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Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae)

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Abstract

The distribution patterns of symbiotic algae are thought to be conferred mainly by their hosts, however, they may originate in algal environmental requirements as well. In lichens, predominantly terrestrial associations of fungi with algae or cyanobacteria, the ecological preferences of photobionts have not been directly studied so far. Here, we examine the putative environmental requirements in lichenized alga *Asterochloris*, and search for the existence of ecological guilds in *Asterochloris*-associating lichens. Therefore, the presence of phylogenetic signal in several environmental traits was tested. Phylogenetic analysis based on the concatenated set of internal transcribed spacer rDNA and actin type I intron sequences from photobionts associated with lichens of the genera *Lepraria* and *Stereocaulon* (Stereocaulaceae, Ascomycota) revealed 13 moderately to well-resolved clades. Photobionts from particular algal clades were found to be associated with taxonomically different, but ecologically similar lichens. The rain and sun exposure were the most significant environmental factor, clearly distinguishing the *Asterochloris* lineages. The photobionts from ombrophobic and ombrophilic lichens were clustered in completely distinct clades. Moreover, two photobiont taxa were obviously differentiated based on their substrate and climatic preferences. Our study, thus reveals that the photobiont, generally the subsidiary member of the symbiotic lichen association, could exhibit clear preferences for environmental factors. These algal preferences may limit the ecological niches available to lichens and lead to the existence of specific lichen guilds.

Keywords: adaptation, algae, *Asterochloris*, coevolution, ecology, fungi, *Lepraria*, lichen guilds, ombrophoby, photobiont, phylogenetic signal, symbiosis

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Introduction

A number of aquatic as well as terrestrial algae and cyanobacteria live in various symbiotic associations. In particular, they play a role of endosymbionts in heterotrophic hosts—protists (ciliates) and invertebrates (scleractinian corals, sea anemones, sponges, green hydras)—or they inhabit the lichen thalli formed by lichen-forming fungi (ascomycetes or basidiomycetes).

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In the case of corals and lichens, the nature of the symbiosis can be described as controlled parasitism whereby the host (exhabitant) actively 'farms' its domesticated autotrophic partner (Ahmadjian & Jacobs 1981; Lücking *et al.* 2009; Wooldridge 2010). Within such an association, a photosynthetic partner (photobiont) releases a substantial part of photosynthates to its heterotrophic partner. Furthermore, some cyanobacteria supply their host with the nitrogen fixed from the atmosphere.

The symbiotic associations exhibit a distinct measure of specificity of their symbiotic partners (the degree of taxonomic difference among partners with which an

organism associates, Smith & Douglas 1987). Typically, marine invertebrates, such as reef corals and sea anemones, associate with unicellular dinoflagellate algae from the genus *Symbiodinium* (e.g. Muller-Parker & Davy 2001; Coffroth & Santos 2005), although an exception to this rule was recently described by Letsch *et al.* (2009). Similarly, autotrophic symbionts of different freshwater protozoa and invertebrates are known to be members of various green algae (Pröschold *et al.* 2010). In lichen-forming fungi, many genera, or even families, were found to be exclusively associated with terrestrial green alga *Trebouxia*, or cyanobacterial genus *Nostoc* (Miadlikowska *et al.* 2006). However, in many cases the specificity of partners has been revealed as low at the level of species or populations. The exhabitant species can associate with multiple lineages (species) of compatible algae or cyanobacteria and they are able to switch between them (e.g. Friedl 1987; Ulstrup & van Oppen 2003; Blaha *et al.* 2006; Guzow-Krzemińska 2006; Abrego *et al.* 2009; Bačkor *et al.* 2010). Simultaneously, particular photobiont lineage was found in more than one species of the host, i.e. several hosts can share the same alga or cyanobacterium (e.g. Piercey-Normore & DePriest 2001; Beck *et al.* 2002; Fabricius *et al.* 2004; Yahr *et al.* 2004; Doering & Piercey-Normore 2009; Finney *et al.* 2010).

