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Research

Environment and host identity structure communities of green algal symbionts in lichens

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Summary

• An understanding of how biotic interactions shape species' distributions is central to predicting host–symbiont responses under climate change. Switches to locally adapted algae have been proposed to be an adaptive strategy of lichen-forming fungi to cope with environmental change. However, it is unclear how lichen photobionts respond to environmental gradients, and whether they play a role in determining the fungal host's upper and lower elevational limits.

• Deep-coverage Illumina DNA metabarcoding was used to track changes in the community composition of *Trebouxia* algae associated with two phylogenetically closely related, but ecologically divergent fungal hosts along a steep altitudinal gradient in the Mediterranean region.

• We detected the presence of multiple *Trebouxia* species in the majority of thalli. Both altitude and host genetic identity were strong predictors of photobiont community assembly in these two species. The predominantly clonally dispersing fungus showed stronger altitudinal structuring of photobiont communities than the sexually reproducing host. Elevation ranges of the host were not limited by the lack of compatible photobionts.

• Our study sheds light on the processes guiding the formation and distribution of specific fungal–algal combinations in the lichen symbiosis. The effect of environmental filtering acting on both symbiotic partners appears to shape the distribution of lichens.

Introduction

Abiotic and biotic factors, and their interactions, organize biological communities into nonrandom assemblages of species (Thrall *et al.*, 2007). Among the abiotic factors, climate is a primary driver governing community composition and range limits along both latitudinal and altitudinal gradients (von Humboldt & Bonpland, 2009; Pettorelli, 2012). For instance, biodiversity increases from polar to equatorial regions (latitudinal biodiversity gradient; Hillebrand, 2004). Altitudinal gradients are particularly suited for studying climate effects on species diversity, because they exhibit steep ecological transitions (e.g. in temperature, precipitation and UV radiation) over short distances (Körner, 2007; Keller *et al.*, 2013). Plant communities, for instance, form characteristic elevational belts with abrupt transitions along altitudinal gradients (D'Odorico *et al.*, 2013; Mayor *et al.*, 2017).

The study of species' distribution boundaries, or range limits, is complicated by the fact that many organisms rely on close interactions with other species. Recent theoretical frameworks suggest that associations with mutualists may increase and shift the ecological niche and the geographic distribution of organisms (Poisot *et al.*, 2011; Peay, 2016). Factors specific to the symbiotic lifestyle, which affect species distribution patterns, include the genetic make-up of the symbionts, mode of symbiont transmission, and host population dynamics (Johnson & Stinchcombe, 2007; Moran, 2007; Chong & Moran, 2016). For example, symbionts may influence the range of their hosts through effects on fitness and population dynamics (Rosenberg *et al.*, 2007; Janson *et al.*, 2010; Joy, 2013; Corbin *et al.*, 2017). The acquisition of obligate symbionts may open up new ecological niches for the host and, thus, drive range expansion (Afkhami *et al.*, 2014; Maher *et al.*, 2017). Symbiont-driven ecological expansion has been documented in a wide diversity of symbiotic systems, from insects (Sudakaran *et al.*, 2017) to corals (Silverstein *et al.*, 2012), sponges (Pita *et al.*, 2016), and angiosperms (Joy, 2013). It is thus important to consider how both abiotic and biotic factors determine range boundaries in mutualists (HilleRisLambers *et al.*, 2013; Louthan *et al.*, 2015).

Lichens are multi-species assemblages in which a fungus (host or exhabitant) forms obligate symbiotic associations with microalgae (including cyanobacteria). The majority of lichen-forming fungi associate with green algae of the family

Trebouxiophyceae. Fungal–algal associations in lichens are influenced, for example, by micro- (Werth & Sork, 2014) and macroclimatic factors (Singh *et al.*, 2017). Shifts in fungus–photobiont interactions can be climate-driven (Fernández-Mendoza *et al.*, 2011), and show increased specificity (i.e. one-to-one interactions) in warmer regions (Singh *et al.*, 2017). Further, switches to locally adapted algae may increase the geographic range and ecological niche of their fungal hosts (Muggia *et al.*, 2014). At the local scale, for instance along altitudinal gradients, this may translate into habitat-specific photobiont switches (Vargas Castillo & Beck, 2012), and/or microclimatic partitioning of ecologically differentiated fungal and algal gene pools (Nadyeina *et al.*, 2014). At the individual scale, a few studies reported that lichen thalli may harbor multiple algal species, which possibly display different environmental tolerances (del Campo *et al.*, 2013; Dal Grande *et al.*, 2014a). However, relatively little is known about how photobiont diversity is structured along environmental gradients and how this diversity affects host distribution and ability to withstand environmental changes.

The reproductive strategy of the fungal host may also affect fungal–algal associations in lichens (Yahr *et al.*, 2004; Dal Grande *et al.*, 2012; Singh *et al.*, 2015; but see Wornik & Grube, 2010). Asexual reproduction via specialized propagules (e.g. isidia and soredia) that co-disperse clonally derived fungal and algal offspring at the local scale (vertical transmission) is expected to result in low photobiont diversity and congruent fungal–algal population structures (Werth & Scheidegger, 2012; Widmer *et al.*, 2012). In contrast, sexual reproduction – independent dispersal of fungal spores, generally over long distances – may lead to the formation of new symbiotic pairs with potentially different viability and physiological capacities (horizontal transmission), and is expected to yield high photobiont diversity and low fidelity (Beck *et al.*, 2002). These assumptions are not always met in nature, as some studies reported no correlation between fungal and algal genotypes in strictly asexual lichen systems (e.g. Ohmura *et al.*, 2006; Nelsen & Gargas, 2008; Wornik & Grube, 2010). Whether the maintenance of symbiotic associations in lichens is always or only sometimes a consequence of joint fungal–algal dispersal remains an open question.

