : 68.8	60.42	<0.0001
Uneven-aged*	197	61	8	69.03	9.76 +/− 2.97	3.52 +/− 1.15	0.90	−0.14	0.48 +/− 0.13	31.0 : 69.0	28.55	<0.0001

N: number of samples; MLGs: number of genotypes per population; R: rarefied allelic richness (N = 188); PR: rarefied private allelic richness (N = 188); Ia: index of association; rBarD: linkage disequilibrium; uH: unbiased genetic diversity. Values in bold indicate significance at $\alpha = 0.05$.
*Burnt** and *logged** represent areas including the surrounding *uneven-aged* plots and *uneven-aged** represents *uneven-aged* plots excluding the plots lying spatially close to *burnt* and *logged* regions.

sequences in a single PCR mixture (Dyer *et al.*, 2001). The PCR mix used 20–50 ng DNA in a total volume of 50 μl containing 18 μl of JumpStart REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich) and 200 nM of each primer. Cycling conditions were (1) 2 min 94°C; (2) 35 cycles of 30 sec 94°C, 30 sec 54°C, 40 sec 72°C; (3) 10 min at 72°C final extension. Axenic fungal culture DNA was used as positive control, whereas algal DNA and no template reactions served as negative controls.

Table 2. Information on the populations used in this study. See footnotes below for definition of terms.

Disturbance	Plot	N	uH	R	PR	MAT ratio	Unique MLGs	rBard
B	W09	22	0.011	1.057	0.182	0	2	–
B	W14	23	0.302	1.737	0.189	0.522	2	1.000
B	W15	4	0.000	NA	NA	0	1	–
B	W21	24	0.145	1.453	0.000	0.333	2	1.000
B	W36	23	0.000	1.000	0.004	0	1	–
B	W37	15	0.093	1.339	0.428	0	2	1.000
B	W46	21	0.180	1.736	0.034	0.286	4	0.686
B	W61	23	0.000	1.000	0.002	0	1	–
B	W71	22	0.341	2.194	0.003	0	6	0.277
B	W79	24	0.031	1.156	0.000	0	2	1.000
B	W93	28	0.000	1.000	0.000	0	1	–
B	W98	41	0.000	1.000	0.000	0	1	–
L	W103	12	0.083	1.417	0.000	0.167	2	1.000
L	W145	24	0.346	2.303	0.082	0.952	8	0.219
L	W151	22	0.283	2.113	0.002	0.182	8	0.329
L	W188	24	0.372	2.141	0.352	0	4	0.709
L	W226	21	0.431	2.969	0.270	0.421	9	0.277
L	W228	21	0.305	1.890	0.125	0.952	5	0.335
L	W229	17	0.461	2.655	0.711	0.706	5	0.416
L	W230	24	0.178	1.756	0.425	0	8	0.215
L	W238	23	0.453	2.608	0.118	0.870	6	0.565
UA	W13	23	0.410	3.162	0.390	0.609	12	0.092
UA	W139	23	0.054	1.272	0.001	0	2	1.000
UA	W185	26	0.189	1.771	0.144	0.231	6	0.567
UA	W202	22	0.426	3.115	0.226	0.727	14	0.164
UA	W210	24	0.072	1.365	0.004	0.083	4	0.324
UA	W221	22	0.213	1.777	0.000	0.167	4	0.570
UA	W32	22	0.229	1.608	0.151	0	3	0.912
UA	W35	28	0.278	1.743	0.217	0.357	5	0.760
UA	W40	24	0.327	2.113	0.000	0.333	5	0.233
UA	W43	23	0.339	2.137	0.073	0.348	5	0.747
UA	W50	2	0.500	NA	NA	1.000	2	–
UA	W51	24	0.352	1.896	0.077	0.083	4	0.661
UA	W52	3	0.000	NA	NA	0	3	–
UA	W55	10	0.025	1.125	0.000	0	2	–
UA	W64	28	0.307	1.755	0.018	0	4	0.715
UA	W75	24	0.326	1.625	0.007	0	2	1.000
UA	W82	24	0.342	2.563	0.123	0.667	9	0.483
UA	W89	24	0.020	1.084	0.019	0	2	–
UA	W90	27	0.316	2.390	0.461	0.889	11	0.371
UA	W96	26	0.385	2.573	0.233	0.769	8	0.262