What are the reasons for switching between symbiotic partners? Different photobionts have been detected in the host species or communities growing in different environmental conditions. In case of the coral-alga associations, differences in irradiance and temperature have been found to affect the composition of the *Symbiodinium* community. The light-dependent distribution of individual *Symbiodinium* lineages within the coral colonies has been reported in several studies (Rowan & Knowlton 1995; Rowan *et al.* 1997; van Oppen *et al.* 2001). Sampayo *et al.* (2007) found two coral species associated with multiple symbiont profiles that showed a strong zonation with depth (irradiance). Similarly, Finney *et al.* (2010) showed that habitat depth and geographic isolation appeared to influence the bathymetric zonation and regional distribution for most *Symbiodinium* species.

Interestingly, analogical patterns have been reported from terrestrial conditions—in lichens. Different lineages of *Trebouxia* algae have been found in tropical and temperate lichens (Cordeiro *et al.* 2005). Fernández-Mendoza *et al.* (2011) revealed that a considerable fraction of the genetic variation in the photobiont of a widespread lichen *Cetraria aculeata* could be explained by climate (they found differences between polar and temperate populations). The occurrence of different photobionts along the gradient of altitude (climate) has been reported for crustose epilithic lichens (Blaha *et al.* 2006; Muggia *et al.* 2008) as well as fruticose epiphytic lichens (Kroken & Taylor 2000). In the lichen family Physciaceae, Helms (2003) revealed the photobiont phylogeny to be more closely correlated with environmental factors than the phylogeny of the host fungi. According to his results, two *Trebouxia* lineages predominantly occurred in the tropics. Moreover, photobionts from basiphilous lichens growing on calcareous rocks formed a single lineage distinct from that of photobionts detected in acidophytic lichens. Interesting pattern in distribution of lichenized cyanobacteria in lichen communities associated with old-growth forests was described by Rikkinen *et al.* (2002), who found that *Nostoc* strains from epiphytic lichens were genetically separated from the strains associated with lichens growing on the ground. Such pattern forms a system of lichen guilds (the communities of lichens growing in the same habitat, sharing the same photobionts).

Thus, the host probably seeks to obtain a photobiont well adapted to local conditions because only a thriving autotrophic partner, exhibiting maximum photosynthetic activity, can nourish its host effectively. The acquisition of such a photobiont can increase the fitness of the host as well as of the whole association (the holobiont).

According to this hypothesis, autotrophic symbionts, generally the subsidiary members of the symbiotic associations could show their own preferences for environmental factors and influence the distribution of their hosts.

The aim of this study was to test the existence of environmental preferences in symbiotic green alga *Asterochloris* associated with lichen-forming fungi *Lepraria* and *Stereocaulon*, and to search for the existence of ecological guilds in these green algal lichens. *Lepraria* and its sister taxon *Stereocaulon* (Stereocaulaceae, Ascomycota) represent two genera known for their prevailing specificity to *Asterochloris* algae (Piercey-Normore & DePriest 2001; Nelsen & Gargas 2006, 2008). The members of *Lepraria* are completely sterile, morphologically simple lichenized fungi with cosmopolitan distribution (Orange & Laundon 2009). Most of the species are variable in their requirements to the substrate type and climate; however, two distinct groups could be defined within the genus, based on their relationship to liquid precipitation: the ombrophiles and ombrophobes. Interestingly, the latter strategy represents the predominant lifestyle within *Lepraria*. Such ombrophobic species grow in fully rain-sheltered sites, often with high air humidity and low illumination where the vapour is the only available source of water (e.g. rock overhangs, some patches on tree trunks). The ability to survive under such specific conditions is probably provided by their morphological adaptation: they posses very simple

thallus lacking complex structures, which is evidently very effective in the absorption of water from the air (such adaptation is known also in other lichens growing under similar conditions, e.g. *Chaenotheca*, *Chrysothrix*, *Psilolechia*).

