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Recombination and clonal propagation in different populations of the lichen *Lobaria pulmonaria*

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Propagation, dispersal, and establishment are fundamental population processes, and are critical stages in the life cycle of an organism. In symbiotic organisms such as lichens, consisting of a fungus and a population of photobionts, reproduction is a complex process. Although many lichens are able to reproduce both sexually and asexually, the extent of vegetative propagation within local populations is unknown. We used six polymorphic microsatellite loci to investigate whether recombination is common in natural populations, and to assess if and how clonal reproduction influences the spatial genetic structure within populations of the epiphytic lichen species *Lobaria pulmonaria*. High genetic diversity within all 12 investigated populations and evidence of recombination, from various tests, indicated that *L. pulmonaria* is a predominantly outcrossing species. Nevertheless, clonality occurred in all populations, but the presence of recurring multilocus genotypes influenced the spatial genetic structure only within low-density populations. This could be interpreted as indicative of genetic bottlenecks owing to increased habitat loss and disturbance. Consequently, for a predominantly outcrossing lichen species, exogenous factors might be substantially altering population processes and hence genetic structure.
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Introduction

Propagation, whether sexual or asexual, is a fundamental step in the life cycle of every single organism. Sexual reproduction enhances the number of different genotypes within populations and increases the probability of survival in a competitive and/or changing environment (Maynard Smith, 1978; Balloux *et al*, 2000), whereas asexual propagation, on the other hand, can be a successful evolutionary strategy for well-adapted genotypes in extreme but stable habitats (Murtagh *et al*, 2000). While our knowledge of mating systems and patterns in animals and plants is broad (eg, Jackson *et al*, 1996; Takebayashi and Morrell, 2001), we have only just started to investigate and understand them in symbiotic life forms such as lichens. Despite a few studies on sexual reproduction in lichens (Murtagh *et al*, 2000; Kroken and Taylor, 2001; Högberg *et al*, 2002), nothing is known about its importance as compared to clonal reproduction (through thallus fragments, soredia, or isidia) in natural populations. Therefore, the objectives of this study were (i) to investigate whether recombination, and hence outbreeding, is common in natural populations of lichens, and (ii) to determine how clonal reproduction influences the spatial genetic structure within populations.

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Recent developments of modern molecular markers allow the population genetic processes of lichens to be investigated. However, the few studies available so far suffer from various limitations (Walser *et al*, 2003). Some have focused on nuclear ribosomal DNA (nrDNA) amplified by fungus-specific primers (Bridge and Hawksworth, 1998) which, although sufficient for the analysis of major fungal lineages (Lutzoni *et al*, 2001), have the disadvantage of detecting rather low levels of intraspecific genetic variation (Bridge and Hawksworth, 1998; Zoller *et al*, 1999; Högberg *et al*, 2002). Greater variation has been detected with random amplified polymorphic DNA markers (RAPDs), allowing studies on breeding systems (Murtagh *et al*, 2000) and on the population structure of lichen-forming fungi (Dyer *et al*, 2001). In these RAPD studies, DNA has been isolated either from axenic fungal cultures or from thallus tissue free of photobionts (Crespo and Cubero, 1998; Heibel *et al*, 1999). As it is not possible to obtain algae-free material in lichen species that are asexual and for which the mycobiont is not cultivatable (Miao *et al*, 2001), anonymous DNA fingerprinting cannot provide specific results for lichenised fungi and is thus not universally applicable in mutualistic endosymbiotic systems. Highly variable, fungus-specific microsatellites would circumvent these problems. For *Lobaria pulmonaria*, several microsatellite loci have recently been developed (Walser *et al*, 2003), which suit the specific requirements of our study. Four of the 12 original primers used in this study were re-designed in order to shorten allele sizes, to avoid indels in the flanking regions, and to amplify loci in multiplex PCR.

Materials and methods

Species

The foliose epiphytic lichen *L. pulmonaria* (L.) Hoffm. is widely distributed in circumboreal regions of the Northern Hemisphere and in South Africa (Yoshimura, 1971, 1998a, b). While *L. pulmonaria* has a large range and is still common in North America (Brodo *et al*, 2001), it is considered endangered in many parts of Europe (Wirth *et al*, 1996). Although it is described as both a sexually and clonally reproducing species, apothecia (fruit bodies with sexual propagules) are formed infrequently and populations with only a limited number of fungal genotypes appear to be nonsexual (Zoller *et al*, 1999). This correlation between genotypic diversity and fertility has been interpreted as evidence that *L. pulmonaria* might be self-sterile (heterothallic; Zoller *et al*, 1999).

