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Standard Paper

Local-scale panmixia in the lichenized fungus *Xanthoria parietina* contrasts with substantial genetic structure in its *Trebouxia* photobionts

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Abstract

Microsatellite markers can provide valuable information about gene flow and population history. We developed and tested new microsatellites for the nitrophilic lichenized fungus *Xanthoria parietina* and studied its genetic diversity and structure within the urban area of Munich, Bavaria. We compared its local genetic pattern with that of its photobiont partner *Trebouxia decolorans*, for which existing microsatellites were applied. For comparison, a reference site with clean air was included in the sampling. We found support for three genetic clusters in the fungus *X. parietina*, which occurred intermingled in collecting sites. There was a high degree of admixture within fungal populations and individuals, and analysis of molecular variance revealed a lack of population structure in the mycobiont. The *Trebouxia* photobiont, in contrast, exhibited structured populations which grouped into two to five genetic clusters, and individuals showed less admixture than in the mycobiont. This indicates that the two lichen partners differ in their ability to move around in the landscape. The microsatellite markers we report are polymorphic and are suitable for population genetic studies.

Key words: gene flow, genetic diversity, genetic marker, *Lecanoromycetes*, lichens, microsatellite markers, population differentiation, population genetics, simple sequence repeats (SSR), *Teloschistales*

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Introduction

Lichens are excellent indicators of air pollution (Hawksworth & Rose 1970; Chaparro *et al.* 2013; Seed *et al.* 2013). Many lichens are substantially affected by air pollution (Hawksworth & Rose 1970) and, depending on their pollution sensitivity with respect to compounds containing nitrogen, they can be classified as nitrophilic or nitrophobic (de Bakker 1989; van Dobben 1996; van Dobben & ter Braak 1998). Since about the 1950s, SO_2 air pollution has been a major concern, causing die-offs of lichens and rendering heavily polluted inner cities virtually lichen-free. Since the 1980s, populations of SO_2-sensitive lichens have been able to recover to some degree in Central Europe (Heibel *et al.* 1999; Vorbeck & Windisch 2001). For example, in surveys in 1968, there were no foliose lichens at all in four sites within the city of Munich. However, by 1973, a small number of species had managed to recolonize the area (Vorbeck & Windisch (2001) and references therein).

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Air pollution in the form of NO_2 has remained constantly high in cities in Central Europe and is affecting lichen communities adversely, leading to decreased abundances of acidophilic lichens and increases of nitrophilic species. NO_2 pollution levels were found to determine the species composition of epiphytic lichen communities on deciduous trees in the urban area of Munich in Germany, and all common species except one were nitrophilic (Sebald *et al.* 2022). In regions with depositions of NO_2 and ammonia, acidophilic lichens have been largely replaced by nitrophilic ones (de Bakker 1989; van Dobben & de Bakker 1996; van Herk 2001; Gadsdon *et al.* 2010). For example, the nitrophilic *Xanthoria parietina* (L.) Th. Fr. has become highly abundant across Central Europe in areas with high levels of air pollution by nitrogen-containing compounds (de Bakker 1989; Ruoss 1999; Gaio-Oliveira *et al.* 2001; van Herk 2001) and in areas with high pollution from alkaline dust (Marmor & Randlane 2007; Degtjarenko *et al.* 2016*a*).

The population genetic structure of rare lichens such as *Lobaria pulmonaria* (L.) Hoffm. has been investigated in much detail, and at different spatial scales (Werth *et al.* 2006, 2007; Dal Grande *et al.* 2012). Nitrophilic lichen species have received comparatively little attention with respect to population genetic analyses. *Xanthoria parietina* (*Lecanoromycetes*, *Teloschistales*) is one of the most widespread and common nitrophilic lichen

species in Central Europe. The species occurs on a wide range of natural and anthropogenic substrata and can be recognized by its characteristic golden yellow colour which originates from its cortical pigment, parietin. The species is distributed from the Caucasus to westernmost Europe and from North to South Africa, and it can also be found in America (Lindblom 1997; Fraser *et al.* 2016). This lichen once possibly had a more restricted distribution on nutrient-rich substrata but appears to have undergone a large population expansion and now dominates epiphytic lichen communities in many urban areas of Central Europe (Wirth *et al.* 2013; personal observations). For example, the lichen flora of four sites in the inner city of Munich were surveyed over the course of several decades (e.g. Vorbeck & Windisch 2001; Sebald *et al.* 2022). In 1968, there were no foliose lichens at all and *X. parietina* was also absent from all sites. *Xanthoria parietina* was found in only one of the surveyed sites in 1973, but by the year 2000 it had recolonized all four inner city sites (Vorbeck & Windisch (2001) and references therein). We were interested to see if we could see signs of this population expansion in genetic data. Two scenarios could be predicted: if sites are colonized by just a small number of individuals and subsequent immigration remains low, one might see distinct genetic clusters in recolonized sites. Alternatively, high migration rates may homogenize the genetic structure among sites.

Large thalli of *X. parietina* are almost always fertile, their central thalline areas being covered by apothecia, the fruiting bodies with which the fungal partner reproduces sexually through ascospores (Itten & Honegger 2010). The two main sexual breeding systems in the filamentous *Ascomycotina* are either heterothallism, in which partners of the opposite mating type are required for sex to occur, or homothallism, in which single isolates are self-fertile whilst often retaining the ability to outcross. The sexual breeding system is determined by mating-type (*MAT*) genes at one or more mating-type (*MAT*) loci in the genome (Dyer *et al.* 2016). *Xanthoria parietina* contains a *MAT* locus characteristic of heterothallic species, with only a single *MAT1-2* gene detected rather than a combination of both *MAT1-1* and *MAT1-2* genes seen commonly in homothallic species (Pizarro *et al.* 2019). However, Honegger *et al.* (2004*b*) found that the progeny of *X. parietina* from a single ascus were genetically homogeneous in terms of phenotypic features and DNA fingerprint patterns. Scherrer *et al.* (2005) also reported that all ascospore progeny contained a *MAT1-2* gene at the *MAT* locus, rather than segregation of *MAT* genes seen in other heterothallic *Xanthoria* species. These results indicated that the species is able to reproduce via selfing, and Pizarro *et al.* (2019) speculated that *X. parietina* might exhibit a novel form of homothallism known as unisexuality (Heitman *et al.* 2014).