The specific water conditions as well as the lower illumination definitely influence the photosynthesis of the symbiotic algae that is fundamental for the life of the lichen. Thus, an adaptation of the photobiont seems to be necessary for the successful survival of the lichen in rain-sheltered habitats. In contrast, a life on surfaces exposed to the rain and direct sun light requires tolerance of the symbionts to desiccation, temperature extremes and high light intensities (Beckett *et al.* 2008). Therefore, we hypothesized that the ombrophilic and ombrophobic lichens should host different algal genotypes. In addition, we searched for the substrate and climatic preferences of selected photobionts.

Material and methods

Taxon sampling

Lichen samples were collected in Europe (predominantly in central part) and North America (California). The sampling sites represented various habitats (diverse rock outcrops, boulder screes, forest and roadside trees, etc.) up to 2440 m above sea level (a.s.l). The sampling was long-term (2003–2008) and occasional, preferring neither habitat type nor lichen taxa (except the tendency to collect ombrophilic as well as ombrophobic *Lepraria* specimens, see below). Lichen specimens were deposited in the herbaria PL (collection of O. Peksa) and PRA (Š. Slavíková-Bayerová, Z. Palice). The data set was expanded by sequences of photobionts from GenBank (see below). Information on all specimens used in the study is included in Table S1, (Supporting information).

Study species

A total of 104 *Lepraria* s.str. and 3 *Stereocaulon* samples were collected. Lichens were identified using conventional lichenological methods; all *Lepraria* specimens were analysed using thin-layer chromatography on Merck silica gel 60 F254 pre-coated glass plates in solvent systems A, B and C, according to Orange *et al.* (2001). Complete data on the secondary chemistry of investigated specimens are available from the first author.

For the present study, we accepted the distinction among the principal *Lepraria* species based on differences in their morphology and secondary chemistry (Saag *et al.* 2009). Within our samples, we distinguished 11 *Lepraria* phenotypic species; the sequences obtained from GenBank represented five other species. Within *Lepraria* taxa identified, variability in secondary product chemistry was detected, especially in *L. caesioalba*. *Lepraria* specimens containing only atranorin and angardianic/roccellic acid as their main substances were denoted *Lepraria* sp.*, reflecting different opinions on the correct taxonomic classification of this chemotype (Leuckert *et al.* 1995; Lohtander 1995; Tønsberg 2004; Saag *et al.* 2007). Ombrophilic *Lepraria* (growing on rain/sun-exposed surfaces) were represented by six closely related species from *L. neglecta* 'core group' *sensu* Fehrer *et al.* (2008): *L. alpina*, *L. borealis*, *L. caesioalba* (var. *caesioalba sensu* Saag *et al.* 2009), *L. granulata*, *L. neglecta*, *L.* sp.*; and the species *L. nylanderiana*. Ombrophobic specimens of *Lepraria* belonged to the unrelated species *L. crassissima*, *L. caesiella*, *L. cupressicola*, *L. incana*, *L. lobificans*, *L. membranacea*, *L. nivalis*, *L. rigidula* and *L. yunnaniana*.

The samples of the *Stereocaulon* (completely ombrophilic) belonged to eight morphospecies, representing five different phylogenetic lineages (Högnabba 2006): *S. botryosum*, *S. dactylophyllum*, *S. paschale*, *S. pileatum*, *S. saxatile*, *S. subcoralloides*, *S. tomentosum* and *S. vesuvianum* (the remaining three samples were only incompletely determined as *Stereocaulon* sp.)