Sampling

In total, 562 thalli of *L. pulmonaria* were sampled from nine populations in British Columbia, Canada, and from three populations in Switzerland (Table 1). We considered a population as a patch of trees colonised by *L. pulmonaria*. Beyond the patch perimeter, the species did not occur locally. The samples from British Columbia were collected within three different zones (hypermari time, maritime, and intermontane; Goward, 1999) on a transect from the south coast of Vancouver Island to the interior of the province. These populations either occurred in old growth forests (eg, CS, PR, DC, CL, BL, or OC) or in dense undisturbed forests (AY, TO, and BC). The samples from Switzerland were collected in comparatively large, but sparse populations in the Pre-Alps and the Jura Mountains, where anthropogenic disturbance has been minimal. Across an entire population, one thallus per tree was randomly taken from selected nearest neighbor trees. The distance between the host trees was measured, the area of the local population was calculated (Table 2), and the number of samples with apothecia was determined.

Microsatellite analysis

DNA was isolated according to Walser *et al* (2003) from approximately 20 mg of cleaned marginal lobes per

Table 1 Locations and their geographic circumscriptions of the 12 studied populations of *Lobaria pulmonaria*

Location	*Code*	*Life zones/area*	*Altitude (m)*	*Latitude*	*Longitude*
Canada, British Columbia					
Ayum Creek, Vancouver Island	AY	Hypermaritime[a]	15	48°23′29″N	123°39′37″W
Chesterman Beach, Tofino	TO	Hypermaritime[a]	5	49°06′47″N	125°53′31″W
Cape Scott Provincial Park	CS	Hypermaritime[a]	5	50°40′27″N	128°16′32″W
Lakelse Lake Provincial Park	PR	Maritime[a]	105	54°22′54″N	128°31′52″W
Date Creek, Kispiox	DC	Maritime[a]	550	55°24′52″N	127°48′52″W
Clayton Falls, Bella Coola	BC	Maritime[a]	5	52°22′12″N	126°48′49″W
Carp Lake Provincial Park	CL	Intermontane[a]	860	54°52′08″N	123°15′39″W
Bowron Lake Provincial Park	BL	Intermontane[a]	910	53°15′19″N	121°21′03″W
Oregana Creek, Tumtum Lake	OC	Intermontane[a]	723	51°59′08″N	119°05′21″W
Switzerland					
Taaren Wald, Toggenburg	TW	Pre-Alps	1350	47°10′50″N	9°18′15″E
Murgtal, Walensee	MT	Pre-Alps	1280	47°03′52″N	9°11′51″E
Marchairuz, Jura Mountains	UZ	Jura Mountains	1200	46°29′57″N	6°10′21″E

[a]Lichen distribution classification system after Goward (1999).

Table 2 Number of samples (*N*), area in acres of the investigated populations of *Lobaria pulmonaria*, local population density (*N*/area), mean number of alleles (*A*), effective mean number of alleles (A_e), and percentage of different genotypes (*M*), and of fertile samples (samples with apothecia)

Population	N	*Area (acres)*	N/*Area*	A	A_e	M *(%)*	*Apothecia (%)*
AY	39	30	1.30	7.8	5.1	79	13
TO	47	80	0.59	7.2	4.3	62	19
CS	50	30	1.67	7.7	4.8	52	42
PR	50	10	5.00	8.7	4.4	90	42
DC	52	10	5.20	7.3	3.4	92	25
BC	50	10	5.00	10.5	5.7	90	10
CL	51	50	1.02	6.3	3.1	53	4
BL	49	10	4.90	7.2	3.3	90	2
OC	52	40	1.30	8.7	4.2	92	10
TW	52	380	0.14	7.8	4.4	52	8
MT	38	680	0.06	5.3	3.6	47	11
UZ	32	80	0.40	6.8	3.8	53	0
Mean±SE CA	48.9	30	2.89±0.68	7.9±0.4	4.3±0.3	78±6	19±5
Mean±SE CH	40.7	380	0.20±0.10	6.6±0.7	3.9±0.2	51±2	6±3
Mean±SE total	46.8	120	2.21±0.62	7.6±0.4	4.2±0.2	71±6	16±4

Means and standard errors are given for British Columbia in Canada (CA), for Switzerland (CH), and for the total sample set (total). For population codes, see Table 1.