While *X. parietina* does not produce specific vegetative propagules, there are ways for the species to disperse both partners vegetatively. Lichenivorous invertebrates feed on the apothecia and thalli of the lichen, and their faecal pellets may contain viable hyphae, ascospores, and *Trebouxia* photobiont cells (Meier *et al.* 2002; Boch *et al.* 2011). Birds may passively carry lichen-containing invertebrate faecal pellets with them and disperse the lichens over long distances (Meier *et al.* 2002).

Xanthoria parietina forms a lichen symbiosis with *Trebouxia*, a genus of green-algal photobionts. Saxicolous or epilithic thalli of *X. parietina* associate with the green alga *Trebouxia arboricola,* while epiphytic thalli form lichen symbioses with *T. decolorans* (Nyati *et al.* 2013, 2014). It is assumed that *Trebouxia* species are exclusively asexual (Friedl & Büdel 2008) and they are unable to reproduce sexually while in lichen symbiosis. *Trebouxia* algae are able to live without their fungal partner; however, free-living *Trebouxia* populations are small and do not seem to be persistent (Bubrick *et al.* 1984; Mukhtar *et al.* 1994; Sanders 2005; Wornik & Grube 2010). Honegger *et al.* (2004*a*) investigated the presence of genetic diversity in worldwide samples of *X. parietina* using RAPD-PCR fingerprinting of axenically cultured ascospore discharge. They found evidence for two main groups based on phylogenetic analysis, namely an Iberian branch from south-west Europe versus all other global isolates that formed a separate grouping. In a subsequent study, Itten & Honegger (2010) found low but significant regional genetic differentiation and high genetic diversity within five western and Central European populations of *X. parietina*, again based on RAPD-PCR fingerprinting of axenically cultured specimens. The *Trebouxia* photobionts of these *X. parietina* lichens also exhibited high genetic variability (Nyati *et al.* 2013). It remains unknown whether populations of *X. parietina* are also structured if smaller geographical areas are investigated, and how much genetic diversity is found in urban areas and along pollution gradients.

We developed new microsatellites for *X. parietina* taking advantage of this fungal species' publicly available whole genome sequence to study genetic diversity and structure at small spatial scales. Moreover, we used existing microsatellites developed for *T. decolorans* (Dal Grande *et al.* 2013, 2014*a*) to investigate the genetic structure of the photobiont in order to compare the genetic structure among the symbionts. We carried out our investigations in the urban area of Munich, Bavaria, and included a site with clean air in southern Bavaria for comparison. Our main research question was whether there is significant genetic differentiation in populations of *X. parietina* and its green-algal photobiont *T. decolorans* at small spatial scales. We discuss the results in the light of air pollution levels and the history of *X. parietina* populations in Munich.

Methods

Sample collection

Xanthoria parietina was collected between May and July 2018 from seven urban sampling sites in the city of Munich (Botanical Garden, Flaucher, Freimann, Landshuter Allee, Lothstraße, Pasing and Stachus), representing parks and other green spaces within the city and in one remote site 110 km away with relatively clean air (< 1.71 µg m^{-3} NO_2, 3.35 µg/m^3 SO_2 according to Sebald *et al.* (2022)) located in Hinterstein (Oberallgäu) (Fig. 1; see Table 1 for latitude and longitude details). From each location, we collected *X. parietina* samples from the trunk of six deciduous trees which were at least 10 m apart (28 *Acer* sp., 10 *Fraxinus excelsior*, 6 *Fagus sylvatica*, 2 *Betula* sp., 1 *Malus* sp., 1 *Prunus* sp., 1 *Platanus* × *hispanica*, 1 *Aesculus hippocastaneum*). From each tree, at least two thalli of *X. parietina* were collected; in total 112 samples were included in the analysis.

Air pollution analyses

For comparative purposes, air pollution data were generated with NO_2/SO_2 diffusion measuring tubes and analyzed by ion chromatography at Gradko Environmental, as described in Sebald *et al.* (2022). In each site except Lothstraße, two devices were analyzed; in Lothstraße, one device went missing. Diffusion tubes were

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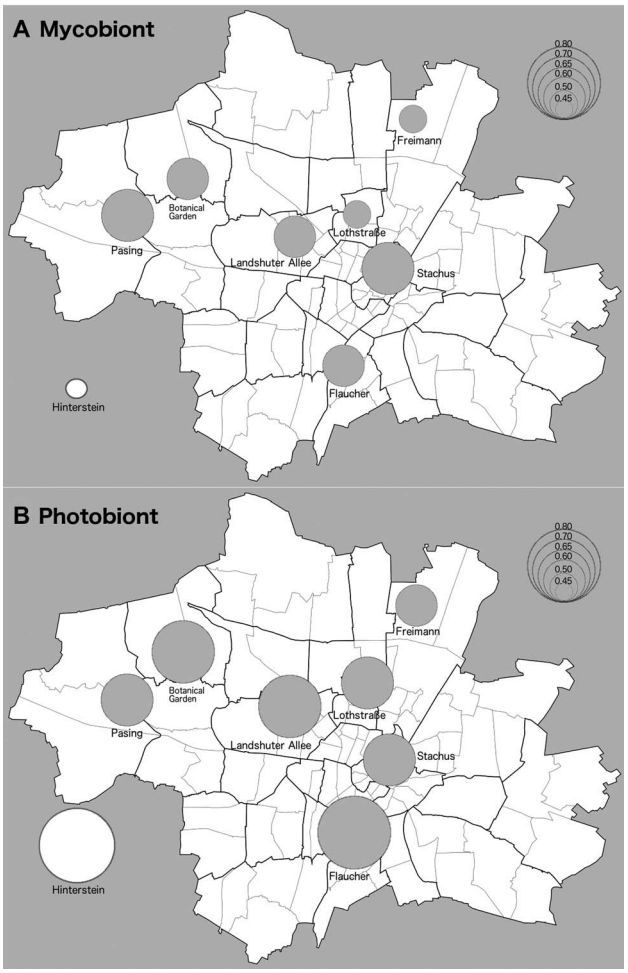

Fig. 1. Map of Nei's gene diversity in populations of *Xanthoria parietina* and its photobiont. Size of circles is proportional to gene diversity at sampling sites as per key in the top right-hand side of figures. Seven of the sampling sites were located in the city of Munich (Bavaria, Germany). The inset represents a geographically distant population located in an area with clean air (Hinterstein valley, Oberallgäu). A, mycobiont. B, photobiont. Base map by Maximilian Dörrbecker, shared via Wikimedia Commons (https://de.m.wikipedia.org/wiki/Datei:M%C3%BCnchen_-_Stadtbezirke_und_Stadtbezirksteile_(Karte).svg: accessed 29 March 2021).

exposed on average for 359 h (range 338.5–383.25). NO_2/SO_2 data from our sampling sites have been reported by Sebald *et al.* (2022).