DNA isolation, polymerase chain reaction (PCR) amplification and sequencing

Total genomic DNA was extracted from 46 algal cultures (isolated from *Lepraria* and *Stereocaulon* specimens; see Table S1, Supporting information) and 61 lichen thalli following the standard CTAB protocol (Doyle & Doyle 1987), with minor modifications. DNA was re-suspended in sterile dH_2O and amplified by PCR. The internal transcribed spacer ITS1-5.8S-ITS2 rDNA region was amplified using the algal-specific primer nr-SSU-1780-5′ (5′-CTGCGGAAGGATCATTGATTC-3′; Piercey-Normore & DePriest 2001) and a universal primer ITS4-3′ (5′-TCCTCCGCTTATTGATATGC-3′; White *et al.* 1990). Actin type I locus (1 complete exon and two introns located at codon positions 206 and 248; Weber & Kabsch 1994) was amplified using the algal-specific primers ActinF2 Astero (5′-AGCGCGGGTA CAGCTTCAC-3′) and ActinR2 Astero (5′-CAGCACT TCAGGGCAGCGGAA-3′; Skaloud & Peksa 2010). All PCR reactions were performed in 20 μL reaction vols (15.1 μL sterile Milli-Q Water, 2 μL 10′ PCR buffer (Sigma), 0.4 μL dNTP (10 μM), 0.25 μL of primers (25 pM/mL), 0.5 μL Red Taq DNA Polymerase (Sigma) (1 U/mL), 0.5 μL of $MgCl_2$ (25 mM), 1 μL of DNA (not quantified). PCR and cycle-sequencing reactions were performed in either a XP thermal cycler (Bioer) or a

Touchgene gradient cycler (Techne). PCR amplification of the algal ITS began with an initial denaturation at 95 °C for 5 min, and was followed by 35 cycles of denaturing at 95 °C for 1 min, annealing at 54 °C for 1 min and elongation at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Identical conditions were used for the amplification of the actin I locus, except that an annealing temperature of 60–62 °C was used. The PCR products were quantified on a 1% agarose gel stained with ethidium bromide and purified using either the JetQuick PCR Purification kit (Genomed) or the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's protocols. The purified amplification products were sequenced with an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730XL) using the PCR primers from Macrogen Corp. (Seoul, Korea). Sequencing readings were assembled and edited using SeqAssem program (SequentiX Software).

Sequence alignment and DNA analyses

Sequences were initially aligned using MUSCLE alignment software (Edgar 2004). Photobiont sequences from 26 *Lepraria* specimens and 14 *Stereocaulon* specimens deposited in GenBank were acquired and included in the alignment. For the *Lepraria*, we included only those GenBank photobiont sequences acquired from lichens determined at the species level. In total, we used 147 ITS rDNA and 60 actin type I sequences (see Table S1, Supporting information). After deleting identical sequences obtained from the same lichen taxa, the resulting concatenated alignment comprised 64 sequences (including 64 ITS rDNA and 38 actin type I locus sequences; missing actin data were replaced with question marks according to Rannala & Yang 2003). ITS sequences were aligned on the basis of their rRNA secondary structure information, the alignment of actin I locus sequences has been improved through comparison of ClustalW alignments produced under different gap opening/extension penalties using SOAP version 1.2 alpha 4 (Löytynoja & Milinkovitch 2001). For detailed information about alignment improvement see Skaloud & Peksa (2010). The resulting concatenated alignment had a length of 1173 characters (ITS, 514; actin, 659; available from the second author upon request). The congruence of data partitions that allows their merging into a concatenated alignment has been previously justified by inspecting bootstrap scores above 70% resulting from separate maximum likelihood (ML) and maximum parsimony (MP) analyses of the ITS and actin data set (Skaloud & Peksa 2010).

Bayesian inference (BI) was performed with MrBayes version 3.1 (Ronquist & Huelsenbeck 2003). The alignment was divided into six region partitions (ITS1, ITS2, 5.8S rRNA, actin intron 206, actin intron 248, actin exon), and for each partition the most appropriate substitution model was estimated using the Akaike Information Criterion with PAUP/MrModeltest 1.0b (Nylander 2004). Posterior probabilities were calculated using a Metropolis-coupled Markov chain Monte Carlo approach (MCMC). Two parallel MCMC runs were carried out for 3 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. The stationary distribution of the runs was confirmed by checking average standard deviations of split frequencies between the two analyses, which approached zero. Convergence of the two cold chains was checked and burn-in was determined using the 'sump' command.