B: *burnt*; L: *logged*; UA: *uneven-aged*; N: total number of samples per plot; uH: unbiased haploid diversity; R: rarefied allelic richness ($N = 10$); PR: rarefied private allelic richness ($N = 10$); MAT ratio: 1- |(MAT1-1 – MAT1-2)/N| unique MLGs: unique multilocus genotypes at eight microsatellite loci; rBarD: multilocus linkage disequilibrium.

Data analyses

Fragment lengths were detected on a 3730xl DNA Analyzer (Life Technologies) and electropherograms were analysed with GENEMAPPER 3.7 (Life Technologies) using LIZ500 as size standard. Multilocus genotypes (MLGs) were defined for the fungus, based on eight microsatellite loci.

Identical MLGs in a population are the result of clonal propagation or sometimes by chance even by recombination especially in small populations. To estimate fungal clonality in each population with N number of samples, we divided the number of samples with clonal fungal MLGs (calculated by subtracting the number of unique MLGs from N) by the total number of samples (N). The estimator of clonality indicates percentage of total recurrent MLGs in a plot and not the frequency of a single genotype.

To infer recombination in the fungal symbiont within populations, we calculated the overall index of association (I_A, rBarD) as implemented in multilocus (Agapow and Burt 2001) (see Table 1).

Unbiased haploid diversity (uH) for the fungal symbiont in each region and plot was estimated using GenAlEx 6.41 (Peakall and Smouse 2006) (Table 1). Rarefaction of total genotypes (MLGs) in the populations was calculated in MOTHUR v1.22.2 (Schloss *et al.*, 2009) to produce estimates of allelic richness that are comparable among populations with different sample sizes (*burnt*, *logged* and *uneven-aged*; Fig. 3). Allelic richness (R) and private allelic richness (PR) were calculated for random subsamples equal to the smallest sample size among the three regions (188 samples in the *burnt* sites) and plots (10 samples) using ADZE 1.0 (Szpiech, Jakobsson and Rosenberg 2008).