Laboratory work

DNA extraction. Total genomic DNA was extracted from small terminal lobes of the lichen thalli, to avoid extracting DNA of multiple individuals intermingled since the lichens tended to grow on top of each other. We extracted the DNA of *X. parietina* following the protocol from Werth *et al.* (2016). DNA samples were stored at −20 °C until processing.

Primer design, PCR and DNA sequencing. We developed and tested new microsatellites for the mycobiont *X. parietina* based on this species' genome (sequenced based on an axenic fungal culture) available through DOE-JGI Joint Genome Institute (https://mycocosm.jgi.doe.gov/Xanpa2/Xanpa2.home.html) (Table 2). To find microsatellites and design primers, the genomic data was analyzed with the software MSATCOMMANDER v. 0.8.2 (Faircloth 2008) which implemented the Primer3 software (Rozen & Skaletsky 2000). Microsatellite markers for the photobiont, *Trebouxia decolorans*, were based on an existing study where the microsatellites were developed on the basis of axenic algal cultures (Dal Grande *et al.* 2013).

For the microsatellite analysis, 10 primer pairs for the mycobiont *X. parietina* (Xpa), each located on a different genomic contig and thus presumably unlinked, were used in the PCR. Moreover, 10 loci were included for the photobiont *T. decolorans*. Primers were ordered from Microsynth, Balgach, Switzerland. After optimizing primer concentrations for the photobiont (primer names starting with 'Tde') with 1:5 diluted DNA samples, the most efficient mix was chosen (Table 3). Primer concentrations had to be optimized because we found that some microsatellites initially produced too strong signals and others were very low. After these preliminary tests, we did not need to dilute the DNA in order to increase the quality of the results.

For the PCR, 4.5 µl PCR-mix (consisting of 0.5 µl of the respective primer mix, 2.5 µl 2× Qiagen Type-IT Master Mix and 1.5 µl nuclease-free water) and 0.5 µl of total genomic DNA (30–70 ng μl^{-1}) was added to 96-V-Thin-Wall-MT-Plates. Amplification was performed in a T100 Thermal Cycler (Bio-Rad, Feldkirchen) with an initial denaturation step at 95 °C

Table 1. Data for eight populations of *Xanthoria parietina* sampled in southern Bavaria, Germany. The table provides Nei's gene diversity *H* of the mycobiont and photobiont (higher values of *H* correspond to greater diversity) as well as the geographical location of the sampling sites (decimal degrees, map datum WGS84). NO_2 data represent averages of two measurements and were taken from Sebald *et al.* (2022).

Population	Region	$H_{Mycob.}$	$H_{Photob.}$	Latitude	Longitude	NO_2 (µg m^{-3})
Hinterstein	Allgäu	0.44	0.82	47.4722	10.4117	< 1.71
Lothstraße	Munich Centre	0.50	0.66	48.1540	11.5544	27.19
Freimann	Munich North	0.52	0.59	48.1904	11.6129	14.38
Flaucher	Munich South	0.60	0.78	48.1082	11.5603	13.66
Landshuter Allee	Munich South	0.62	0.75	48.1454	11.5337	42.87
Stachus	Munich South	0.65	0.63	48.1405	11.5686	14.96
Botanic Garden	Munich West	0.62	0.73	48.1644	11.4981	2.21
Pasing	Munich West	0.64	0.65	48.1521	11.4592	5.35

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Table 2. Newly developed microsatellites for the lichenized fungus *Xanthoria parietina*. The table shows the forward and reverse primer names and sequences (5′–3′), microsatellite motif, number of repeats (Rep), the genomic scaffold on which the locus is located, and alignment coordinates on the scaffold (start_bp, end_bp), as well as the product size in the sample used for genome sequencing, the number of alleles (N_A) and the percentage of missing data (% Missing). Scaffold names and alignment coordinates refer to the Xanpa1 genome assembly of *Xanthoria parietina* (DOE-JGI Joint Genome Institute). Note that locus Xpa15 did not amplify in our final PCRs and was therefore excluded.

Locus	Forward primer	Reverse primer	Motif	Rep	Scaffold	start_bp	end_bp	Size	N_A	% Missing
XPa03	TTGACCGAGGATTATACCAC	AGTTAATCAGTCTGACCGAG	AAGAGC	6	scaffold_3	1459330	1459366	442	7	33.3
XPa04	TTCTATGAATCGTTGCAAGG	GGGATTGAGAGGATGGTATG	AGG	8	scaffold_4	670639	670663	399	4	0.9
XPa05	TTTGATGGCTTACGTGAAAC	ATAGGAATGGGTAGAGCAAC	AGC	15	scaffold_5	645483	645528	419	12	4.6
XPa06	CACCACCAAATACTCATTCC	TCGTAATCTCTCAACCAGTC	AC	10	scaffold_6	262327	262347	432	6	0.9
XPa07	AGGAATATGCTCACTTCTCG	AAGGGTGGGCTTATTAGATC	AAG	12	scaffold_7	1541472	1541508	154	12	35.2
XPa10	CGCAGATTGAACGACTATTC	AATCTCATGTCGCCAAATTG	ACCAG	10	scaffold_10	486659	486709	271	9	5.6
XPa12	ACAGTAATCCGTTCTCTGAG	GAACGAGGGAAAGTTGATTC	ATCC	6	scaffold_12	741299	741323	417	9	54.6
XPa14	GCAGATTTCCCTTCTCTTTG	GACCGAAGTAATAACGATGG	AGC	9	scaffold_14	918665	918692	128	3	19.4
XPa15 *	TATCCGTCTCTGGCTTTAAG	CTTGAAAGAAAGAACCGAGG	GT	9	scaffold_15	177799	177835	299	0	100
XPa16	TAGCAAATGTGTGAATCAGC	CTGTTTCCAATGATTCCACC	AC	10	scaffold_16	50013	50033	122	8	0.9

*No successful amplifications

for 3 min, followed by 35 cycles of 1 min at 95 °C, 10 s at 95 °C, 60 s at 55 °C and 30 s at 72 °C, with a final elongation at 72 °C for 5 min.