thallus. Six microsatellite loci were studied (*LPu03*, *LPu09*, *LPu15*, *LPu16*, *LPu20*, and *LPu27*; Walser *et al*, 2003), and four of the original primers were re-designed in order to shorten allele sizes or to avoid indels in the flanking regions (Table 3). The loci were amplified in two multiplex polymerase chain reactions (PCRs) with each containing three primer pairs (*LPu03/LPu09/LPu15* and *LPu16/LPu20/LPu27*). Subsequent amplification and fragment analysis, using an automated capillary sequencer, followed protocols given by Walser *et al* (2003). As DNA was isolated from thallus material, the proportion of fungal DNA was unknown and varied among samples. In each 30-μl amplification reaction, 3 μl of genomic DNA extract was used. In addition to Walser *et al* (2003), the lengths of the 19 alleles at locus *LPu09* that were longer than 400 bp were partially checked with the size standard ROX-1000 (Applied Biosystems) and subsequently extrapolated. Samples with missing microsatellite data were excluded ($n = 3$).

Statistical analysis

Mean allele number (A) and mean effective number of alleles (A_e) were calculated following Hartl and Clark (1997).

Since the fungal partner of a lichen is haploid and has only one allele per locus, tests for recombination involve the examination of allelic associations between different loci (Maynard Smith *et al*, 1993). Panmixia would imply that alleles at all loci, except for physically proximate loci, were randomly associated with each other. However, even genes on different chromosomes may deviate from random association due to different factors such as genetic drift, selection, clonal reproduction, and/or nonrandom mating (Lewontin, 1988). We used a χ^2 test with P-values computed by Monte Carlo permutations with 10 000 replicates and adjusted with Hommel's method for multiple comparisons (Hommel, 1988) to test for the presence of a significant association between pairs of loci per population (with and without recurring genotypes). We also calculated the normalized measure for total disequilibrium described by Hedrick (1987) and the extended Fisher exact probability test under the null hypothesis of no association between the two tested loci per population as implemented in ARLEQUIN (Schneider *et al*, 2000).

We also used the standardized index of association (I_A) (Maynard Smith *et al*, 1993) to check for recombination or clonality within populations. From the distribution of mismatch values, an observed variance (V_D) was calculated and compared with the expected variance (V_e) under linkage equilibrium (Maynard Smith *et al*, 1993; Haubold *et al*, 1998; Haubold and Hudson, 2000). The null hypothesis ($I_A = 0$, ie, random association of alleles) was tested by 1000 permutations and P-values again adjusted with Hommel's (1988) method. These calculations were performed once with and once without recurring genotypes.

If a lichen-forming fungus reproduces clonally (through mitosis) or by homothallic mating, the entire genome is effectively linked since there is no segregation and no re-assortment of alleles. We therefore also calculated pairwise comparisons of microsatellite loci in addition to the multilocus associations described above. A lack of association between any pair of loci may be interpreted as being due to recombination, whereas linkage or homoplasy might cause deviations from expected genotypic frequencies under random mating in some pairs of loci.

The spatial genetic structure within populations was analyzed based on multilocus genotypes (Smouse and Peakall, 1999) using GENALEX (Peakall and Smouse, 2001) to investigate the effects of the dispersal of vegetative and sexual propagules. We performed a spatial autocorrelation test for distance classes with equal sample sizes and used the stepwise mutation model based on sum-of-squared-number-of-repeat differences between two genotypes (Slatkin, 1995) as a measure of genetic distance. Spatial autocorrelation analysis was carried out twice: once with and once without recurring genotypes. Deviation from the null hypothesis H_0: $r = 0$ was determined by 1000 permutations. In addition to this analysis, we also tested for correlation between geographic and genetic distances within populations of *L. pulmonaria* using Mantel tests with 1000 permutations calculated with the R package.

Spearman's rank correlation coefficients were used to estimate the associations between local population area (log-transformed) and percentage of different genotypes (M) or fertile samples, and between percentage of fertile samples and mean allele number (A) or mean effective number of alleles (A_e).

Table 3 Microsatellite loci of *Lobaria pulmonaria* included in this study

Locus	*Primer sequence (5′–3′)*	T_a (°C)	*Label*	*Allele size range* (bp)	*Number of alleles*
LPu03	F: GGCTGCAATGATGACTAGGA R: CACCCTGGTGTTGACTGCTA	55	NED	187–193	4
LPu09	F: AGCCTGGAGTTCAGACAACC R: ATCTTGTCTTGGCGCTTCTG	55	6FAM	181–640	34
LPu15	F: CAAAATACCTGAATGGATGT R: CTGAGGCAACACTCTACAGC	55	HEX	149–203	20
LPu16	F: GCCTGCCAAAGAATACAGCA **R: TGTCGATGTCTTGCCTGAAC**	57	HEX	186–260	23
LPu20	F: CTCTGCCGCTCGGGTTACAT **R: TGCCAGTACTGCAATGTGGT**	57	NED	161–241	32
LPu27	**F: GCTCATGCTCCACATCTGAC** **R: CATGCTCTTCCATTCACAGC**	57	6FAM	170–206	14