Much of our current knowledge about fungus–alga associations, distribution patterns, and diversity in lichens relies on studies based on Sanger sequencing. This direct sequencing approach might, however, lead to overly simplistic inferences, as only the primary photobiont is retrieved (Voytsekhovich & Beck, 2016; Moya *et al.*, 2017). Thus, ecological and biogeographic studies on lichen-associated microalgae using Sanger sequencing may underestimate the complexity of natural samples as a consequence of the inability to detect less abundant co-occurring photobionts. Improved assessment of photobiont diversity in lichens is possible with metagenomic analyses of entire algal communities based on high-throughput sequencing technology (i.e. DNA metabarcoding). DNA metabarcoding is rapidly transforming community ecology, especially the study of cryptic biodiversity (Bálint *et al.*, 2015; Evans *et al.*, 2016). Further, this approach has been shown to be invaluable for the study of interactions between host organisms and their symbiotic communities, accurate profiling of entire symbiont communities, and the investigation of ecological convergence at the community level (reviewed in Baker *et al.*, 2016; Bittleston *et al.*, 2016). Metabarcoding of algal communities is increasingly used for its ability to detect rare but potentially functionally important genetic variants in complex DNA mixtures in both marine (e.g. phytoplankton; Chain *et al.*, 2016) and terrestrial ecosystems (e.g. endolithic algae; Sauvage *et al.*, 2016). For lichens, pyrosequencing analyses of whole-thallus DNA extracts have shown that metabarcoding is a promising tool to uncover cryptic microalgal diversity in natural samples (Bates *et al.*, 2012; Park *et al.*, 2014; Moya *et al.*, 2017). Although metabarcoding technology is a promising tool for quickly producing taxonomically comprehensive data sets of microbial communities, several technical and methodological challenges remain. The main drawbacks are those related to primer biases, sequencing artifacts, clustering of operational taxonomic units, and contamination (Thomsen & Willerslev, 2015; Guardiola *et al.*, 2016). Thus, care should be taken during sample collection and preparation, followed by the application of strict bioinformatic filtering steps (Krohn *et al.*, 2016). This is particularly relevant in the case of lichens and their photobionts, to avoid or minimize the inclusion of epiphytic algae that may have landed on or within the thallus during the life cycle of the lichen but that are not a functional part of the symbiosis. Deep-coverage sequencing has so far not been applied to studies of lichen-associated microalgal diversity in an ecological context.

In this study, we examined photobiont communities associated with two congeneric fungal hosts along an altitudinal gradient. We assessed the effects of altitude and host identity in shaping photobiont communities in lichens. The selected fungal host species, *Lasallia pustulata* L. (Mérat) and *Lasallia hispanica* (Frey) Sancho & A. Crespo, gradually replace one another along altitudinal gradients in the Mediterranean, with a zone of sympatry at *c.* 1200–1700 m above sea level (asl). Using deep-coverage Illumina DNA metabarcoding on an extensive set of lichen thalli, we assessed to what extent closely related fungal hosts with different environmental preferences and reproductive modes share algal communities, and how these communities change along the gradient. In particular, we aimed to answer the following questions: How does the lichen-associated photobiont community shift along an altitudinal gradient? To what extent does host genetic identity alter the responses of photobiont communities to altitude? Can differences in the composition of the photobiont communities explain current elevational range limits of the hosts?

Materials and Methods

Host species

The two focal host species of this study are rock-inhabiting, lichen-forming fungi with foliose, umbilicate growth forms that differ in their distribution, habitat preference, and reproduction. *Lasallia hispanica* is a high-elevation species found on mountains in the Mediterranean region, usually above 900 m. Despite this restricted distribution, it is one of the most frequent macrolichens

of the subalpine and alpine zones on mountains of the Central System in the Iberian Peninsula. It has a Western European distribution, occurring on the Atlantic slope of the Iberian Peninsula and in southern Italy, and is also distributed in northern Morocco (Sancho & Crespo, 1989). *Lasallia hispanica* is sympatric with *L. pustulata* in the montane zone (900–1800 m), but does not occur in either lowland or submontane zones. In contrast, *L. pustulata* is widely distributed all over Europe and spans a broad elevation range. In Mediterranean mountains it extends from lowland to submontane zones, but is usually not found above 1800 m (i.e. in the subalpine/alpine zones). Water uptake in *L. hispanica* is mostly from fog and low-lying clouds at high altitude, whereas in *L. pustulata* it is mostly from surface run-off. Thus, *L. pustulata* typically prefers less inclined slopes with trickling water. Both host species have a mixed strategy of asexual and sexual cycles. However, reproduction in *L. pustulata* is predominantly vertical and asexual, by means of macroscopic, coral-like propagules (isidia) that co-disperse fungi and algae over short distances. Reproduction in *L. hispanica* is predominantly horizontal and sexual, by means of fungal ascospores dispersed independently from the photobionts (Sancho & Crespo, 1989). The latter reproductive strategy requires frequent new combinations of fungal and algal partners.

Study site and sample collection

For the present study, we established a transect consisting of nine sampling stations evenly distributed along an elevational gradient ranging from *c.* 700 to 2100 m asl on the south-facing slope of the 'Sierra de Gredos' mountain range (Sistema Central, Spain; Supporting Information Table S1). The transect covered a linear distance of *c.* 6.5 km and an altitudinal interval of *c.* 1400 m.

In order to investigate spatial relationships among algal communities, Global Positioning System (GPS) coordinates and altitude were taken using a handheld GPS (Garmin, Olathe, KS, USA) at each sampling site. The area encompasses different climates including warm temperate, cold temperate and boreal/alpine ecozones. Annual precipitation ranges from *c.* 400 mm at 700 m asl to *c.* 850 mm at 2100 m asl, and the annual mean temperature difference is *c.* 7.3°C between the highest and lowest sampling locations (Table S1). To summarize the climate along the gradient, we used altitude, and 19 bioclimatic variables from the WorldClim database, drawn at the highest spatial resolution (*c.* 1 km) of each sampling site (Hijmans *et al.*, 2005). Altitude strongly covaried with all bioclimatic variables (Fig. S1), and was thus used as proxy for climatic conditions in all subsequent analyses.

Sample collection

Throughout the paper, the term 'population' refers to groups of lichen thalli (i.e. sampling units) belonging to a given fungal host species that were collected at a given site. The term 'community' refers to assemblages of photobionts associated with one or both of the fungal hosts (within and across samples). Populations were located on horizontal or gently sloping, fully sun-exposed rock faces in scattered granitic outcrops. From each site, 20 thallus pieces of both host species (if present) of *c.* 8 mm in diameter were collected over an area of *c.* 50 m^2 in July 2015. This resulted in a total of six populations (i.e. 120 thalli) for each host species. The host *L. pustulata* occurred between 706 and 1699 m asl, while *L. hispanica* occurred between 1258 and 2096 m asl. In order to capture the maximal diversity present at the sites, thalli were sampled with a minimum distance of 0.5 m from the nearest sampled thallus of the same host species. Samples were collected with sterile tools and transferred into sterile 2-ml tubes. Each sample was genotyped at the fungal single-copy protein coding gene *MCM7* (DNA replication licensing factor mini-chromosome maintenance complex component 7) following Sadowska-Deś *et al.* (2013). This locus was chosen because it is the population genetic marker with the highest resolution among commonly used loci in *L. pustulata* (Sadowska-Deś *et al.*, 2013). Samples were grouped into fungal haplotypes using the software Tcs (Clement *et al.*, 2000) with 95% connection limit on each host data set.