Bootstrap analyses were performed by ML and weighted parsimony (wMP) criteria using PAUP* version 4.0b10 (Swofford 2002). ML analyses (100 replicates) consisted of heuristic searches using the neighbour-joining tree as the starting tree, tree bisection reconnection swapping algorithm and number of rearrangements limited to 10 000. The analysis was conducted using unpartitioned alignment with GTR + Γ + I model. The wMP analyses (1000 replicates) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences (the number limited to 10 000 for each replicate), and gap characters treated as missing data. Bootstrap percentages and posterior probabilities were interpreted as weak (<50%), moderate (50–94% for BI; 50–79% for ML and MP) or high (>94% for BI; >79% for ML and MP).

Analyses of ecological relationships

The following environmental data were collected for each lichen sample: exposure to rain (exposed/sheltered), altitude (m.a.s.l.) and type of substrate (woodbark; basic type of bedrock—basalt, gneiss, granite, sandstone, shale and serpentine—coded as a set of dummy variables). To reconstruct the evolution of ombrophoby (see below), we assigned the samples obtained from GenBank using the common knowledge of this character in the investigated lichens (e.g. Laundon 1992; Aptroot *et al.* 1997; Slavíková-Bayerová & Fehrer 2007; Saag *et al.* 2009; Smith *et al.* 2009).

To analyse possible ecological preferences of particular photobiont lineages, we conducted three different tests for the existence of phylogenetic signal in our data (according to Blomberg *et al.* 2003, the phylogenetic signal is the tendency for related species to resemble each other). All calculations were performed in the program R, version 2.9.2 (The R Foundation for Statistical Computing 2009, http://www.r-project.org/). First, we tested the phylogenetic signal using Pagel's λ (Pagel

1999). This test uses a tree transformation parameter that has the effect of gradually eliminating phylogenetic structure. The maximum-likelihood optimization of λ value was performed using the 'fitDiscrete' or 'fitContinuous' functions of the Geiger package (Harmon *et al*. 2008). To test for the existence of phylogenetic signal in the data set, we compared the negative log likelihoods obtained from a tree without phylogenetic signal and the original topology, using likelihood ratio test. Second, the phylogenetic signal was tested using the *K* statistic (Blomberg *et al*. 2003). This statistic quantifies the phylogenetic signal by estimating the accuracy of the original phylogeny to describe the variance-covariance pattern observed in the data test. The *K* value and randomization test were calculated by '*K*calc' and 'phylosignal' functions of the Picante package (Kembel *et al*. 2010).

Finally, the existence of phylogenetic signal was tested by searching for significant ecological similarity in selected sets of closely related organisms (organisms with short genetic distance), using our simple customized *R* script (see Appendix S1, Supporting information). The ecological similarity was evaluated as the sum of Euclidean distances of the environmental data. The small value of sum of Euclidean distances signified high ecological similarity of the examined samples (if all samples had the same value of ecological factor, the sum of Euclidean distances would be zero).The genetic distances were calculated using Kimura 2-parameter substitution model on the concatenated data using MEGA4. The distances of environmental data were calculated using PAST, version 1.90 (Hammer *et al.* 2001) using Euclidean distances in a similarity/distance tool.

First, we specified the genetic distance which delimits closely related strains by analysing the histogram of frequency distribution of pairwise genetic distances. The apparent gap in the histogram around the distance of 0.04 led us to select this value to define the closely related strains belonging to one, or seldom two, phylogenetic lineages as revealed by Bayesian phylogenetic analysis (Fig. S1, Supporting information). Next, the sum of Euclidean distances of environmental data was calculated for the set of photobiont pairs whose genetic distances were lower than the selected value delimiting the closely related strains. Finally, the existence of phylogenetic signal (i.e. significant ecological similarity in closely related strains) was tested by non-parametric permutation of all photobiont pairs (100 000 replicates).