Primer sequence (F: forward; R: reverse), annealing temperature (T_a) in multiplex-PCR, dye used for 5′ fluorescent labelling of forward primer, range of allele sizes, and number of alleles detected. The six loci are described in Walser *et al* (2003), but four primers (given in bold) were re-designed in order to shorten allele sizes or to avoid indels in the flanking regions.

Results

Microsatellite diversity and frequency of apothecia

Genetic diversity within local populations of *L. pulmonaria*, based on the six microsatellite loci, was high. Among the 562 individuals analyzed, we found 405 different genotypes, 75% of which occurred only once. The number of alleles per locus and population ranged from one for locus *LPu27* in population DC to 22 for *LPu20* in population BC. The mean number of alleles per population (A) ranged from 5.3 to 10.5 and the mean effective number of alleles (A_e) ranged from 3.1 to 5.7 (Table 2). The percentages of different genotypes (M) were uniformly low within the Swiss populations ($\approx$50%), but varied from 52 to 92% among the populations from British Columbia (Table 2). The percentages of fertile specimens (bearing apothecia) ranged from 0% (population UZ) to a maximum of 42% (populations CS and PR; Table 2). There was a significant negative association (Spearman coefficient, $r_s = -0.766$, $P = 0.005$) between M and the local population area. In contrast, we found neither a significant correlation between the percentage of fertile samples (apothecia) and the mean allele number (A) or the mean effective number of alleles (A_e), nor between the percentage of fertile samples and local population area.

Recombination and clonality

All three measurements of linkage disequilibrium, the χ^2 test, Hedrick's normalized measure for total disequilibrium, and the extended Fisher exact probability test, yielded similar results. Only the results for the χ^2 test are presented (Table 4). About 5% of all pairwise comparisons in British Columbia and 7% in Switzerland rejected the null hypothesis of independence of loci. The results were not congruent across loci and suggested linkage disequilibrium between loci *LPu*16/*LPu*27 and *LPu*20/*LPu*27 (Table 4).

In the pairwise and the multilocus index of association I_A analysis (Maynard Smith *et al*, 1993) and when all samples were considered, the null hypothesis (ie, no association between two loci) was rejected in 32 and 69% of all pairwise comparisons in the populations of British Columbia and Switzerland, respectively (Table 5a). Excluding recurring genotypes, the null hypothesis was rejected in only 5% of the pairwise comparisons for British Columbia and in 4% for Switzerland (Table 5b). In accordance with the linkage disequilibrium analysis given above, a somewhat higher number of I_A-values was significantly different from zero for the loci *LPu*16, *LPu*20, and *LPu*27 (Table 5b).

Spatial genetic structure

The spatial autocorrelation analysis showed different genetic structures in the Swiss and British Columbia populations of *L. pulmonaria*. In the Swiss populations, positive autocorrelation values (r) were found in the first distance classes when all samples (with recurring genotypes) were used in the analysis (Figure 1). In population MT, a particularly high coefficient of $r > 0.6$ was detected in the shortest distance class. However, this positive autocorrelation in the Swiss populations disappeared when the recurring genotypes were excluded from the analysis (Figure 1). In contrast, we found no significant genetic structure in any of the nine *L. pulmonaria* populations from British Columbia, irrespective of whether recurring genotypes were taken into account or not. As an example, the respective correlograms for population TO are presented in Figure 1.

In accordance with these results, significant and positive Mantel correlations were only found between the geographic and genetic distances of Swiss populations when all samples were included (data not shown). However, correlations were not significant when the reduced data set without recurring genotypes was used.