Fig. 1 Phylogeny of *Trebouxia* operational taxonomic units (OTUs) associated with the lichen-forming fungi *Lasallia pustulata* and *Lasallia hispanica*. This is a maximum likelihood tree based on seven *Trebouxia* OTUs and 17 unique top 10 NCBI (National Center for Biotechnology Information) GenBank NT BLAST hits. *Trebouxia* OTUs retrieved for this study are in bold face. Thickened branches indicate maximum likelihood bootstrap support values (1000 replicates) > 70%. Colored boxes indicate host preference. An expanded version of this tree is available as Supporting Information Fig. S4.

Illumina metabarcoding of photobiont communities in individual lichen thalli

Trebouxia communities associated with the two host species were assayed using Illumina high-throughput sequencing of the second

part of the internal transcribed spacer region (ITS2) of the rRNA operon. Thalli were thoroughly washed twice in sterile water before extraction. Genomic DNA was extracted separately using a cetyl trimethylammonium bromide (CTAB)-based method (Cubero & Crespo, 2002). Alga-specific primers were developed to target the ITS2 region in a custom database of Sanger-sequenced ITS sequences of *Trebouxia* sp. associated with the two lichen hosts (this study; G. Rolshausen *et al.*, unpublished). We selected the ITS2 region because it showed higher variability than ITS1. ITS2 has been proposed as a universal barcode across eukaryotic kingdoms (Coleman, 2009) because it generally provides good species-level resolution, it is easy to amplify, and it has a relatively conserved secondary structure among eukaryotes. Furthermore, changes in conserved pairing positions of the ITS2 transcript secondary structure have been correlated to the biological species concept (Müller *et al.*, 2007). We designed high-coverage PCR primers at conserved nucleotide positions of the aligned consensus sequences using PRIMER3 (Untergasser *et al.*, 2012; Fig. S2). Primer sequences were: FDGITS2-f: AGCGAAATGCGATACGTAGTGT; FDGITS2-r: GGGTGTTCTTGCCTGACCTC. The amplified PCR fragment length and the primer specificity were simulated using an *in silico* approach as implemented in the software ECOPCR (Ficetola *et al.*, 2010). The virtual PCR tests confirmed the specificity of the primers for the algal phylum Chlorophyta and showed that they amplify a large number of algal species belonging to different families (Fig. S2). Optimal annealing temperatures for the selected ITS primer pair were explored *in vitro*.

All samples were treated simultaneously and identically in order to minimize bias in the representation of the algal community. Two PCR replicates with different annealing temperatures (50°C and 63°C) were pooled to ensure a more exhaustive sampling of community diversity (Schmidt *et al.*, 2013). The fragments in each sample were combinatorially labeled at both ends (Table S2). The 25-μl reactions contained 0.65 U TaKaRa ExTaq (Clontech Laboratories Inc., Palo Alto, CA, USA), 2.5 μl of buffer, 18.5 μl of water, 0.5 μl of bovine serum albumin (BSA; 10 mg ml^{-1}), 2.0 μl of dNTP mixture (2.5 mM each), *c.* 5 ng of total DNA (0.5 μl), and 0.22 μM (0.5 μl) labeled forward and reverse primers. We used multiplexing labels published by Gloor *et al.* (2010). The reactions were performed with the following cycle conditions: initial denaturation at 95°C for 4 min followed by 35 cycles of 95°C for 30 s, 50/63°C for 20 s and 72°C for 20 s, and a final elongation at 72°C for 5 min. The end-labeled amplicons were solid phase reversible immobilization (SPRI)-purified, concentration-normalized (using Qubit; Life Technologies, Darmstadt, Germany), SpeedVac-dried (SpeedVac Concentrator, Farmingdale, NY, USA) and paired-end-sequenced (2 × 300 bp) on an Illumina MiSeq sequencer at StarSeq (Mainz, Germany).

Bioinformatic analyses

All reads with an average quality score < 26 phred and shorter than 200 bp were removed using the script READS_QUALITY_LENGTH_DISTRIBUTION.PL (Bálint *et al.*, 2014). The read pairs were assembled with default settings in PANDASEQ (Masella *et al.*, 2012). All reads that contained unknown nucleotides were discarded. Reads were then reoriented into 5′–3′ directions. Primer artifacts were removed using the script REMOVE_MULTIPRIMER.PY (Bálint *et al.*, 2014). Reads were demultiplexed with FQGREP (https://github.com/indraniel/fqgrep, accessed on 10 July 2016). No mismatches in the label and primer sequences were allowed. Complete ITS2 regions were extracted from the reads using ITSX (Bengtsson-Palme *et al.*, 2013) to remove conserved flanking regions that may interfere with downstream sequence clustering. Sequences were dereplicated using VSEARCH v.2 (Rognes *et al.*, 2016). Sequences were clustered into operational taxonomic units (OTUs) with SWARM v.2.0 using $d = 1$ and the FASTIDIOUS option (Mahé *et al.*, 2015). SWARM is a *de novo*, single-linkage clustering method that overcomes the problem of choosing an arbitrary global clustering threshold (e.g. 97% similarity). This is done by using a local clustering threshold (d) and taking into account sequence abundance values to define OTUs. Potentially chained, small OTUs are subsequently eliminated by grouping them into bigger ones if the existence of an intermediate amplicon can be postulated (FASTIDIOUS option; Mahé *et al.*, 2015). OTU centroids were searched for chimeric sequences with the UCHIME method (Edgar *et al.*, 2011) as implemented in VSEARCH v.2 (Rognes *et al.*, 2016) using the –uchime_denovo command, and their OTUs were removed even if they occurred in multiple samples. OTUs were compared with a BLAST search (Altschul *et al.*, 1997) against the entire GenBank NT database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/nt*, downloaded on 31 October 2016). The BLAST outputs were parsed in MEGAN 5 (Huson *et al.*, 2011) to avoid the inclusion of taxa that did not belong to the phylum Chlorophyta.

As spurious taxa, that is, OTUs derived from erroneous sequences or artifacts of the data collection process, may persist to this point in the analysis, we applied stringent filtering on the OTU table to avoid exaggerated community diversity estimates. As we did not include mock communities in the sequencing runs, we followed the general recommendation to filter the final OTU table by removing OTUs that represented < 0.005% (i.e. $n = 1194$) of the total read abundance on a per-sample basis and that were present in < 5% (i.e. $n = 12$) of the total samples (Bokulich *et al.*, 2013; Callahan *et al.*, 2016; Krohn *et al.*, 2016). Only OTUs assigned to the algal genus *Trebouxia* passed the filtering process and were thus retained for downstream analysis.