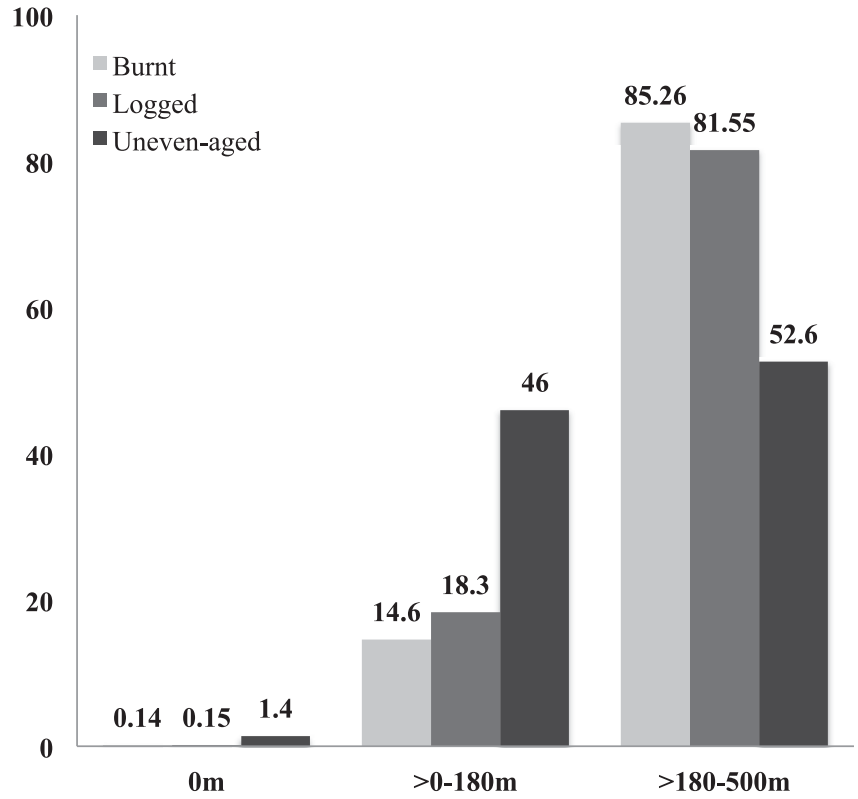

Figure 2. Histogram of pairwise average distance between MAT1-1 and MAT1-2 thalli in the three areas: light grey (*burnt* sites), black (*uneven-aged* sites) and grey (*logged* sites). Pairs at 0 m represent percentage of compatible thalli present on the same tree, whereas compatible pairs at > 0–180 m represent percent of thalli present in the vegetative dispersal range; compatible pairs from > 180–500 m represent isolated mating pairs. Numbers at the top of the bar represent percentage.

All populations were then analysed for MAT genotype ratio, and significant deviation from 1:1 ratio was calculated using the GraphPad QuickCalcs (http://www.graphpad.com/quickcalcs/ConfInterval1.cfm, August 2013, date last accessed).

Regression analysis was carried out using linear regression followed by ANOVA (Field 2007) to test for significant differences in uH, R, MAT ratio, clonality and number of unique MLGs among the three different regimes. The analysis was performed using the functions *lm* and *anova* in R (R Development Core Team 2013).

To partition molecular variance at different hierarchical levels, we performed AMOVA using 1-ha plots nested within areas at the plot level and among plots using the *F*-statistics approach in Arlequin version 3.11 (Excoffier, Laval and Schneider 2005). Plots with less than 10 samples (3 plots; W15 from *burnt*, W50 and W52 from *uneven-aged*) were excluded from the plot-level analysis, excluding 9 out of 887 thalli. Thirty-seven plots with 878 samples were included in the analyses with 12 plots from the *burnt* region, 9 plots from the *logged* region and 20 plots from the *uneven-aged* region.

The distribution of mating types in the three regions was plotted using gnuplot (Williams and Kelley 2011) through R (R Development Core Team 2013) (Fig. 1).

To correlate the differences in clonality and population structure among the three areas with the spatial distribution of compatible mating types, we calculated the average distance between compatible MATs, i.e. MAT1-1 and MAT1-2, for each area. In order to quantify the percentage of compatible MAT pairs connected by effective vegetative propagation, we grouped the compatible MAT pairs into three distance classes (Fig. 2): (i) 0 m, number of compatible MAT pairs lying on the same tree, (ii) > 0 up to 180 m, numbers of possible pairs connected by vegetative dispersal and (iii) > 180 m up to 500 m. The distance class up to 180 m represents the effective vegetative dispersal range of *L. pulmonaria* (Walser 2004; Öckinger, Niklasson and Nilsson 2005; Werth *et al.*, 2006a; Jüriado *et al.*, 2011; Werth, Cheenacharoen and Scheidegger 2014), i.e. dispersal resulting in the successful establishment of vegetative propagules. Population genetic studies have indicated that *L. pulmonaria* has the potential to disperse over greater distances, e.g. > 200 m (Werth *et al.*, 2000b), but establishment and photobiont limitation may hinder colonization at larger distances.

RESULTS

We analysed 887 samples in the pasture-woodland landscape in the Parc Jurassien Vaudois estimating genetic diversity (uH), allelic richness (R), index of association (I_A, rBarD), percent clonality, MAT allele frequency and spatial distribution in relation to disturbances in the past at both disturbance- and plot-level (Tables 1 and 2). At disturbance-level, uH, R and PR were lowest at the *burnt* sites (uH = 0.45 ± 0.11; R = 6.20 +/− 1.55; PR = 1.15 +/− 0.42), followed by *logged* (uH = 0.49 +/− 0.1; R = 8.22; PR = 2.57 +/− 1.15). Genetic diversity, allelic richness and private allelic richness were highest in the *uneven-aged* region (uH = 0.51 +/− 0.11; R = 9.09; PR = 2.76 +/− 1.02).