Discussion

Recombination

Our data confirm that *L. pulmonaria*, like many other lichens, has the ability to disperse both by means of sexual and asexual propagules (Murtagh *et al*, 1999; Zoller *et al*, 1999; Kroken and Taylor, 2001). Although the occurrence of apothecia *per se* is evidence for sexual

Table 4 Linkage disequilibrium among pairs of loci within populations of *Lobaria pulmonaria*: χ^2-values with $P < 0.05$ (based on Monte Carlo permutations with Hommel's adjustment) are in bold

Population	*Locus*														
	LPu03					*LPu09*				*LPu15*			*LPu16*		*LPu20*
	LPu09	*LPu15*	*LPu16*	*LPu20*	*LPu27*	*LPu15*	*LPu16*	*LPu20*	*LPu27*	*LPu16*	*LPu20*	*LPu27*	*LPu20*	*LPu27*	*LPu27*
AY	31.13	13.51	16.58	30.73	0.88	54.92	53.61	117.90	24.14	46.44	86.41	7.82	143.59	28.35	38.98
TO	8.88	4.43	5.29	13.62	1.92	43.53	60.18	110.23	27.82	39.89	101.15	12.82	145.00	**70.08**	59.15
CS	30.33	14.38	15.89	20.40	3.30	35.10	50.31	91.49	8.78	64.93	107.85	**24.52**	132.46	**48.40**	**52.00**
PR	14.25	10.79	9.22	7.97	3.14	94.46	222.33	127.32	49.07	53.68	60.26	7.04	**162.98**	8.67	6.49
DC	12.16	2.37	17.07	2.91	NA	86.29	89.89	70.59	NA	64.67	52.60	NA	58.45	NA	NA
BC	13.12	37.56	35.36	32.74	1.45	118.83	147.33	248.39	5.29	165.72	141.70	5.27	314.52	19.68	36.96
CL	4.57	4.57	27.00	27.00	12.98	77.70	128.25	27.12	3.67	44.16	14.88	1.97	69.53	16.07	19.71
BL	15.04	6.27	6.93	3.86	0.13	64.18	95.34	57.08	13.98	66.89	84.68	21.49	130.45	44.00	44.00
OC	6.26	14.61	6.96	11.40	0.14	70.93	209.64	206.17	**83.47**	62.47	51.13	4.93	168.17	52.67	**96.00**
TW	14.05	46.13	27.65	43.88	19.15	39.02	45.55	52.26	18.21	**116.04**	87.38	62.45	99.28	**97.63**	65.93
MT	6.04	21.43	2.98	14.50	1.39	25.33	20.10	59.19	4.90	9.85	40.21	1.93	29.16	**10.27**	14.68
UZ	7.43	10.42	13.62	29.10	12.21	45.47	55.66	80.99	36.43	52.21	40.87	42.74	66.87	37.78	41.56

NA: not assessed since population DC was monomorphic at locus *LPu*27. For population codes see Table 1.

Table 5 Standardized indices of association (I_A) between pairs of loci and over all loci in populations of *Lobaria pulmonaria*