Phylogenetic placements

Trebouxia OTUs were classified according to the method proposed by Leavitt *et al.* (2015). For this, we used the alignment of 69 OTUs from Leavitt *et al.* (2015) representing the major, well-supported *Trebouxia* clades (i.e. A, *T. arboricola/gigantea*; I, *T. impressa/gelatinosa*; S, *T. simplex/letharii/jamesii* group; G, *T. galapagensis/usneae* group) to which the seven *Lasallia*-associated *Trebouxia* OTUs found in our study were added using the MAFFT 'add-in' function (Katoh & Frith, 2012). To infer phylogenetic relationships of the seven *Lasallia*-associated *Trebouxia* OTUs, a second data set was built to include the 10 highest scoring non-identical NCBI (National Center for Biotechnology

Information) NT BLAST hits recorded for each OTU. Two sequences of *Trebouxia* clade 'G' were used as an outgroup. Sequences were aligned with MAFFT v.7 (Katoh *et al.*, 2005; Katoh & Toh, 2008) using the FFT-NS-i alignment algorithm and '200PAM/K = 2' scoring matrix, with an offset value of 0.0, 'unalignlevel' = 0.4 using the 'Leave gappy regions' setting and the remaining parameters set at default values. Phylogenetic relationships and their confidence values were inferred using maximum likelihood (ML) as implemented in RAxML (Stamatakis, 2006). All ML searches followed a GTRGAMMA model of molecular evolution with 1000 ML bootstrap pseudo-replicates. Additionally, the secondary structure of each OTU sequence was reconstructed using RNAFOLD with default parameters and temperature settings of 37°C (http://rna.tbi.univie.ac.at).

Statistical analyses

Changes in overall community composition along the elevation gradient were examined with multispecies generalized linear models (GLMs) assuming a Poisson distribution for read number counts (MVABUND package in R; Wang *et al.*, 2012). More specifically, we analyzed overall algal community responses (i) within each host separately, including altitude, host *MCM7* haplotype, and the altitude × host *MCM7* haplotype interaction as predictors, as well as (ii) across both hosts, including only altitude as a predictor. The former approach investigated how the overall algal community within host species (*L. pustulata* or *L. hispanica*) responded to the respective predictors, whereas the latter approach investigated the single impact of altitude on the overall algal community across hosts (*L. pustulata* and *L. hispanica*) independently of host genotype. To take into account the sequencing bias inherent in high-throughput sequencing, all models incorporated the summed read numbers per sample as an offset. In addition to the multispecies GLMs, the similarity of the host populations based on their photobiont composition was examined using nonmetric multidimensional scaling (NMDS). NMDS is commonly used in community ecology to compare diversity patterns among samples. Typically, objects are plotted onto a two-dimensional ordination space using a community dissimilarity matrix based on species composition. For this purpose, overall community responses were visualized based on Jaccard distances among samples grouped by sampling site. Weighted bipartite networks were constructed between each fungal host population and the most frequent *Trebouxia* OTU in each sample using the R package *bipartite* (Dormann *et al.*, 2008).

To further evaluate the (statistical) importance of predictor variables (altitude, host haplotype, and altitude × host haplotype) for abundance changes in each algal OTU, separate generalized linear mixed models (GLMMs, weighted for total read numbers and assuming a Poisson distributed response) that incorporated the above predictor set together with samples nested within locations as a random effect structure were fitted. From these GLMMs, relative within-model effect sizes, calculated as the standardized weighted regression coefficients for each predictor, were inferred in order to evaluate the importance of the predictor within the respective model.

Data accessibility

Illumina MiSeq sequence data were deposited in the Sequence Read Archive (accession no. SRP111342).

Results

Eight fungal *MCM7* haplotypes in *L. pustulata* (P1–8) and four in *L. hispanica* (H1–4) were identified (Table S2). *Lasallia pustulata* haplotypes P3 and P4 were more abundant at low-altitude sites (up to 1082 m asl). Similarly, *L. hispanica* haplotype H3 was only present in the two low-altitude sites for this host (1258–1480 m asl).

Algal metabarcoding, phylogenetic placement, and diversity of *Lasallia*-associated *Trebouxia* OTUs

A total of 31 516 521 paired-end Illumina MiSeq reads were generated. Of these, 26 220 997 reads passed the demultiplexing step, and 23 874 887 quality-filtered reads could be assigned to the genus *Trebouxia*. This represented a mean of 99 479 (median 95 621) algal reads per sample. Seven unique algal OTUs were retained, representing a total of 22 018 669 reads (average OTU length 232 bp; Table S3).

All OTUs belonged to *Trebouxia* clade 'S' (*simplex/lethariijamesii* group) *sensu* Leavitt *et al.* (2015; Fig. S3). Five of the seven OTUs were host-specific; namely, OTU3 was only found in *L. pustulata*, while OTU4, OTU5, OTU15, and OTU16 were restricted to *L. hispanica* (Fig. 1). Based on sequence identity with the type strain and with lichen-associated green algae from other studies, OTU1 was identified as *Trebouxia angustilobata*. OTU2 and OTU3 were previously detected only in *L. pustulata*. In a previous study, OTU3 was always sequenced from thalli collected in the Mediterranean region (Sadowska-Deś *et al.*, 2014). OTU4 corresponds to OTU S03 *sensu* Leavitt *et al.* (2015) and is found in association with five other epiphytic and vagrant/terricolous lichen hosts from temperate, boreal and alpine regions in Europe and North America (Table S4). OTU5 corresponds to the generalist *Trebouxia suecica* which forms associations with a broad range of lichen hosts in temperate, boreal and alpine climates (Singh *et al.*, 2017). OTU15 and OTU16, although closely related to OTU4 and in the case of OTU16 having identical secondary structure, did not match any sequence in the database (Figs 1, S4; see Fig. S5 for the ITS2 secondary structures of all OTUs).