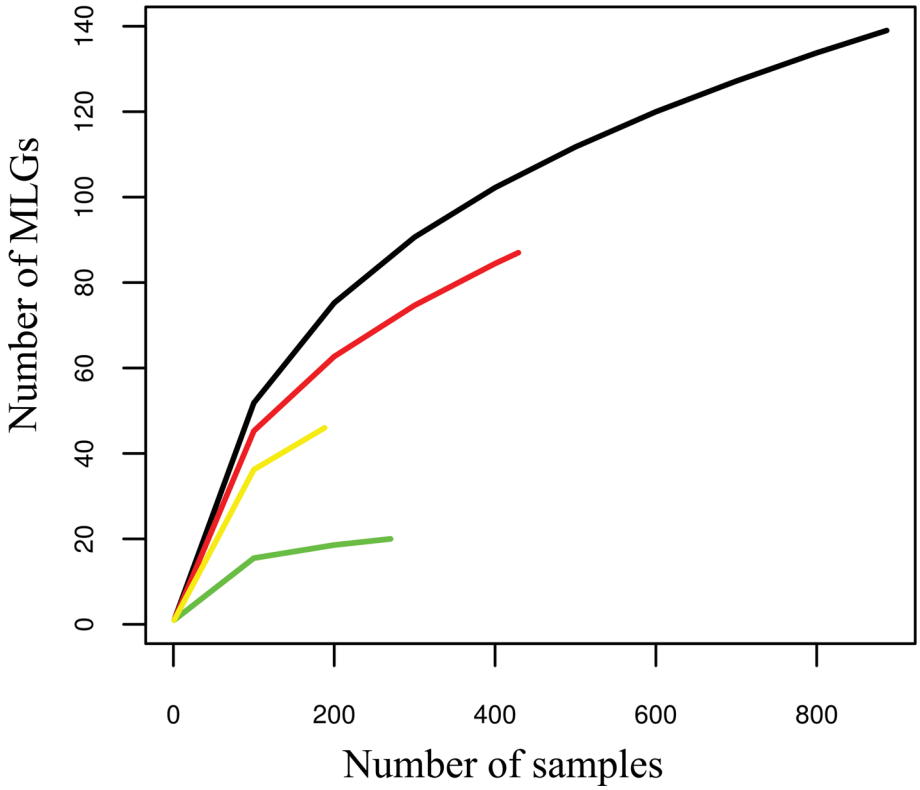

Figure 3. Rarefaction curves of total genotypes (MLGs) in the population: overall population of three areas (black), *burnt* site (green), *logged* site (yellow) and *uneven-aged* site (red).

At the *burnt* sites (excluding uneven-aged sites), 92.2% individuals were clones, i.e. 92.2% MLGs were present more than once in the area. For 270 thalli, 21 unique fungal MLGs combinations were recovered. The frequency of mating types was unbiased (44.4% MAT1-1 and 55.6% MAT1-2) in this area. Including the uneven-aged sites around the *burnt* sites (see Fig. 1A), the clonality was still the highest (~91%), whereas the MAT frequency was significantly biased towards MAT1-2.

The *logged* sites displayed an unbalanced frequency of MAT genes with 62% belonging to MAT1-1. The percent clonality in this area was the lowest among areas (75.53%), with 46 fungal unique MLGs from 188 thalli.

The *uneven-aged* sites had a highly skewed frequency of mating types (31% MAT1-1, 69% MAT1-2). The percent clonality in this area was 79.49%, with 88 fungal unique MLGs in 429 samples. Including the uneven-aged sites closer to the *burnt* and *logged* sites (see Fig. 1A) did not significantly alter clonality and MAT frequency estimates. The *uneven-aged* sites had the highest genetic diversity, highest allelic richness and lowest index of association among the three areas.