A total of 39 µl of ddH_2O were added to the PCR product, and 1 µl of the dilution was taken and combined with 8.5 µl Hi-Di Formamide and 0.5 µl LIZ 500 (Applied Biosystems), an internal size standard. Fragment length analysis was performed at the Genetics Sequencing Service located in the Faculty of Biology at the Ludwig Maximilian University in Munich, with dye set G5 on an ABI 3730 capillary sequencer (Applied Biosystems). Microsatellite genotyping was performed with Geneious v. 6.1 (https://www.geneious.com) with which fragments were sized with the aid of the internal size standard LIZ500.

During the data analyses, we noticed that one of the primer pairs for *T. decolorans* (Tde-3a-FAM) had high amounts of missing data and it was therefore excluded from further analyses. In addition, locus Xpa-15 from the mycobiont did not yield products and was excluded. Thus, the total number of markers was nine for the *Trebouxia* photobiont and nine for the *Xanthoria parietina* mycobiont. The final dataset contained data for 102 samples of *T. decolorans* and 112 of *X. parietina*.

Data analysis

Nei's gene diversity was calculated in Arlequin v. 3.5 (Excoffier & Lischer 2010). Univariate linear regression was used to test for a relationship between gene diversity of the mycobiont and photobiont and NO_2 pollution levels. Principal coordinates analysis (PCoA) and multilocus match analysis were performed with GenAlEx v. 6.5 (Peakall & Smouse 2012) in order to see how different the microsatellite genotypes were from each other, and if there was a grouping of similar genotypes by population. An analysis of molecular variance (AMOVA) was performed with GenAlEx v. 6.5 to test if there was population structure at different hierarchical scales. For this purpose, the populations were divided into five regions (West, Central, North, South Munich, and Allgäu; see Fig. 1, Table 1).

Population structure was analyzed with STRUCTURE v. 2.3.4 (Pritchard *et al.* 2000) using an admixture model without prior population information, where allele frequencies were correlated among populations. Different values of F_{ST} were assumed for different subpopulations (mean prior: 0.01). The allele frequency parameter was constant (1.0). In each run, the parameter for the degree of admixture, α, was inferred, starting with an initial value of 1.0 (maximum 10). Using these settings, STRUCTURE was run 20 times for K = 1 to K = 8 (number of populations) with a burn-in period of 5000 and 50000 MCMC reps after burn-in. To compute the most likely number of clusters, the ΔK statistic was used as implemented in STRUCTURE HARVESTER, web v. 0.6.94 (Earl & von Holdt 2012). The bar plots were created with the CLUMPAK web software (http://clumpak.tau.ac.il; accessed 29 March 2021) (Kopelman *et al.* 2015).

Results

Mycobiont

Nei's gene diversity of the *Xanthoria parietina* mycobiont ranged from 0.44 to 0.65 (Table 1, Fig. 1A). Stachus and Pasing were the populations with the highest mycobiont gene diversity (average 0.65). The control area with high air quality in the Hinterstein

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Table 3. Primer mixes for the lichenized fungus *Xanthoria parietina* (Xpa) and its photobiont *Trebouxia decolorans* (Tde). NED, VIC, FAM and PET refer to the fluorescent dye labels located at the 5′ end of each reverse primer. Each primer stock solution was 10 μM. Note that two primers included in the mix marked with an asterisk (*) did not amplify successfully.

Photobiont		Mycobiont	
Locus	Volume (Concentration)	Locus	Volume (Concentration)
Tde02a-F, Tde02a-R (NED)	2 μl (0.110 μM)	Xpa03a-F, Xpa03a-R (VIC)	2 μl (0.108 μM)
Tde03a-F, Tde03a-R (FAM) *	2 μl (0.110 μM)	Xpa04-F, Xpa04-R (FAM)	2 μl (0.108 μM)
Tde07a-F, Tde07a-R (VIC)	2 μl (0.110 μM)	Xpa05-F, Xpa05-R (NED)	2 μl (0.108 μM)
Tde09-F, Tde09-R (PET)	2 μl (0.110 μM)	Xpa06a-F, Xpa06a-R (VIC)	1.5 μl (0.810 μM)
Tde10-F, Tde10-R (FAM)	1.5 μl (0.083 μM)	Xpa07-F, Xpa07-R (PET)	1 μl (0.054 μM)
Tde13-F, Tde13-R (FAM)	1.5 μl (0.083 μM)	Xpa10-F, Xpa10-R (PET)	2 μl (0.108 μM)
Tde15-F, Tde15-R (NED)	2 μl (0.110 μM)	Xpa12-F, Xpa12-R (PET)	2 μl (0.108 μM)
Tde16-F, Tde16-R (PET)	1 μl (0.055 μM)	Xpa14-F, Xpa14-R (FAM)	0.5 μl (0.027 μM)
Tde19-F, Tde19-R (NED)	1 μl (0.055 μM)	Xpa15-F, Xpa15-R (FAM) *	2 μl (0.108 μM)
Tde20-F, Tde20-R (VIC)	4 μl (0.221 μM)	Xpa16-F, Xpa16-R (NED)	0.5 μl (0.027 μM)
TE buffer 1×	162 μl	TE buffer 1×	169 μl
Total	181 μl	Total	184.5 μl

valley had the lowest gene diversity in the mycobiont (0.44). There was no statistically significant relationship between mycobiont gene diversity and NO_2 pollution levels as assessed by a univariate linear regression model.

PCoA was performed to visualize similarities among mycobiont genotypes (Fig. 2A). The first PCoA axis explained 24.2% of the variance in the data, the second axis 18.22%. Some genotypes clustered together, such as those in the lower right corner of the PCoA plot. These points, however, did not consist of samples originating from the same population, as one would expect if these contained similar genotypes. The samples from different populations were found more or less scattered across the plot, indicating an absence of population structure.

There were four repeated multilocus genotypes in the mycobiont: A, B, C and D (see Supplementary Material Table S1, available online). The remaining samples had different genotypes. Repeated genotypes A and C consisted of samples from locations that were geographically close (Stachus, Lothstraße and Landshuter Allee). The samples from the repeated genotypes B and D, on the other hand, came from locations geographically further away from each other.

There was no population structure in the mycobiont, with AMOVA resulting in no significant genetic differentiation between regions ($\phi_{RT} = 0.028$, $P = 0.169$) or populations ($\phi_{PT} = 0.032$, $P = 0.094$). Nor was any structure found between regions or populations when changing the grouping of populations to consider Landshuter Allee as a 'central' population (data not shown). Nevertheless, three genetic clusters were inferred by Bayesian analysis of population structure with the delta K method (Supplementary Material Figs S1 & S2, available online), but none of these mycobiont genetic clusters showed any geographical tendencies (Fig. 3A). Instead, there was high admixture in the studied individuals, with most individuals having ancestry in more than one genetic cluster (Fig. 3A). Sampling sites in the inner city area (e.g. Stachus, Lothstraße, Landsberger Allee) that were situated within the lichen desert zone in the late 1960s (Vorbeck & Windisch 2001) were not populated by a single genetic cluster or genetically similar individuals, but by several genetic clusters and genetically admixed individuals. Moreover, genetic distance-based population trees showed no clustering of nearby populations (Supplementary Material Fig. S7, available online). For example, the spatially proximate Pasing and Botanical Garden populations did not group together.