As all the above-mentioned tests demonstrated the existence of phylogenetic structure in our data, we used the program BayesTraits (Pagel & Meade 2006), which combines Bayesian and maximum-likelihood based approaches, to test the contingency of character evolution. First, the evolution of ombrophoby was reconstructed using BayesMultiState in an ML framework over all common ancestors (using the 'addNnode' command). We adjusted the 'Mltries' parameter to 100 to increase the number of optimization attempts. The BayesTraits output was mapped onto the reference tree with TreeGradients version 1.03 (Verbruggen 2009). This program plots ancestral state probabilities on a phylogenetic tree as colours along a colour gradient. Second, the ancestral state probabilities of selected environmental parameters (types of substrate) were calculated for the most common ancestors of all highly supported clades.

Some relationships among ecological factors and photobionts were also examined using descriptive statistics (box plots) in Statistica version 8 (Statsoft Inc.) (Hill & Lewicki 2007) and Principal Component Analysis in Canoco for Windows version 4.5 (ter Braak & Šmilauer 1998).

Results

Phylogenetic analysis

Data on length, variability and base composition of the molecular markers as well as the evolutionary models estimated for each partition can be found in Table S2, (Supporting information). Substantial differences were revealed in the sequence variability and estimated substitution models among the individual partitions. Whereas the whole ITS rDNA data set comprised only 48 parsimony informative sites, both actin intron partitions were quite rich in variable sites (112 and 162 parsimony informative sites, respectively).

The concatenated alignment contained sequences from 130 *Lepraria* and 17 *Stereocaulon* specimens (only single photobiont genotype was obtained from each lichen specimen). The phylogram resulting from Bayesian analysis of ITS rDNA and actin type I sequences is presented in Fig. 1. All *Lepraria* and *Stereocaulon* samples were found to associate with green algae from the genus *Asterochloris*. The most of analysed lichen photobionts were clustered in 13 moderately to well-supported clades (see Discussion), 11 samples remained unclassified. Three of the clades could be assigned to the formally described phenotypic species: *A. phycobiontica* (clade A1), *A. glomerata* (clade A12) and *A. irregularis* (clade A13); the unclassified sequence AM905993 originates from the type strain of *A. excentrica* isolated from *S. dactylophyllum*.

The most frequently occurring photobionts belonged to the clades A7 and A10, containing 19% and 20%, respectively, of all samples. On the other hand, the clades A4, A6 and A9, comprised of sequences from two to three lichen samples, represented the least