In addition to different genetic diversity estimates based on microsatellite data, we found profound differences in linkage disequilibrium estimates among the areas. In particular, the *burnt* sites displayed high linkage disequilibrium. Lower but significant linkage disequilibrium was found in the *logged* sites, while in the *uneven-aged* sites the loci were unlinked (Table 1).

We found that few MLGs were shared among disturbance regimes: one MLG between *burnt* and *logged* sites, eight MLGs between *logged* and *uneven-aged* sites, three MLGs between *burnt* and *uneven-aged* sites and one MLG among *burnt*, *logged* and *uneven-aged* sites. In nine cases, clonal MLGs displayed both MATs. This could be due to the fact that eight microsatellite markers are not able to fully resolve the genotypic diversity at the studied scale as shown by the MLGs rarefaction analysis (Fig. 3). More samples and more markers would be needed to reveal the full diversity of the fungus in the studied populations.

AMOVA analysis showed that only 46.78% of the genetic diversity of the fungus was partitioned within disturbance regimes, with 49.44% of the total genetic diversity residing within plots (Table 3).

The results of regression and ANOVA analyses showed that uH ($P = 0.0012$), R ($P = 0.0021$), MAT ratio ($P = 0.038$), clonality ($P = 0.0026$) and number of unique MLGs within plot ($P = 0.005$) were significantly related to disturbance category (Table 4 and Fig. 4). The uH and R were found to be strongly correlated. Plot sample size was not significantly associated with the disturbance regimes ($P = 0.27$).

Pairwise distance comparison (Fig. 2) revealed that only 0.14% of MAT1-1 and MAT1-2 pairs in the *burnt* sites were on the same tree, whereas 14.6% of the pairs were within the effective dispersal range of vegetative propagules and which were all located within the same plots. At the *logged* sites, 0.15% of the pairs within 500-m radius were on the same tree, whereas 18.3% within the effective vegetative dispersal range. At the *uneven-aged* sites, 1.4% of the pairs lying within 500-m radius were on the same tree, whereas 46% within the effective vegetative dispersal range.

DISCUSSION

This is the first study where effects of disturbance on genetic population structure and distribution pattern of mating types of a symbiotic fungus with mixed vegetative and sexual reproduction mode have been investigated.

Table 3. Hierarchical analysis of molecular variance (AMOVA), showing the mean statistics for eight fungal loci of *L. pulmonaria*.

AMOVA Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value (10 000 permutations)
Among areas	2	22.025	0.01890	3.78	0.007
Among plots within areas	38	200.264	0.23387	46.78	< 0.001
Within plots	846	209.117	0.24718	49.44	< 0.001
Total	886	431.406	0.49996		

Table 4. ANOVA table showing differences in uH, R, MAT ratio, clonality and number of unique MLGs among the three different regimes. See footnotes below for the definition of terms.

		d.f.	Sum Sq	Mean Sq	F value	Pr(>F)
uH	Disturbance	2	0.278	0.139	8.19	0.0012
	## Residuals	35	0.594	0.017	–	–
R	Disturbance	2	4.22	2.108	7.42	0.0021
	## Residuals	35	9.94	0.284	–	–
MAT ratio	Disturbance	2	0.68	0.339	3.6	0.038
	## Residuals	35	3.29	0.094	–	–
Clonatity	Disturbance	2	0.210	0.1051	7.09	0.0026
	## Residuals	35	0.519	0.0148	–	–
uMLGs	Disturbance	2	104.34	52.172	6.191	0.005
	## Residuals	35	294.52	8.415	–	–

uH: unbiased genetic diversity; R: rarefied allelic richness; MAT ratio: MAT1-1:MAT1-2; uMLGs: unique MLGs.