In some thalli we detected more than one photobiont. A single photobiont (i.e. 100% of the reads) was found in 59 individuals (49.2%) in *L. hispanica* and in two individuals (1.7%) in *L. pustulata*. A dominant photobiont (representing > 90% of the reads) was found in 14 individuals (11.7%) in *L. hispanica* and in 52 individuals (43.3%) in *L. pustulata*. Two dominant photobionts (one represented by at least 40–75% of the reads, and the second represented by at least 20% of the reads) were present in 20 individuals (16.7%) in *L. hispanica* and in 25 individuals (20.8%) in *L. pustulata*. Three predominant photobionts (each represented by at least 20% of the reads) were

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only found in a single case. This was also a sample with strongly reduced read numbers as a result of the filtering process and might represent an artifact. In three populations of *L. hispanica* (VI, VII, and VIII), most of the thalli contained a single photobiont.

Some OTUs displayed a clear altitudinal trend (Fig. 2). For example, OTU3 was only detected in the first two sampling sites, belonging to the warm temperate biome; OTU2 occurred up to 1699 m, constituting the upper limit of sympatric occurrence of the lichen hosts. Conversely, OTU1 was detected at all altitudes and in all thalli.

Trebouxia communities respond to host genetic identity and altitude

Altitude had a significant effect on the overall community composition of the *Trebouxia* communities along the gradient ($P < 0.001$; Table 1). Both host-specific *Trebouxia* communities were significantly affected by altitude, host genetic identity and their interaction, with the altitude predictor producing the best fit to the community structure data (based on deviance from the intercept-only model) in both host species ($P < 0.001$; Table 1).

Furthermore, individual *Trebouxia* OTUs reacted significantly to either one or both predictors, as evident from separate GLMMs. In *L. pustulata*, OTU abundance changes were mostly explained by altitude or the altitude × host haplotype interaction, except for OTU3 for which abundance changes were mainly explained by host haplotype (Fig. 3; Table 2). In *L. hispanica*, in contrast, OTU abundance changes were mostly explained by host haplotype, except for OTU2, for which abundance changes mostly followed changes in altitude (Fig. 3; Table 2). For the OTUs that were shared between the two hosts (OTU1 and OTU2), the response in abundance was host-specific: in *L. pustulata*, frequency changes in OTU1 and OTU2 were mainly driven by altitude and the altitude × host haplotype interaction, whereas in *L. hispanica*, abundance changes in OTU1 were mainly driven by host haplotype, and changes in OTU2 were mainly driven by altitude (Fig. 3, Table 2).

The effect of altitude on the *Trebouxia* community found in *L. pustulata* was also evident when comparing the similarities of the sampling sites based on their algal composition using NMDS (Fig. 4). Algal communities of *L. pustulata* from the two sites at low altitude (< 900 m asl) were clearly separated from the remaining sites, whereas in *L. hispanica* there was some overlap between communities located at the altitudinal extremes of the gradient (Fig. 4). Host-specific community responses were also evident in the NMDS plots comparing algal community composition between sites of sympatric host occurrence (Figs 5, S6). In

Table 1 Variation in the composition of the *Trebouxia* community found along the whole altitudinal gradient (706–2096 m above sea level), and within each host species separately explained by multispecies generalized linear models (GLMs)

	Res.Df	Df.diff	Dev	*P* (<)
Whole gradient				
(Intercept)	239	NA	NA	NA
Altitude	238	1	2623 592	0.001
L. hispanica				
(Intercept)	119	NA	NA	NA
Altitude	118	1	3769 633	0.001
Host haplotype	115	3	2216 074	0.001
Altitude × host haplotype	113	3	907 788	0.001
L. pustulata				
(Intercept)	119	NA	NA	NA
Altitude	118	1	7326 975	0.001
Host haplotype	111	7	1072 410	0.001
Altitude × host haplotype	108	7	1068 979	0.001

Dev, deviance; Df.diff, difference in degrees of freedom; NA, not applicable; Res.Df, residual degrees of freedom.

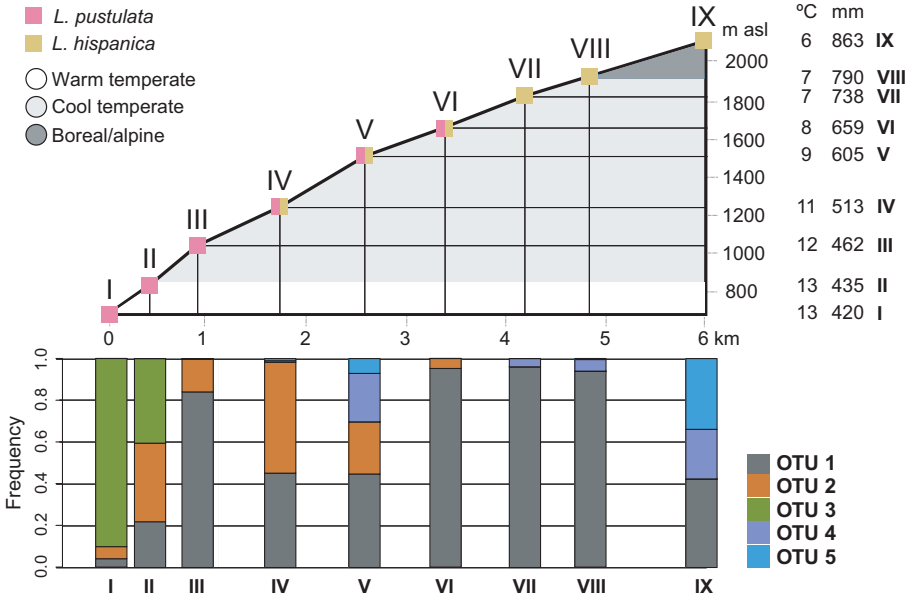

Fig. 2 Elevation profile of the study area showing the nine sampling sites of *Lasallia pustulata* (pink) and *Lasallia hispanica* (yellow). The *x*-axis scale is kilometers and the *y*-axis (elevation) scale is meters above sea level. The gray shading indicates the bioclimatic profile of the gradient according to Metzger *et al.* (2013), spanning three biomes: warm temperate (white), cool temperate (light gray), and boreal/alpine (dark gray). The bars at the bottom of the figure show the relative abundance of the *Trebouxia* operational taxonomic units (OTUs) detected in our study. OTUs exhibiting < 1% relative abundance across all sets of OTUs are not shown. Values on the right indicate annual mean temperature (°C; BIO1) and annual precipitation (mm; BIO12) for each sampling site.