Photobiont

Nei's gene diversity of the photobiont *T. decolorans* ranged between 0.59–0.82 (Table 1, Fig. 1B). Hinterstein had by far the highest gene diversity in the photobiont (0.82). The urban sites in Munich Botanic Garden, Flaucher and Landshuter Allee also showed high photobiont gene diversity with values > 0.7. The photobiont population in Freimann had the lowest gene diversity (0.59). We found no significant relationship between gene diversity and air pollution levels in univariate linear regressions (Table 1).

There was substantially more genetic structure in the photobiont of *X. parietina* than in its mycobiont. In PCoA, the first axis explained 34.7% of the variance in the data, the second axis 24.4%. The Hinterstein, Stachus and Flaucher populations showed some distinct genotypes, while the samples from the remaining Munich populations largely overlapped (Fig. 2B).

Altogether 10 repeated multilocus genotypes were found (A–J; Supplementary Material Table S2, available online). There was no tendency for recurrent photobiont genotypes to be found only in nearby sites; they appeared to be rather widespread within Munich.

In the photobiont, there was substantial genetic differentiation among regions in AMOVA (8%; $\phi_{RT} = 0.080$; $P = 0.005$) and between populations (13%; $\phi_{PT} = 0.128$; $P = 0.001$). When the grouping was changed to consider Landshuter Allee as a central

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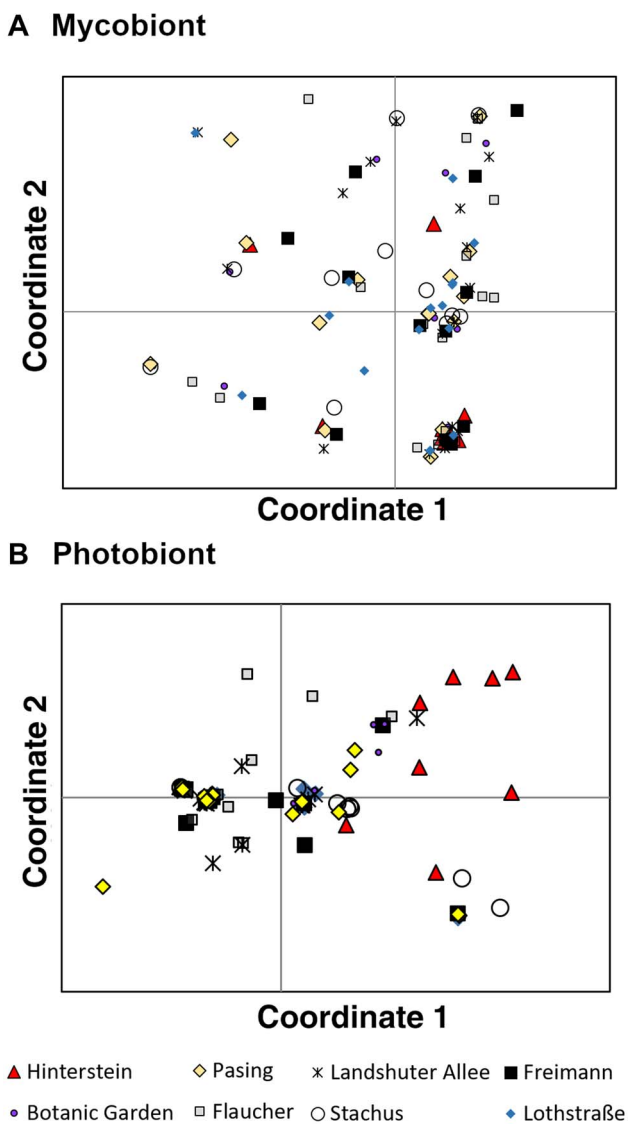

Fig. 2. Principal coordinates analysis of eight populations of *Xanthoria parietina* and its photobiont in Munich (Bavaria, Germany). Patterns displayed for the first two axes. Each point represents a thallus and symbols represent different populations. A, mycobiont. B, photobiont. In colour online.

population, there was still significant differentiation between geographical regions (data not shown). The genetic differentiation was mainly due to the Hinterstein population; when this population was removed, no significant genetic differentiation between populations remained (data not shown). Bayesian analysis of population structure revealed either two or five genetic clusters: delta K values had a bimodal distribution with maxima at K = 2 and K = 5 (Supplementary Material Figs S3–S6, available online). However, in contrast to the mycobiont data, some photobiont genetic clusters had a tendency to be restricted to specific locations (Fig. 3B). As expected in a predominantly clonal organism, only a small number of photobiont individuals were admixed (see bar plots from STRUCTURE analysis; Fig. 3). In a genetic distance-based population tree, spatially proximate populations mostly did not group together, with the exception of Lothstraße and Landshuter Allee populations which were geographically close and clustered together (Supplementary Material Fig. S7B, available online).

Discussion

In the present study, we developed nine new polymorphic microsatellite markers (Table 2) and investigated genetic diversity and population differentiation in the homothallic, sexually reproducing lichen-forming fungus *Xanthoria parietina*. The new fungal microsatellites were found to exhibit suitably high levels of genetic variability, and our results showed that they are appropriate for the study of population genetic diversity and genetic differentiation since they were able to detect distinct genetic clusters within local populations of *X. parietina*. We also studied the photobiont's genetic variation based on a set of existing microsatellite markers. This revealed that the photobionts had high variability within populations (Table 1). Finally, we compared the mycobiont's population genetic structure with that of its green-algal photobiont.