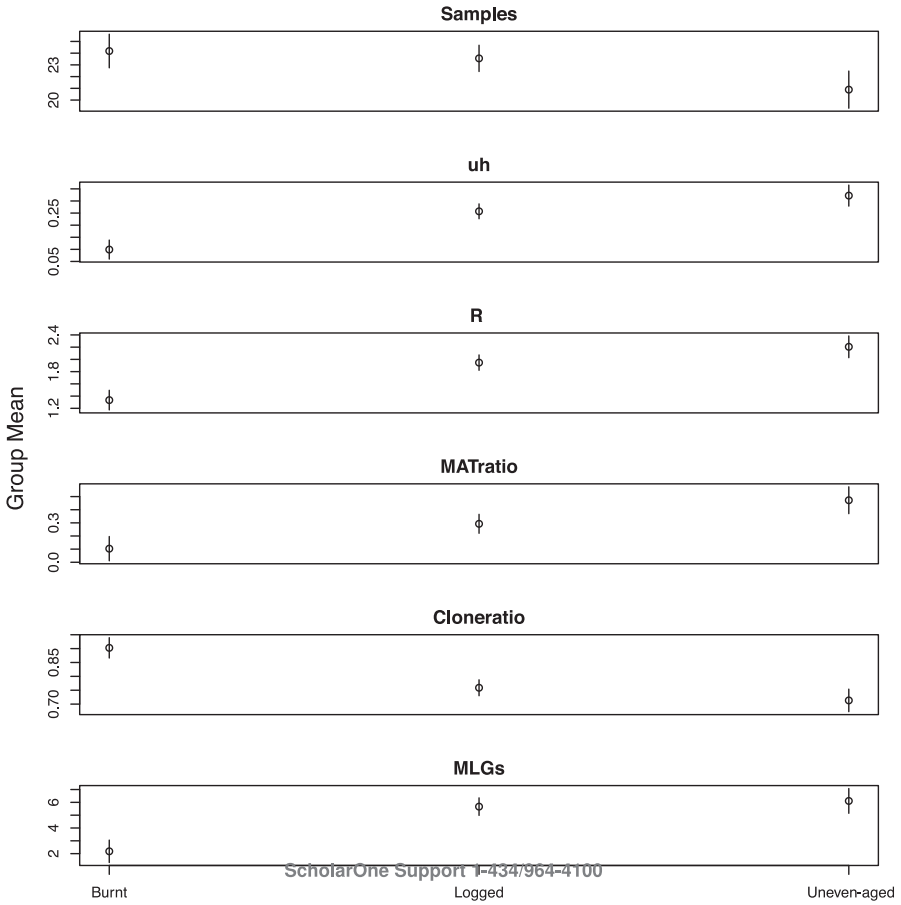

Figure 4. Results of ANOVA showing differences in uH, R, MAT ratio, clonality and number of unique MLGs among the three different regimes. The analyses showed that disturbance regimes to be significantly related to uH, R, MAT ratio, clonality and number of unique MLGs within plot.

Disturbance effect on genetic diversity

Previous studies suggested that genetic diversity of *L. pulmonaria* depends on the type of disturbance (Werth *et al.*, 2006a). Clonal propagation serves as a predominant mode of propagation during early stages of population re-establishment leading to rapid population spread and colonization of the area. Hence, one would expect clonal population structure and lower genetic diversity during initial stages of population establishment. Sexual reproduction usually occurs at later stages (~10–25 years) and introduces genetic variation in populations imparting higher adaptive potential to the populations (Zoller, Lutzoni and Scheidegger 1999). The genetic structure of populations in facultative sexual fungi is shaped by both clonal and sexual reproduction (Zoller, Lutzoni and Scheidegger 1999; Siah *et al.*, 2010). Previous studies have shown that the *burnt* area has been recolonized a few times only and has by now reached a rather large population size, given the severity of the disturbance event (Kalwij, Wagner and Scheidegger 2005; Werth 2005; Werth *et al.*, 2006a; Bolli *et al.*, 2008). However, in spite of a large population size, there is low genetic and allelic diversity and high index of association among alleles, likely due to founder effect, indicating a long-term clonal propagation in this area. Zoller, Lutzoni and Scheidegger (1999) reported genetic variability to be much higher in the populations where the fungus is reproducing sexually than in populations reproducing strictly asexually, regardless of the population size. This clearly indicates that even the small populations tend to maintain high genetic variability. Our results thus indicate that stand-replacing disturbances significantly influence the sexual potential of populations and thus have profound effects on the population structure and reproductive strategies of *L. pulmonaria*.