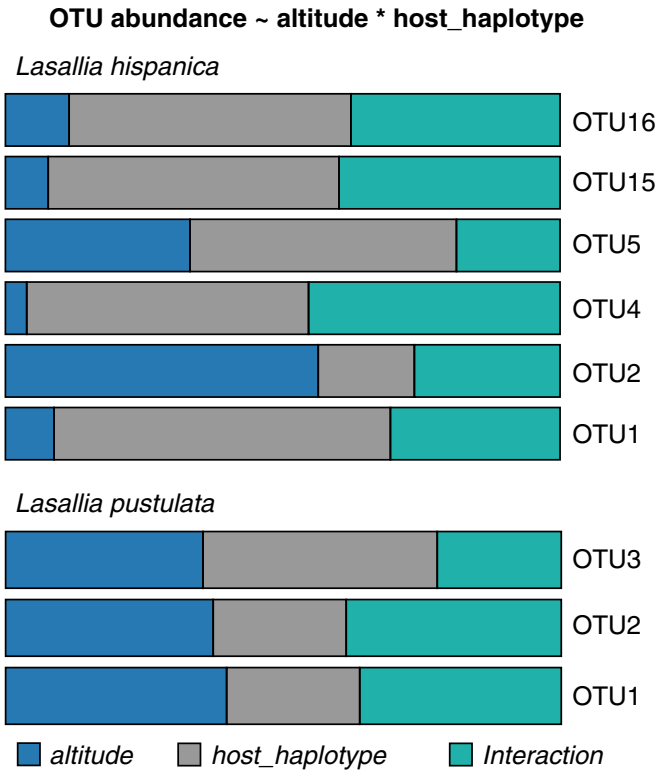

Fig. 3 Relative contribution of predictors (altitude, host haplotype, and their interaction) based on standardized weighted regression coefficients from general linear mixed models (operational taxonomic unit (OTU) abundance ~ altitude × host_haplotype) explaining variance in frequency of each of the *Trebouxia* OTUs detected in six *Lasallia pustulata* (bottom) and six *Lasallia hispanica* (top) populations.

particular, the site located at 1480 m asl was remarkably different between the two hosts, suggesting a highly dissimilar composition of the local photobiont communities between sympatric fungal hosts.

Discussion

We studied the algal communities associated with two phylogenetically closely related but ecologically distant lichen-forming fungal hosts along a steep altitudinal gradient in the Mediterranean region using deep-coverage community DNA barcoding.

Central role of the abiotic environment in shaping *Trebouxia* community assembly

Our results show that altitude plays a prominent role in shaping *Trebouxia* community structure. At the OTU level, some *Trebouxia* taxa showed clear altitudinal preferences while others (e.g. OTU1) appeared to be indifferent to altitude. For example, OTU2 was most frequent at *c.* 1250 m asl, but diminished or disappeared at lower and higher elevations, and OTU3 was detected only at the lowest sampling sites up to *c.* 900 m asl. This altitude (*c.* 900 m) represents the ecotone between warm and cool temperate biomes on the analyzed gradient. Interestingly, *Trebouxia*

Table 2 Variation in the composition of each *Trebouxia* operational taxonomic unit (OTU) found in six *Lasallia hispanica* and six *Lasallia pustulata* populations along an elevation gradient explained by general linear mixed models

L. hispanica	st.beta	abs.beta	st.z	*P*
OTU1				
Altitude	8.781	3.673	10.724	0.116
Host haplotype	60.630	74.489	63.043	**< 0.001**
Altitude × host haplotype	30.588	21.838	26.233	**0.003**
OTU2				
Altitude	56.400	27.990	52.372	**< 0.001**
Host haplotype	17.329	49.670	29.019	0.076
Altitude × host haplotype	26.271	22.339	18.608	0.080
OTU4				
Altitude	3.846	1.881	5.329	0.369
Host haplotype	50.812	60.830	52.402	**< 0.001**
Altitude × host haplotype	45.342	37.290	42.269	**< 0.001**
OTU5				
Altitude	33.279	15.769	36.628	**< 0.001**
Host haplotype	48.040	68.409	46.814	**0.001**
Altitude × host haplotype	18.682	15.822	16.557	0.259
OTU15				
Altitude	7.718	3.861	10.109	0.255
Host haplotype	52.448	62.474	53.188	**< 0.001**
Altitude × host haplotype	39.835	33.665	36.703	**0.001**
OTU16				
Altitude	11.494	5.682	14.574	0.080
Host haplotype	50.794	62.294	50.269	**< 0.001**
Altitude × host haplotype	37.712	32.024	35.157	**0.002**
L. pustulata				
OTU1				
Altitude	39.777	15.849	22.328	**< 0.001**
Host haplotype	23.948	54.106	38.268	**0.001**
Altitude × host haplotype	36.275	30.045	39.404	**< 0.001**
OTU2				
Altitude	37.359	18.093	23.573	**0.001**
Host haplotype	23.935	44.806	30.657	0.074
Altitude × host haplotype	38.707	37.101	45.770	**< 0.001**
OTU3				
Altitude	35.515	14.836	22.630	**< 0.001**
Host haplotype	42.162	66.978	52.006	**0.016**
Altitude × host haplotype	22.323	18.186	25.364	**0.010**

abs.beta, absolute beta coefficient; st.beta, standardized beta coefficient; st.z, standardized z.
Significant *P*-values ($P < 0.05$) are marked in bold.

OTUs occurring at high-altitude sites had larger altitudinal ranges. This is apparently in agreement with the hypothesis by Stevens (1992), who extended the latitudinal Rapoport's rule to elevational distributions of species, suggesting that highland sites support species with larger altitudinal ranges. The origin of these altitudinal range differences has been attributed to the more variable climatic conditions encountered at high elevations. Rapoport's rule has been shown to apply also to microbes (marine bacteria: Sul *et al.*, 2013; fungi: Tedersoo *et al.*, 2014; Cox *et al.*, 2016; marine benthic algae: Santelices & Marquet, 1998). Future studies based on extensive taxon sampling from replicate gradients are needed to test this hypothesis for lichen-associated algae.