Prior studies of *X. parietina* found a pattern reminiscent of isolation by ecology (for definition, see Sexton *et al.* (2014)): when populations growing on bark and rock substrata were investigated, substantial genetic differentiation was found between them, but not within (Lindblom & Ekman 2006, 2007). Therefore, it is conceivable that the environment creates a selection pressure favouring specific, substratum-adapted mycobiont genotypes. Since we focused on bark substrata in our study, less genetic differentiation was expected and indeed we found a lack of population structure (i.e. no distinct populations) in the lichen fungus *X. parietina* in the studied populations in southern Bavaria. Populations of lichen mycobionts which were not structured at all have been found by others, for example in *Ramalina menziesii* Taylor (Werth & Sork 2010), interestingly even in the predominantly clonal lichen species *Usnea subfloridana* Stirt. (Degtjarenko *et al.* 2018, 2019), and sometimes even at large spatial scales, (i.e. across the Mediterranean and Macaronesia in *Parmelina carporrhizans* (Taylor) Hale; Alors *et al.* 2017). Similar to our findings, other studies conducted at a larger spatial scale and employing other marker types found little genetic differentiation between populations of the mycobiont of *X. parietina* growing on bark substrata (Lindblom & Ekman 2006, 2007; Itten & Honegger 2010).

Population genetic structure depends on numerous factors. Particularly important is gene flow, that is the number of migrants exchanged per generation between populations (Wright 1931). Gene flow wipes out genetic structure by homogenizing allele frequencies among populations and it takes only a small number of migrants per generation to remove any genetic differentiation (Wright 1931). Another factor influencing population structure is population size. Lichenized fungi forming small populations tend to have higher levels of genetic differentiation (e.g. Werth *et al.* 2007, 2021). Populations which are genetically isolated change their allele frequencies over time in a process known as random genetic drift. In small populations, fewer generations are necessary for allele frequencies to drift apart than in large populations. The aforementioned examples of lichen species showing panmixia (random mating) such as *Parmelina carporrhizans* or *Ramalina menziesii* represent common species forming large populations, which might partly explain their lack of population structure. In one of these common lichens, the mycobiont showed panmixia at the local scale (Werth & Sork 2008), but there was considerable population structure when the entire geographical range of the fungus was investigated and several genetic lineages were found (Sork & Werth 2014).

We expected clonal propagation to be of low importance in determining population structures because *X. parietina* lacks

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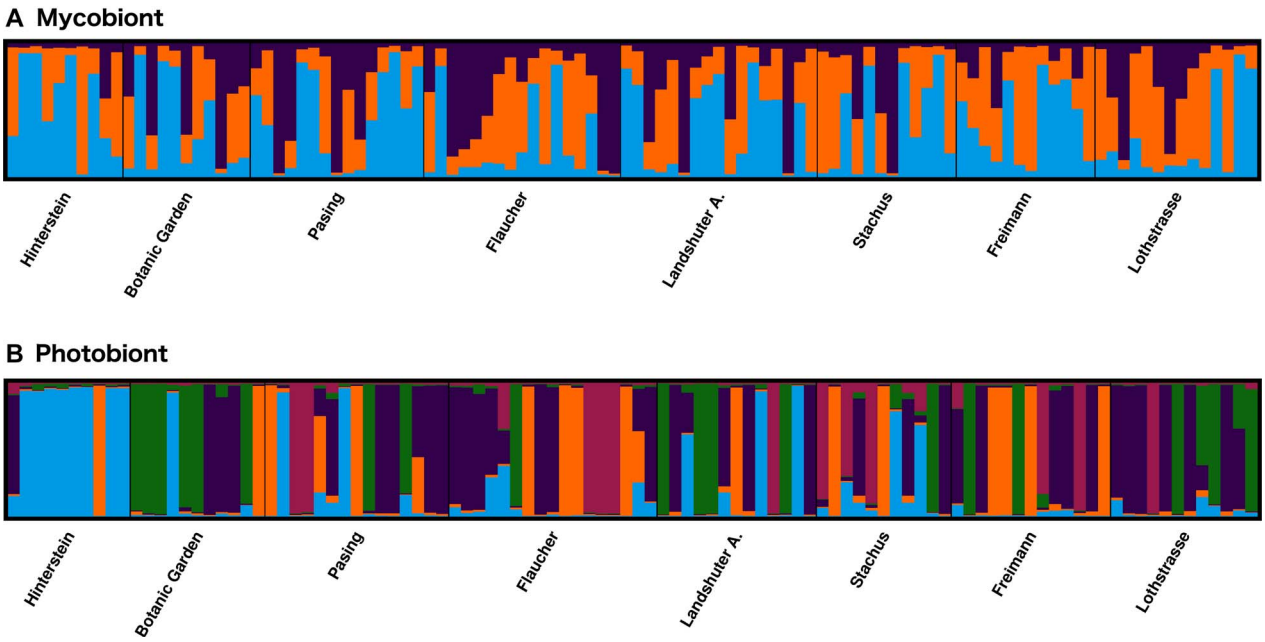

Fig. 3. Bayesian analysis of population structure for nine microsatellite markers of *Xanthoria parietina* and nine microsatellite markers of its photobiont, showing the affiliation of individuals (vertical bars) to genetic clusters (STRUCTURE software). A, mycobiont with three clusters evident (shown in blue, orange, purple). B, photobiont with five clusters evident (shown in blue, green, orange, purple, red). In colour online.

easily dispersed clonal propagules such as soredia or isidia with which mycobiont and photobiont are co-dispersed. Vegetative propagation by symbiotic propagules does not seem to be an important reproductive mode in the studied populations of *X. parietina*, as indicated by the incongruence between fungal and algal genetic patterns and the scarcity of repeated fungal multilocus genotypes. *Xanthoria parietina* is not known to form vegetative propagules but the species could in principle disperse vegetatively when eaten by invertebrates whose faeces are known to contain viable fungal and algal cells (Meier *et al.* 2002), or when thallus fragments are dispersed. Co-dispersal of the mycobiont and photobiont mediated by vegetative propagules must be rare, and dispersal in *X. parietina* may occur mainly through the mycobiont's microscopic ellipsoid ascospores (size range 12.9–16.0 × 5.9–8.7 µm; Lindblom 1997). At least in the studied areas in Munich and Allgäu, almost all large-sized individuals of *X. parietina* were fertile (i.e. apotheciate and thus able to produce ascospores). We know from a study of the lichen *Lobaria pulmonaria* that large and heavy symbiotic propagules disperse over a distance two orders of magnitude shorter on average than microscopic ascospores (Ronnås *et al.* 2017). One possible reason why there was no association between geography and genetic structure in our data might therefore be that the mycobiont disperses mainly with its presumably far-reaching, microscopic ascospores. Large symbiotic propagules (e.g. thallus fragments) should lead to more genetic structure than smaller propagules because they do not reach as far and consequently there should be less gene flow (but see Werth *et al.* (2014)).