Logged and *uneven-aged* populations have higher genetic diversity as compared to the *burnt* sites in spite of the overall skewed representation of mating types. This could be attributed to more uniform distribution of mating types, which ensures availability of compatible partners for sexual reproduction. This is evident from the presence of higher number of compatible pairs on the same tree and within vegetative dispersal range in *logged* and *uneven-aged* as compared to *burnt* sites.

MAT distribution

In organisms with a dual reproductive mode, the trade-off between clonality and recombination can be highly influenced by the distribution and availability of compatible mating partners. A recent study has shown that clonality in some natural populations of *Magnaporthe oryzae* is related to the regional absence of compatible MAT types (Saleh *et al.*, 2012), although compatible strains are able to complete their sexual cycle *in vitro*. This indicates that the absence of compatible MAT thalli may inhibit sexual reproduction altogether, thus imposing clonality (Kema *et al.*, 1996; Siah *et al.*, 2010).

In the *burnt* sites, trees with both MAT thalli were absent, except for a few trees that survived the forest fire in 1871 (Bolli *et al.*, 2008). Therefore, the absence of recombination in this region might be attributed to spatial isolation of mating types. Analysis of MAT distribution showed a wide separation of compatible mating types in the *burnt* sites. Patchy distribution of mating types, coupled with low effective dispersal range may hinder sexual reproduction for several generations, i.e. for more than a century, and at a long term may compromise the adaptive potential of the populations (Muller 1963; Charlesworth 1990). Stand-replacing disturbances and subsequent founder effects in the *burnt* sites thus have led to almost complete fixation of a single mating type in different, disconnected patches.

The *uneven-aged* sites with high allelic richness and low to absent linkage disequilibrium showed even spatial distribution of both mating types, with small distances between compatible mating types. The pairwise distance matrix analysis showed that out of three areas, *uneven-aged* sites have the highest number of compatible pairs present on the same tree and also almost half of the pairs lie within the effective vegetative dispersal range of the species. Hence, in the *uneven-aged* sites the connectedness between mating types and the recombination potential are highest, followed by the *logged* sites and the *burnt* sites.

1:1 MAT ratio as an index of ongoing recombination

Vegetative dispersal leads to an unbalanced MAT ratio, as all offspring belong to the same MAT, whereas sexual reproduction produces offspring with 1:1 proportion of the two MAT. In heterothallic fungi, balanced MAT frequency has been widely accepted as a feature of recombining populations (Notteghem and Siluè 1992; Douhan, Murray and Dyer 2002; Viji and Uddin 2002; Zhan *et al.*, 2002; Linde *et al.*, 2003), provided frequency-dependent selection is operating on MAT genes (May *et al.*, 1999). This frequency-dependent selection has been thought to counteract the effect of genetic bottlenecks by increasing the frequency of rare mating types in populations (May *et al.*, 1999). However, there can be many other factors influencing the MAT ratio in natural populations that could lead to unbalanced MAT representation in spite of ongoing recombination. For example, (i) individuals in the population may be of different age and may not be sexually active together (Leslie and Klein 1996); (ii) during population establishment, clonal propagation leads to rapid, local spread of propagules, potentially biasing the MAT ratio depending on the mating type of the founders; (iii) one mating type may have selective advantage over the other; (iv) processes such as habitat or symbiotic partner selection may also influence MAT ratio in natural populations (establishment limitations, founder effect, etc.; Lachance, Nair and Lo 1993); (v) balancing selection on MAT might not be operating in clonal organisms with facultative sex, as the rate of sexual reproduction varies greatly across time and space (Lachance, Nair and Lo 1993). All these factors may together or independently influence the proportion of MAT types in populations. Therefore, in heterothallic, facultative sexual fungi, frequency-dependent selection might well not be operating to balance the frequency of MAT genes.