	Locus																
	LPu03					*LPu09*				*LPu15*			*LPu16*		*LPu20*		
	LPu09	*LPu15*	*LPu16*	*LPu20*	*LPu27*	*LPu15*	*LPu16*	*LPu20*	*LPu27*	*LPu16*	*LPu20*	*LPu27*	*LPu20*	*LPu27*	*LPu27*	*Over all loci*	
(a) With recurring genotypes																	
AY	0.082	0.113	0.065	0.056	−0.161	**0.143**	0.080	**0.141**	0.141	**0.160**	**0.136**	0.025	0.130	0.154	0.033	**0.382**	
TO	0.046	0.019	**0.060**	0.107	0.150	**0.178**	**0.406**	**0.438**	**0.183**	**0.254**	**0.275**	−0.012	0.615	0.377	**0.327**	**1.015**	
CS	0.041	−0.030	0.003	−0.019	−0.011	**0.266**	**0.296**	**0.363**	**0.138**	**0.362**	**0.505**	**0.327**	0.549	0.369	**0.348**	**1.215**	
PR	−0.003	0.058	−0.031	−0.046	0.047	0.042	0.059	−0.007	0.019	−0.021	0.013	−0.024	0.321	−0.061	−0.027	0.118	
DC	0.025	−0.095	−0.035	0.000	NA	−0.002	−0.029	−0.076	NA	−0.011	0.058	NA	0.039	NA	NA	−0.039	
BC	−0.014	0.097	−0.045	0.006	−0.102	0.035	0.001	−0.011	−0.152	0.056	−0.010	0.026	**0.092**	0.036	**0.088**	0.004	
CL	0.047	0.117	**0.111**	**0.252**	**0.778**	**0.302**	0.266	−0.001	−0.015	0.118	−0.031	0.059	**0.303**	0.104	**0.331**	**0.825**	
BL	0.040	0.034	−0.035	0.058	−0.035	0.067	−0.023	0.001	0.054	−0.004	0.066	0.089	**0.206**	0.073	0.156	**0.268**	
OC	0.006	0.078	−0.001	0.015	−0.047	0.010	**0.107**	0.008	**0.115**	−0.010	−0.065	−0.022	**0.154**	0.101	**0.226**	**0.223**	
TW	0.023	**0.242**	0.146	0.102	**0.347**	**0.372**	**0.367**	**0.287**	**0.134**	**0.459**	**0.345**	**0.236**	**0.423**	**0.214**	**0.240**	**1.266**	
MT	0.027	0.124	−0.099	0.117	−0.138	**0.322**	**0.349**	**0.362**	0.149	**0.199**	**0.375**	**0.199**	**0.377**	**0.423**	**0.245**	**0.947**	
UZ	−0.042	0.125	0.049	**0.193**	0.186	**0.186**	**0.318**	**0.490**	**0.247**	**0.184**	**0.168**	0.170	**0.357**	**0.387**	**0.211**	**1.043**	
(b) Without recurring genotypes																	
AY	0.118	0.026	0.007	0.002	−0.140	0.106	0.017	−0.009	0.098	0.043	0.011	−0.048	−0.023	0.094	−0.047	0.084	
TO	0.023	−0.048	−0.048	−0.033	−0.024	0.063	0.050	0.057	0.021	0.037	0.044	−0.025	**0.214**	**0.483**	**0.281**	**0.299**	
CS	0.098	0.052	0.015	−0.032	−0.026	0.076	−0.002	0.055	−0.060	0.067	0.130	0.143	0.035	**0.359**	**0.205**	**0.398**	
PR	0.009	0.036	−0.034	−0.072	0.058	0.010	0.040	−0.007	0.000	−0.050	−0.009	−0.025	**0.302**	−0.068	−0.022	0.057	
DC	0.004	−0.081	−0.002	0.039	NA	−0.007	−0.047	−0.081	NA	−0.020	0.029	NA	0.040	NA	NA	−0.039	
BC	−0.032	0.082	−0.049	−0.008	−0.092	0.050	−0.015	−0.045	−0.147	0.035	−0.039	−0.007	0.040	0.011	0.078	−0.059	
CL	−0.047	0.061	0.098	0.314	0.663	0.055	−0.030	−0.035	−0.077	−0.066	−0.050	−0.035	0.209	0.066	0.437	0.475	
BL	0.060	0.016	−0.046	0.035	−0.039	0.069	−0.043	−0.011	0.055	−0.034	0.052	0.093	0.180	0.076	0.157	0.215	
OC	−0.017	0.105	−0.017	0.010	−0.051	0.007	0.065	−0.002	0.108	−0.017	−0.078	−0.021	0.102	0.089	0.230	0.169	
TW	−0.018	0.209	0.017	0.034	0.152	0.065	0.146	−0.024	−0.016	**0.235**	−0.007	0.065	0.119	**0.271**	0.070	0.444	
MT	−0.075	0.025	−0.109	−0.060	−0.142	−0.050	0.076	−0.052	−0.077	0.011	−0.003	−0.004	−0.067	0.277	0.080	−0.046	
UZ	−0.212	0.006	−0.093	0.180	0.171	−0.065	0.024	−0.069	0.120	0.145	−0.103	0.154	0.080	0.065	−0.035	0.145	

Significance values ($P < 0.05$; based on 1000 permutations with Hommel's adjustment) are in bold. NA: not assessed owing to monomorphism at locus *LPu*27. For population codes see Table 1.

reproduction, it provides neither information pertaining to the effective establishment of sexually derived individuals nor any indication about the relative importance of sexual versus asexual reproduction under natural conditions. While sexual processes can occur between genetically identical cells or hyphae in self-fertile (homothallic) fungi, recombination in self-sterile (heterothallic) lichen species leads to segregation and reassortment of alleles at different loci. When recurring multilocus genotypes are included and the hypothesis of panmixia is rejected, this suggests nonrandom associations among samples within populations. Conversely, acceptance of the hypothesis, when recurring genotypes are not present, is usually regarded as evidence for random mating (Maynard Smith *et al*, 1993; Xu *et al*, 1999). Together, this would indicate that both recombination and clonal growth influence population genetic structure. In the present study, the index of association, as well as the conventional linkage disequilibrium tests, pointed to substantial rates of recombination in *L. pulmonaria* both in local populations from British Columbia and Switzerland. Therefore, we can conclude that *L. pulmonaria* is primarily outcrossing. A recent study of six *L. pulmonaria* populations in Switzerland (Zoller *et al*, 1999) came to the same conclusion and also showed that populations with higher genetic variation in the nuclear ribosomal DNA (nrDNA) region were more likely to form sexual propagules than less variable populations. In contrast, our data did not reveal any significant correlation between the percentage of fertile samples and the number of alleles or the percentage of different genotypes per population. For example, in population BL, 90% of all samples showed different genotypes, but only 2% were fertile, and in population UZ, despite the absence of apothecia, evidence for recombination based on the microsatellite markers was found. However, our samples were not necessarily representative of the whole populations as there may well be fertile thalli within the tree canopy that were not collected. A recent study showed that multiple colonization of a single tree may occur in dense populations of *L. pulmonaria* (Walser *et al*, 2003), and therefore the number of fertile samples within a local population might be underestimated because only one randomly taken thallus per tree was assessed for the presence of apothecia.