While exhibiting a *MAT* locus characteristic of heterothallic species (Pizarro *et al.* 2019), Honegger *et al.* (2004*a*) reported that *X. parietina* appears to show breeding behaviour consistent with selfing (homothallism). This was supported by mating-type (*MAT*) gene analysis, with all progeny shown to be of the same *MAT* genotype as seen elsewhere in homothallic ascomycete species (Scherrer *et al.* 2005; Dyer *et al.* 2016). If a haploid fungus reproduces via selfing, its offspring will be identical to the parent, leaving a genetic footprint resembling clonality (Murtagh *et al.* 2000). By contrast, outcrossing would be predicted to shuffle the alleles originating from the maternal and paternal individuals due to random chromosomal segregation. However, it is important to note that many fungal species that are able to undergo selfing are not restricted to self-fertility, and can instead outcross if a suitable opportunity arises, and indeed outcrossing might be favoured (Dyer *et al.* 2016). Several lines of evidence from the present study indicate that outcrossing does seem to occur in *X. parietina*: i) the finding that most multilocus genotypes occurred only once points towards their origin by sexual reproduction involving outcrossing; ii) most fungal individuals were found to be admixed (i.e. having ancestry in several genetic clusters), noting that matings involving outcrossing among individuals belonging to different gene pools would create such an admixed pattern. Thus, our data suggest that outcrossing has occurred in the *X. parietina* mycobiont and seems to have resulted in large within-population variability. There are various ways in which species containing a single heterothallic form of a *MAT* locus may become self-fertile (homothallic). It may be achieved via secondary homothallism, also referred to as pseudohomothallism. One way this is enabled is by the occurrence of two genetically different nuclei in each ascospore, one of each mating type, as is the case for example in *Neurospora tetrasperma* Shear & B.O. Dodge (Boddy 2016; Wilson *et al.* 2021). Since this type of secondary homothallism results in dinucleate hyphae from the onset, the resulting mycelia are dinucleate and in microsatellite data one should frequently detect two alleles per locus. However, we found no such evidence of two alleles per microsatellite locus in a given sample, and therefore our data exclude this possibility of pseudohomothallism. A second type of pseudohomothallism occurs in some fungal species whose cells can switch their mating type, which enables a single culture to be self-fertile (Lin & Heitman 2007). However, Scherrer *et al.* (2005) found no

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evidence of a *MAT1-1* gene elsewhere in the genome to allow such switching. Although we found some repeated genotypes, there was also clear evidence of outcrossing (i.e. admixed fungal individuals), which further makes mating-type switching unlikely. The third way a genetically heterothallic species can undergo selfing is by 'unisexuality', where an individual of a single mating type can undergo selfing, which is thought to be enabled by mutations in specific genes involved in the initiation of sexual reproduction (Wilson *et al.* 2021). Our data overall is therefore consistent with the suggestion of unisexuality, as proposed by Pizarro *et al.* (2019). However, additional studies are required to quantify the frequency of outcrossing in *X. parietina* and to screen for possible causal mutations typical for unisexual fungi (Wilson *et al.* 2021).

Xanthoria parietina mycobionts were genetically admixed (i.e. with each individual having ancestry in multiple gene pools). By contrast, most individuals of the photobionts predominantly had ancestry in only one genetic cluster. This latter pattern implies that sexuality involving recombination between individuals is rare in the *Trebouxia* photobionts of *X. parietina*. These findings are also in line with the hypothesis that photobionts should be clonal while in lichen symbiosis (Honegger 1993).

If mycobiont and photobiont were mainly co-dispersed, their genetic structures should be congruent, such as in Swiss *Lobaria pulmonaria* and its *Symbiochloris reticulata* photobiont (Werth & Scheidegger 2012). Conversely, if there was little co-dispersal of lichen symbionts, differences in mycobiont and photobiont genetic structures would be expected. This is also what we observed for *X. parietina*: the degree of genetic differentiation was fundamentally different in the mycobiont and photobiont. While the mycobiont showed no population structure, populations of the photobiont of *X. parietina* were substantially structured. For example, gene pools of the mycobiont did not show geographical separation in a Bayesian analysis of population structure and individuals were admixed, indicating an absence of population structure, while there was more of a geographical pattern in the photobiont, with also far less admixture of individuals. Moreover, genotypes in the principal coordinates analysis showed no clustering by population in the mycobiont, while there was some distinctiveness of the Hinterstein, Stachus and Flaucher populations in the photobiont. Analysis of molecular variance showed significant differentiation between populations in the photobiont but not the mycobiont. Previous studies have found a similar pattern of more genetic structure in photobiont than in mycobiont populations (Werth & Sork 2010; Lutsak *et al.* 2016). Some studies indicate that *Trebouxia* photobionts can be structured by ecological factors such as the tree species they are growing on, or other habitat-related factors (Werth & Sork 2010, 2014). When lichen-forming fungi disperse to new sites with the tiny ascospores resulting from sexual reproduction, they are able to form a novel symbiosis with a locally adapted photobiont (Werth & Sork 2010, 2014; Lutsak *et al.* 2016). This should be highly advantageous for spores dispersing over long distances and that reach sites outside of the species' climatic optimum, because in this way, at least one partner of the symbiosis is adapted to the environment. In our study, the genetic differentiation between populations of the photobiont was mainly due to the remote Hinterstein population, indicating that at very fine spatial scales there was no detectable genetic structure in the photobiont.

Genetic diversity was higher in the photobiont of *X. parietina* than in its mycobiont. A similar pattern of higher photobiont than mycobiont genetic diversity has been found previously for the lichen *Cetraria aculeata* (Schreb.) Fr. sampled from different continents (Lutsak *et al.* 2016). In lichens, one mycobiont species can associate with more than one photobiont species. Although some improvements have been made in recent years to generate more realistic species concepts of photobionts (Škaloud & Peksa 2010; Malavasi *et al.* 2016; Škaloud *et al.* 2016), the species concepts of several lichen photobiont groups still need to be clarified and algal morphospecies may represent multiple phylogenetic species (Kroken & Taylor 2000). The photobionts of *X. parietina* belong to the *Trebouxia arboricola–T. decolorans* species complex, for which 34 OTUs have been reported which may represent different species (Leavitt *et al.* 2015). Microsatellites usually only amplify the species they were developed for, or closely related species. We were nevertheless able to generate microsatellite data for the *Trebouxia* photobiont based on the markers from Dal Grande *et al.* (2013), but these data did not allow us to distinguish different species of *Trebouxia*. Other lichens might share algal strains with *Xanthoria parietina* in the area of Munich. *Trebouxia decolorans* has been reported as a photobiont of various other lichen species or genera, including *Anaptychia ciliaris* (L.) Körb. ex A. Massal. (Dal Grande *et al.* 2014*a*), *Punctelia subrudecta* (L.) Körb. ex A. Massal., *Lecanora* (Blaha *et al.* 2006) and *Xanthomendoza* (Nyati *et al.* 2014), and of species of *Candelaria*, *Ramalina* and *Xanthoria* (Werth 2012; Dal Grande *et al.* 2014*a*). *Candelaria concolor* Arnold frequently co-occurs with *Xanthoria parietina* in the Munich area (Sebald *et al.* 2022) and it is conceivable that these species might share algal strains, but further investigations need to confirm this hypothesis.