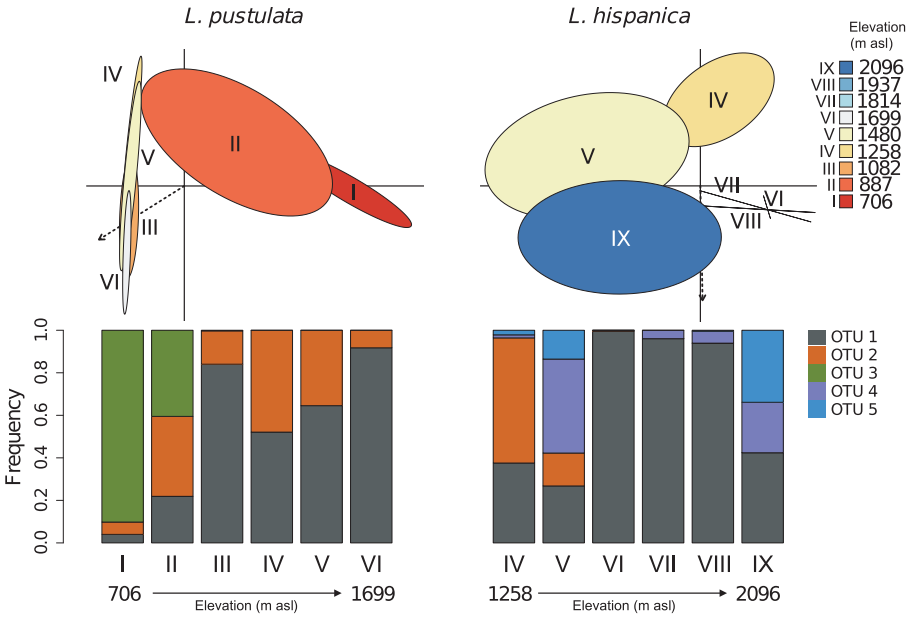

Fig. 4 Top: nonmetric multidimensional scaling (NMDS) ordinations with fitted altitude vector that describe photobiont (*Trebouxia* operational taxonomic unit (OTU)) community shifts in six *Lasallia pustulata* (left) and six *Lasallia hispanica* (right) populations along the altitudinal gradient (706 m above sea level (asl) (red) to 2096 m asl (blue)). Bottom: barplots showing the relative abundance of the *Trebouxia* OTUs detected in six *L. pustulata* (left) and six *L. hispanica* (right) populations. OTUs exhibiting < 1% relative abundance across all sets of OTUs are not shown.

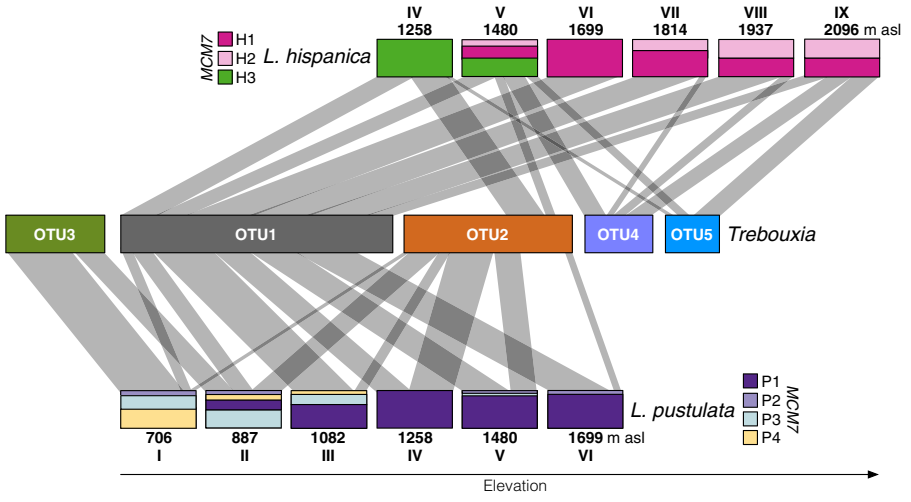

Fig. 5 Bipartite association network between the most frequent *Trebouxia* operational taxonomic unit (OTU) in each sample and its fungal hosts in six *Lasallia pustulata* and six *Lasallia hispanica* populations. Populations are represented by colored boxes showing the relative frequency of *MCM7* (DNA replication licensing factor) fungal haplotypes. OTUs exhibiting < 1% relative abundance across all sets of OTUs and fungal haplotypes having a single occurrence are not shown.

Our results suggest that the distribution of *Trebouxia* species across the landscape is shaped by the abiotic environment (environmental filtering). Changes in symbiont species abundance and community composition along latitudinal and/or altitudinal gradients have been documented for many organisms; for example, in hoopoe-associated bacteria (Ruiz-Rodríguez *et al.*, 2014), arctic-alpine vegetation (Ruotsalainen & Kytöviita, 2004), and fungal endophyte communities (Giauque & Hawkes, 2013). Our findings are also in agreement with reports indicating that lichen photobionts may display clear ecological preferences and niche differentiation. Peksa & Škaloud (2011), for instance, showed that rain and sun exposure can be used as predictors for distinguishing *Asterochloris* lineages, sister taxa to *Trebouxia*. Nadyeina *et al.* (2014) reported strong altitudinal partitioning in the distributions of different gene pools of *Symbiochloris reticulata* (Trebouxiophyceae; Škaloud *et al.*, 2016), the photobiont of the lungwort lichen. In general, the strong genetic association of lichen-associated algae, including *Trebouxia* (e.g. Yahr *et al.*, 2006; Vargas Castillo & Beck, 2012; Fernández-Mendoza *et al.*, 2011), with climatic factors and substrate (i.e. phorophyte) (*Ramalina menziesii*-associated *Trebouxia*; Werth & Sork, 2014) has been interpreted as evidence for ecological specialization (Muggia *et al.*, 2014).

Host identity and reproductive strategies affect the response of *Trebouxia* communities to altitude

Studies on symbiont diversity and community composition in lichens and other symbiotic associations have highlighted that fungal–algal community structure may not be predicted from niche properties and environment alone. Biotic factors, such as partner choice and population genetic processes of the host, may in fact play an important and, in some cases, even predominant role in determining the distribution of photobionts (Leavitt *et al.*,

2015; Magain *et al.*, 2017; Singh *et al.*, 2017). In accordance with this prediction, altitude was not the only driver of algal community assembly in our study. Our data showed that host genetic identity also played a significant role in determining algal community shifts along the gradient. This is consistent with Leavitt *et al.* (2015), who reported that host genetic identity can in some cases be the major player in shaping fungal–algal association patterns in lichens. Similar examples have been reported in other host–microbe interactions, for instance, in coral–*Symbiodinium* symbioses (e.g. Tonk *et al.*, 2013), in the foliar fungal communities of the balsam poplar (*Populus balsamifera*; Bálint *et al.*, 2013), and, recently, in the human upper airway microbiome (Igartua *et al.*, 2017). These results highlight the importance of considering host genotype-by-environment interactions when studying patterns of symbiont community shifts.