Generally, populations from Switzerland had lower densities of thalli than those from British Columbia and might well represent the remains of formerly larger, denser, and more fertile populations. Furthermore, the relation between fertile samples and measures of genetic diversity is influenced by the perennial life cycle of lichens and by the fact that apothecia in *L. pulmonaria* occur at later life stages (Ramstad and Hestmark, 2001; Denison, 2003). It is therefore possible that different age structures within populations studied led to the differences in the observed frequency of apothecia. However, the data given in Table 2 suggest that the percentage of fertile samples per population might be different between Switzerland and British Columbia or even between the zones within British Columbia. These

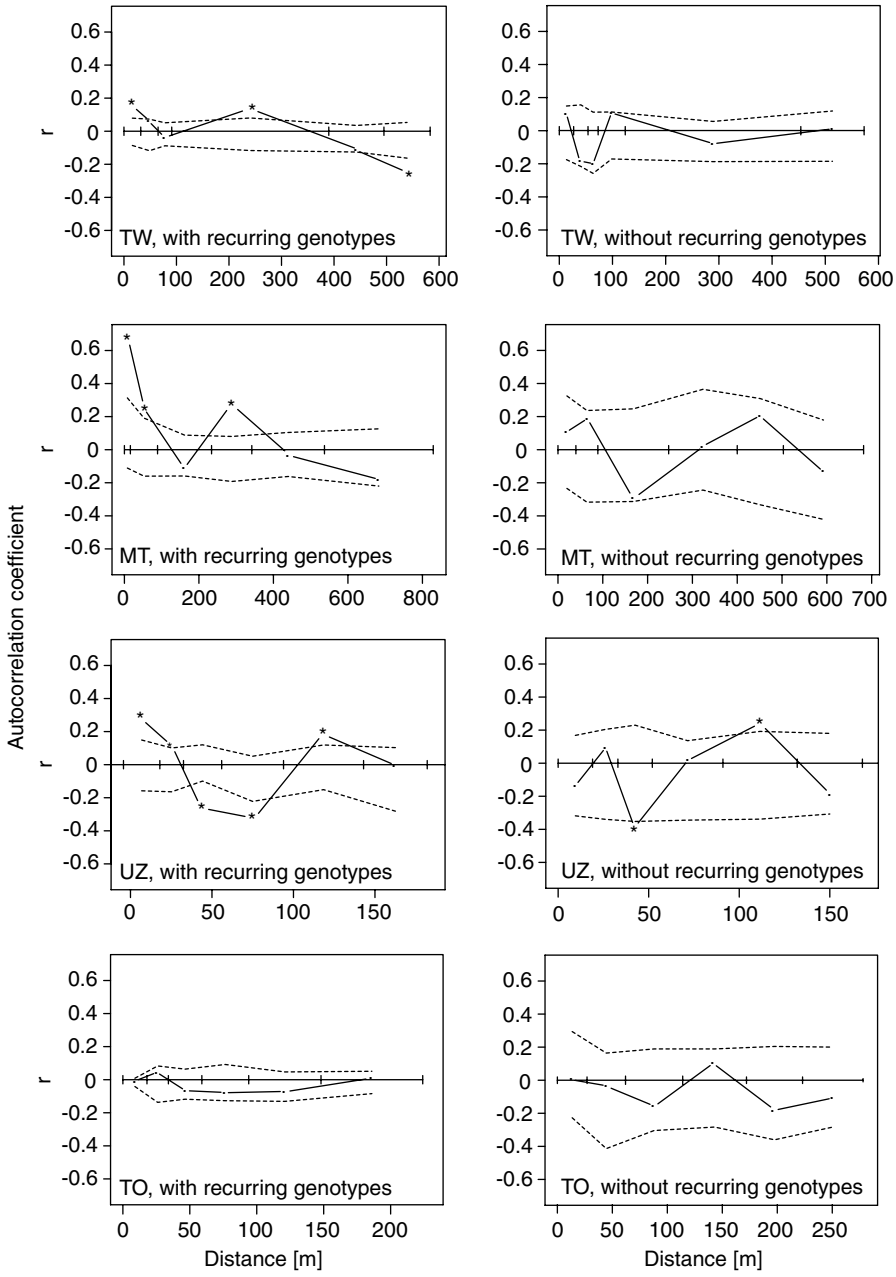

Figure 1 Examples of multilocus spatial autocorrelation in *Lobaria pulmonaria*; populations from Switzerland (TW, MT, and UZ) and British Columbia, Canada (TO). For each population, two correlograms with and without recurring genotypes are shown. The 95% confidence intervals are indicated by dotted lines, and values significantly different from zero (based on 1000 permutations) are presented by asterisk. Note the different scales of the *x*-axis owing to equal sample size within distance classes but, therefore, different scales per population.

findings suggest that the frequency and the development of apothecia are governed by exogenous factors, for example, oceanity, which is, however, confounded with altitude in our study.

Genetic structure

A recent study on *Letharia vulpina* showed that samples from North America had a higher genetic variation at eight single-nucleotide polymorphism sites (SNPs) than did samples from Sweden or Italy. It was thus concluded that *L. vulpina* should be outcrossing in North America, but effectively clonal in Europe (Högberg *et al*, 2002). Similarly, our data suggested that populations of *L. pulmonaria* from British Columbia also harbored a significantly larger number of genotypes than did Swiss populations (Table 2). This lower genotypic diversity in the Swiss populations may be attributed to historical bottlenecks (Zoller *et al*, 1999). In addition, we also found that populations from Vancouver Island, with the exception of population CL, had a lower genotypic diversity than did populations from the mainland. This could be due to the spatial isolation of these populations (Walser *et al*, unpublished data). Interestingly, the number of different genotypes showed a negative association with the local population area, which might

indicate that denser populations support a higher number of genotypes.