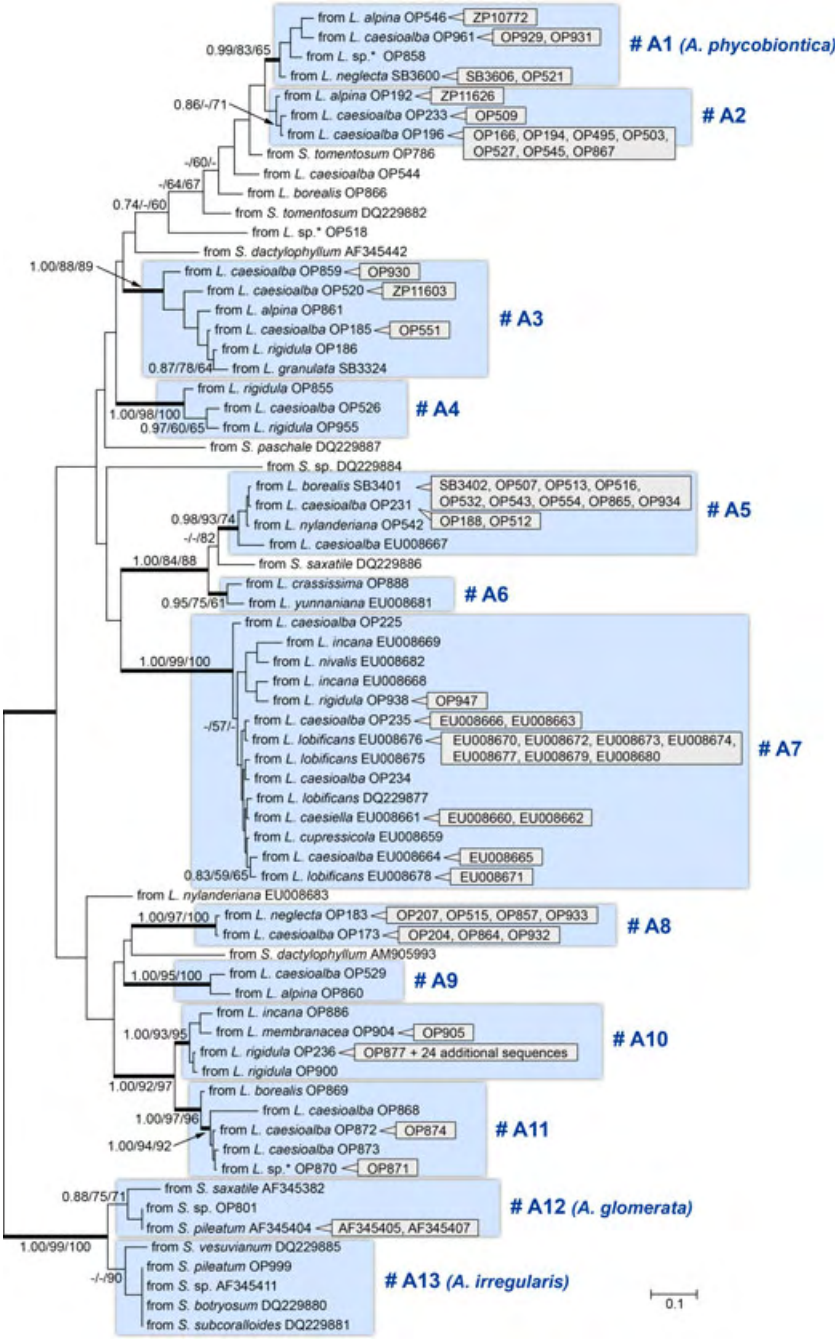

Fig. 1 Unrooted BI analysis of *Asterochloris* photobionts based on the combined ITS + actin data set. The analysis used a HKY + I model for ITS1 and ITS2, F81 model for 5.8 rRNA partition, a HKY + G model for the actin-intron 206, GTR + G model for the actin-intron 248 and K80 + I model for the actin-exon partition. Values at the nodes indicate statistical support estimated by three methods—MrBayes posterior node probability (left), maximum-likelihood bootstrap (middle) and maximum parsimony bootstrap (right). Support values are displayed only for nodes with BI/ML/MP supports of ≥0.70/50/50. Thick branches represent nodes with a posterior probability ≥0.95. The affiliation of strains to the 13 lineages is indicated (the clade labelling does not correspond to that in our previous study; see Table S1, Supporting information). The additional, identical photobiont sequences are shown in grey boxes to the right of the sequence used for the analysis. Our samples are labelled by initial letters of the lichen collectors followed by their collection number: OP—O. Peksa, SB—Š. Slavíková-Bayerová, ZP—Z. Palice (cf. Table S1, Supporting information); additional sequences are labelled by GenBank Accession nos of ITS rDNA sequences. Scale bar—expected number of substitutions per site.

abundant algae. The remaining clades contained nine samples on average.