Despite being phylogenetically closely related (F. Dal Grande *et al.*, unpublished), *L. hispanica* and *L. pustulata* display clear differences in niche preference and reproductive strategies (Sancho & Crespo, 1989). These differences seem to influence the algal communities of the two hosts at both the local and whole-gradient levels. At the local level, *Trebouxia* community structure differed between the hosts. Despite abundant sharing of the most frequent OTUs present in the zone of sympatry, *L. hispanica* formed associations with a greater number of different *Trebouxia* OTUs. At the whole-gradient level, the *Trebouxia* community associated with *L. hispanica* displayed weaker altitudinal differentiation. Taking these findings together, it is tempting to propose a scenario of a *Lasallia*-*Trebouxia*-mediated guild in which the abundant, vertically dispersed, asexual reproductive units (isidia) produced by the source species (*L. pustulata*) locally scatter compatible algae on the rock surfaces (Rikkinen *et al.*, 2002; Dal Grande *et al.*, 2014b; Hestmark *et al.*, 2016). These algae are then likely to be taken up through horizontal transmission by the sink species (*L. hispanica*) during the germination of the meiotic spores. Horizontal transmission of potentially free-living *Trebouxia* directly from the environment, or of photobionts associated with adjacent thalli or propagules of other lichens (Muggia *et al.*, 2013; Dal Grande *et al.*, 2014a), may also explain the higher algal diversity and weaker altitudinal differentiation associated with *L. hispanica*. In fact, the light and highly mobile fungal spores may explore broader geographic areas during dispersal and make contact with more, potentially compatible, algae.

Although analyzing the diversity of photobionts within individual thalli was not the main goal of the present study, we observed that the majority of lichen thalli harbored more than one algal OTU. This has been interpreted as the result of two processes: the lichenization from a heterogeneous algal pool collected from the substratum (Ott, 1987) by loose hyphal webs of germinating spores or propagules; and/or the acquisition of different photobionts during the lifetime of the thallus in regions of actively dividing fungal and algal cells, for example the margins of wounds caused by lichen grazers (e.g. Asplund *et al.*, 2010). Very little is known about the mechanisms leading to intra-thalline diversity in lichens. Although a few studies have previously reported this phenomenon (e.g. del Campo *et al.*, 2013; Dal Grande *et al.*, 2014a; Moya *et al.*, 2017), lichen thalli are still predominantly considered as pair-wise interactions between a lichen-forming fungus and a single alga. Our results suggest that this view may oversimplify the complexity of lichen thalli. As the recent discovery of a potentially symbiotic lichen-associated basidiomycete (*Cyphobasidium* sp.; Spribille *et al.*, 2016) suggests the presence of more than one fungus in a lichen thallus, the presence of multiple algal species might be the rule rather than the exception. This hypothesis warrants further investigation with the aim of evaluating the impact of the sequencing platform and library preparation on the estimation of intra-thalline algal abundance, and exploring the role of intra-thalline algal plurality as a possible driver of population differentiation in lichens.

Lack of compatible photobionts is not limiting altitudinal ranges in *Lasallia* spp.

The spread of hosts arriving at new localities may be constrained by the unavailability of compatible symbionts. Thus, the inability to associate with locally adapted symbionts may determine a host's range limits (e.g. Wolfe & Pringle, 2012; Afkhami *et al.*, 2014). It has been suggested that the local lack of appropriate photobionts in lichens ('photobiont limitation') can limit the fungal host's persistence across landscapes and thus influence the distribution of lichen-forming fungi (e.g. Werth *et al.*, 2006). Our results do not support this hypothesis with respect to altitude. The distribution of the most frequent *Trebouxia* strain (OTU1) spanned the entire gradient, and there were no particular *Trebouxia* community shifts associated with the range limits of the sympatric hosts.

Environmental filtering affects both partners and thus constrains the number of possible fungal–algal combinations

The distribution of hosts and photobionts might not be related to symbiont-driven ecological expansion, but rather to environmental filtering of both hosts and photobionts combined with their individual demographic history and adaptive potential. This might result in the formation of geographically structured host–symbiont combinations experiencing different selection pressure as a result of differences in regional biotic and abiotic factors (geographic mosaic of coevolution; Thompson, 2005). In support of this hypothesis, we showed in a recent study that the fungal partners in a metapopulation of *L. pustulata* along an altitudinal gradient consisted of locally adapted ecotypes (Dal Grande *et al.*, 2017). Interestingly, the ecotone between warm and cool temperate biomes corresponds to the upper limit of the distribution for both the warm-adapted *Lasallia* ecotype and *Trebouxia* OTU3. Furthermore, the warm-adapted *Lasallia* ecotype and *Trebouxia* OTU3 occupy the same ecological niche at the continental scale (G. Rolshausen *et al.*, unpublished).

Here we studied patterns of lichen-associated algal community shifts along an altitudinal gradient. Consistent with findings in other symbioses such as sponges and their microbiome (Webster & Thomas, 2016), our results support the hypothesis that the abiotic environment may act as a filter for both partners of the symbiosis and that demographic processes in the host population

may additionally shape algal association patterns. Establishment and maintenance of these symbiotic partnerships might thus be the result of the interplay between species-specific tolerances to environment and host–symbiont genetic background. These factors may lead to the formation of a geographic mosaic of host and symbiont populations across different environments (Thompson, 2005; Fedrowitz *et al.*, 2012).

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Author contributions

F.D.G. and I.S. designed the research; F.D.G., J.O., P.K.D. and A.C. collected the data; G.R. and M.S. contributed scripts; F.D.G., G.R. and M.S. analyzed data; F.D.G. and I.S. wrote the manuscript with contributions from the other authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Principal component analysis (PCA) of axes 1 (PC1, horizontal) and 2 (PC2, vertical) of 19 BIOCLIM climatic variables and elevation for the nine sampling sites of *L. pustulata* and *L. hispanica*.

Fig. S2 Organization of the ITS locus and target regions of the newly designed primers FDGITS2-f/r.

Fig. S3 Phylogenetic placement of the seven *Lasallia*-associated *Trebouxia* OTUs (using 69 *Trebouxia* OTUs).

Fig. S4 Phylogeny of the seven *Lasallia*-associated *Trebouxia* OTUs (using the top 10 NCBI NT BLAST hits; $n = 54$).

Fig. S5 ITS2 secondary structure of the seven *Lasallia*-associated *Trebouxia* OTUs.

Fig. S6 NMDS ordinations of *Trebouxia* OTU community shifts in nine sampling sites (left) or 12 populations (six for each host).

Table S1 Climatic data for 19 variables from the WORLDCLIM database for the nine sampling sites

Table S2 Per-sample read number, multiplex label, and associated fungal *MCM7* haplotype with accession number

Table S3 *Trebouxia* OTU abundances associated with *L. hispanica* and *L. pustulata*

Table S4 List of lichen-forming fungal hosts associated with identical *Trebouxia* strains

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