Furthermore, our data points to substantial clonal propagation at a small spatial scale within the three Swiss populations (Walser, in press). The different spatial genetic structures between populations on the two continents may be attributed to population density and history. For example, the populations from British Columbia occur in less disturbed forest ecosystems compared to the Swiss populations, which could have led to higher local densities, overlapping generations, and higher numbers of different genotypes even at a small spatial scale. Interestingly, Gu *et al* (2001) found that the spatial patterns of *L. pulmonaria* in Finland were more clumped in managed than in virgin forests. Although they did not examine spatial genetic structure, their results, together with ours, indicate that population history can influence the within-population spatial patterns.

Conclusions

Earlier studies on sexual reproduction in lichens examined the correlation between sexual reproduction and recombination (Zoller *et al*, 1999; Murtagh *et al*, 2000; Kroken and Taylor, 2001; Högberg *et al*, 2002). Our research has extended these studies by investigating clonal reproduction and its influence on spatial genetic structure within natural populations. The high genetic diversity within local populations and evidence of recombination from the association of alleles suggests that *L. pulmonaria* is a predominantly outcrossing species, despite the small number of apothecia that are present in a population. Nevertheless, clonality occurs in all investigated populations, with autocorrelation analysis indicating that only the genetic structure of the Swiss populations was influenced by clonal propagation. Certain population characteristics, namely density and levels of disturbance, are different between the Swiss populations and those from North America, and might explain the contrasting extent and prevalence of clonal propagation between continents. Such an intercontinental difference was also found in a recent study on another lichen species, with greater evidence for clonal spread in European populations compared to populations from North America (Högberg *et al*, 2002). This was interpreted as evidence for population genetic bottlenecks, and it was hypothesized that the European populations originated from their North American counterparts via long-distance dispersal of vegetative propagules. For *L. pulmonaria*, analysis of genetic differentiation between populations from British Columbia and Switzerland and a study on dispersal capacity of vegetative propagules do not support such a hypothesis (Walser *et al*, 2003; Walser *et al*, unpublished data). However, given that *L. pulmonaria* has suffered a significant decline in Europe within the last few decades (Hallingbäck and Martinsson, 1987) and that the Swiss populations are of lower density, our results could also be interpreted as indicative of genetic bottlenecks within Switzerland, owing to increased habitat loss and disturbance. Consequently, for predominantly outcrossing lichen species, exogenous factors, such as disturbance or fragmentation, might substantially alter population processes and hence genetic structure.

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