Leslie and Klein (1996) proposed that in natural populations, MAT frequency in facultative sexual organisms could depend on the ratio between sexual and asexual reproduction (see also Kaltz and Shykoff 1997). Thus in *L. pulmonaria*, where populations result from sexual as well as asexual reproduction, MAT ratio can be highly skewed despite the ongoing sexual reproduction. Our analysis supports the theory of Leslie and Klein (1996), as in the *uneven-aged* sites we found signatures of recombination, i.e. high genetic diversity and negative index of association, in spite of highly skewed MAT frequency. Moreover, in spite of a balanced MAT distribution, the *burnt* sites showed positive association among alleles, clearly indicating that the balanced representation of MAT genes in an area may not be a reliable indicator of ongoing recombination. This has also been shown for example in *Tuber melanosporum*, where significant difference in MAT frequency was found despite ongoing recombination (Linde and Selmes 2012).

Clonality: a gift and a curse

Clonal propagation preserves locally adapted genetic combinations and is therefore advantageous in a stable environment. Moreover, clonality is the preferred mode of reproduction during colonization for rapid population establishment with low energy and time expenditure. For lichen-forming fungi, an additional advantage of clonal propagation is represented by the co-dispersal of symbionts (Dal Grande *et al.*, 2012), avoiding the complications of finding a suitable partner and sustaining the well-adapted symbiosis, as long as the environment is stable. Clonal propagation is thus beneficial for rapid population establishment and for achieving a large population size.

Sexual propagation in facultative sexual fungi occurs under unfavorable environmental conditions or overcrowding, as the costs of sex are lower (Bell 1982; Agrawal 2006). The sexual spores are capable of avoiding harsh conditions until the environment improves and also serve as a means of long-distance dispersal (Barton and Charlesworth 1998). Sexual reproduction reshuffles the genotypic combinations of symbionts (horizontal transmission; Dal Grande *et al.*, 2012). The association with locally adapted algae may broaden the ecological range of the fungal symbiont, thus increasing its adaptive potential (Piercey-Normore and DePriest 2001; Rikkinen 2003; Yahr, Vilgalys and DePriest 2006; Peksa and Škaloud 2011; Dal Grande *et al.*, 2014a; Werth and Sork 2014). Therefore, in populations having only a single mating type, clonality is imposed, and such populations have lower adaptive potential than populations with both mating types.

Previous analyses showed that *L. pulmonaria* populations have high genetic diversity and allelic richness in spite of high clonal propagation (Zoller, Lutzoni and Scheidegger 1999). This clearly indicates that reproduction in undisturbed populations of *L. pulmonaria* is asexual with intermittent sexual reproduction, thereby keeping the genetic diversity high. However, the observed low allelic richness in *burnt* and *logged*, even more than 140 years after the disturbance events, is probably due to hindrance of sexual reproduction. Many cycles or generations of clonal propagation in a heterothallic population may lead to the loss of one mating type (because of a fitness disadvantage or by drift) and thus eventually compromise sexual reproduction (Zeyl 2009). Our analysis, revealing the spatial isolation of compatible MAT thalli, indicates that high clonality in this area is imposed and could be attributed to spatial isolation of the two mating types after two different types of disturbance. Clonality in this case is clearly imposed and is therefore a curse, compromising the adaptive potential of a population.

CONCLUSIONS

Our analysis shows that disturbance may influence the MAT distribution in the study area, thus affecting the potential for sexual reproduction and eventually the adaptive and evolutionary potential of populations. We showed that after disturbance, the distribution of mating types in the re-established populations greatly influences the population structure of heterothallic fungi.

Our analysis further suggests that 1:1 MAT ratio is not a reliable indicator of ongoing recombination in natural populations of facultatively sexual fungi as all individuals in a population may not enter the sexual cycle together making the overall MAT ratio in population completely independent from recombination events.

Conservation by thallus transplantation has been suggested as a management strategy to augment declining populations of threatened lichens (Scheidegger 1995; Scheidegger, Frey and Zoller 1995; Scheidegger and Werth 2009). Our results emphasize the need for amending the current management strategies for heterothallic lichen species, in particular by considering the restoration of both population size and connectivity for compatible mating types.

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