Other investigations have found multiple genotypes of the photobiont within thalli of *X. parietina* (Dal Grande *et al.* 2014*a*) and there have been similar reports for *Parmotrema tinctorum* (Despr. ex Nyl.) Hale (Mansournia *et al.* 2011). Our study was not designed to investigate intrathalline genetic variability in *X. parietina* since we investigated only small marginal pieces of our specimens and these were found to be genetically homogeneous, both for the mycobionts and the photobionts. Molins *et al.* (2021) recently reported that photobiont diversity depended on thallus growth stage in *Ramalina farinacea* (L.) Ach., with young thalli containing multiple photobionts and larger thalli typically containing only one. Moreover, basal parts of the lichen tended to contain more photobiont types than apical parts. Since in our study we sampled small marginal pieces of rather large thalli, this sampling strategy might have caused us to find a single genotype in each sample, but the focus of our study was not on intrathalline photobiont or mycobiont diversity.

Many studies have investigated the pollution sensitivity of lichens and lichen communities (e.g. Hawksworth & Rose 1970; Richardson 1992; Garty *et al.* 1993; Geiser & Neitlich 2007). A large portion of lichen biota in Central Europe have declined tremendously over the last 150 years and air pollution has been identified as one of the key factors causing this dramatic loss of diversity (Hauck *et al.* 2013). However, few studies have investigated how air pollution affects the genetic diversity of lichens. Populations of *Usnea subfloridana* had lower genetic diversity in polluted than in unpolluted sites (Degtjarenko *et al.* 2016*b*). We did not find a significant relationship between genetic diversity of the mycobiont or photobiont and NO_2 pollution levels in our study sites but this might partly be an effect of the relatively small number of populations. Future studies of more sites are needed to conclusively test for a relationship. However, of all studied locations, an unpolluted, remote site in Allgäu had the highest

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photobiont gene diversity. The effects of nitrogen pollution are dosage dependent, and in high dosage treatments, even the nitrophilic species *X. parietina* suffered, with several treatments eventually causing mortality (Munzi *et al.* 2010). In polluted urban sites, there is accumulating evidence that nitrogen pollution levels are influencing the lichen symbioses. In lichen cells, NO_x leads to the production of nitrate (NO_3^-) and toxic nitrite (NO_2^-) accumulates. The NO_3^- may endanger the symbiotic balance by providing a nutrient for growth of the algal partner with the risk that it can grow faster than the mycobiont is able to control (Vorbeck & Windisch 2001). The NO_2 level at the remote Allgäu site was much lower than in the urban area of Munich, and it has not been disturbed as much by the fertilizing effect of NO_2, so that a larger number of pollution-sensitive lichens are able to grow there.

Guilds of lichen fungi sharing photobionts via release from vegetative propagules have been reported for cyanobacterial lichens (Rikkinen *et al.* 2002) and for *Symbiochloris* green-algal lichens (Dal Grande *et al.* 2014*b*). Consequently, the local availability of algal strains may depend on how many symbiotic propagules are deposited at a given site by other lichens with a compatible photobiont species. This, in turn, would depend on the species diversity and abundance in a given site. The *Trebouxia arboricola–T. decolorans* species complex includes some very common lichen photobionts (Dal Grande *et al.* 2014*a*; Leavitt *et al.* 2015), associating with many ecologically different lichenized fungi, for example with *Ramalina menziesii* found in foggy climates of coastal western North America (Werth & Sork 2010, 2014; Werth 2012), with *Caloplaca* species in the Atacama Desert in Chile (Vargas Castillo & Beck 2012), or with *Tephromela atra* (Huds.) Hafellner in high elevation habitats of European mountain regions (Muggia *et al.* 2010). The *Trebouxia arboricola–T. decolorans* species complex is also found in some lichens which produce symbiotic propagules. The photobiont could therefore be dispersed with the soredia/isidia of other lichen species, followed by uptake by germinating *X. parietina* spores. Very little is known about the initial stages of lichen establishment but sharing of the same photobiont pool has been reported for other lichen communities (Beck *et al.* 2002; Werth 2012). In *X. parietina*, growing sporelings are apparently able to initially associate with suboptimal *Pseudotrebouxia* algae, which are later replaced by the compatible partner (Ott 1987).

The high genetic diversity of *Trebouxia* photobionts associating with *X. parietina* in Allgäu may reflect the presence of a diverse local community of lichens that share the same *Trebouxia* photobionts. Ongoing studies have found a very high diversity of epiphytic lichens in the Hinterstein valley (I. Buschmann, S. Dehos, T. Karlowski & S. Werth, unpublished data; S. Werth, personal observation). The higher genetic diversity of the photobionts at Allgäu could also be a consequence of there being more algal strains available in unpolluted sites. In contrast, we found very low genetic diversity in the mycobiont of *X. parietina* in the unpolluted, remote site in Allgäu. This may reflect a relatively small local population size, which is confirmed by our field observations.

One interesting question is whether inner city areas which used to be lichen deserts because of high air pollution are recolonized by one specific gene pool or genotype, or from multiple sources. The recolonization of a formerly heavily polluted city in Germany by the lichen *Usnea filipendula* Stirt. (*U. dasopoga* (Ach.) Nyl.) occurred from different source populations, and individuals were not genetically closely related (Heibel *et al.* 1999). The city of Munich was never a complete lichen desert but nitrophilic species such as *X. parietina* were largely restricted to the most basic, eutrophicated substrata until a few decades ago and they have since increased dramatically in local population size (S. Werth, personal observation). Given the admixture found in our data, it seems plausible that the population in the urban area of Munich originates from individuals with different genetic backgrounds after the area became suitable for mass colonization by *X. parietina* thanks to decreased SO_2 air pollution levels. Our data on *X. parietina* show that urban areas can host substantial genetic diversity in the symbiotic partners of nitrophilic lichens.

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