Specificity of lichen associations

We analysed photobionts from 16 *Lepraria* phenotypic species (including *Lepraria* sp.*; see Material and methods) and 8 *Stereocaulon* species (excluding three incompletely determined samples *Stereocaulon* sp.). The degree of specificity varied among both photobiont lineages and fungal species. Each algal lineage was shared by at least two (clades A2, A4, A6, A8, A9), and up to eight (clade A7) fungi (see Fig. 1). Photobionts from the clades A1, A2, A8, A9 and A11 were found exclusively in closely related fungi from *L. neglecta* 'core group' (see Material and methods); in addition to this group the clades A3 and A5 associated with another *Lepraria* species (*L. rigidula* and *L. nylanderiana*, respectively). Each of the clades A4, A6, A7 and A10 contained photobionts of two or more unrelated *Lepraria* species. Clades A12 and A13 were found to be associated with several *Stereocaulon* species. Thus, we did not observe any sharing of algal lineages between the two analysed fungal genera *Lepraria* and *Stereocaulon*.

The majority of fungal species were associated with a polyphyletic assemblage of algae from several clades (e.g. different samples of *L. alpina* with clades A1, A2, A3 and A9). Only three *Lepraria* species (from those represented by at least 10 specimens) were found to be associated with one individual algal clade with an apparently higher frequency: *L. borealis* (clade A5), *L. lobificans* (clade A7) and *L. rigidula* (clade A10).

Environmental preferences of photobionts

To analyse possible environmental preferences of photobionts, the presence of phylogenetic signal in three environmental traits was examined (exposure to rain, altitude and substrate type). For each trait, Pagel's λ and *K* statistics were calculated to show the influence of inferred phylogeny (Fig. 1) on trait variance across photobiont strains. Both methods revealed significant phylogenetic signal in all traits (Table 1).

Moreover, we devised additional methods to test for the existence of phylogenetic signal by searching for significant ecological similarity in closely related strains (for details of the method see Material and methods). In the presence of phylogenetic signal, the photobionts having short genetic distances from each other (i.e. closely related) should be ecologically similar. Accordingly, environmental preferences of photobionts were detected by comparing the similarity of environmental data of genetically close photobiont pairs to that of genetically distant pairs (the value of genetic distance distinguishing closely related and distant algal strains was identified at 0.04). We detected significant ecological similarity in the set of photobiont pairs with short genetic distances (Table 1).

All environmental traits showed significant phylogenetic signal, whichever method was employed. In other words, closely related photobionts tend to be similar in each environmental characteristic: they occurred in lichens growing in habitats characterized by similar water regime (rain-exposed or rain-sheltered surfaces), similar climate (limited range of altitudes) and similar type of substrate.

To illustrate the ecological preferences of *Lepraria* photobionts more clearly, we mapped the evolution of a selected ecological character—the relationship to precipitation—onto the phylogenetic tree (Fig. 2). The relationship of the lichen to liquid water was chosen as an ideal character for the evolutionary mapping because it can have usually only two aspects: a lichen thallus grows either on exposed or sheltered surface, i.e. it is either ombrophilic or ombrophobic (rarely, some species can grow in intermediate conditions,

Table 1 Statistics for randomization tests showing the significance of phylogenetic signal for three environmental traits investigated. For each trait, Pagel's λ, *K* statistics, and ecological similarity among closely related strains (our method) were calculated to show influence of inferred phylogeny on trait variance across *Asterochloris* strains. λ values could vary from 0 (no influence of phylogeny) to 1 (strong phylogenetic influence). Likelihood ratio indicates comparison of the log-likelihoods of a model with the maximum-likelihood estimate of λ for a given trait to the log-likelihood of a model where λ was set to zero. The *K* values indicate how closely the species trait correlated to its phylogeny, as expected under Brownian motion (higher *K* values mean better correlation). Ecological similarity was tested in the set of photobiont pairs with short genetic distances (lower than 0.04)

	Pagel's λ			*K* statistics		Ecological similarity
Trait	λ	Likelihood ratio	*P*-value	*K* value	*P*-value	*P*-value
Exposure to rain	0.946	1.53	<0.0001	0.2126	0.001	<0.0001
Altitude	0.045	1.01	<0.0001	0.0832	0.005	<0.0001
Substrate type	0.652	1.05	0.0011	0.1168	0.002	<0.0001

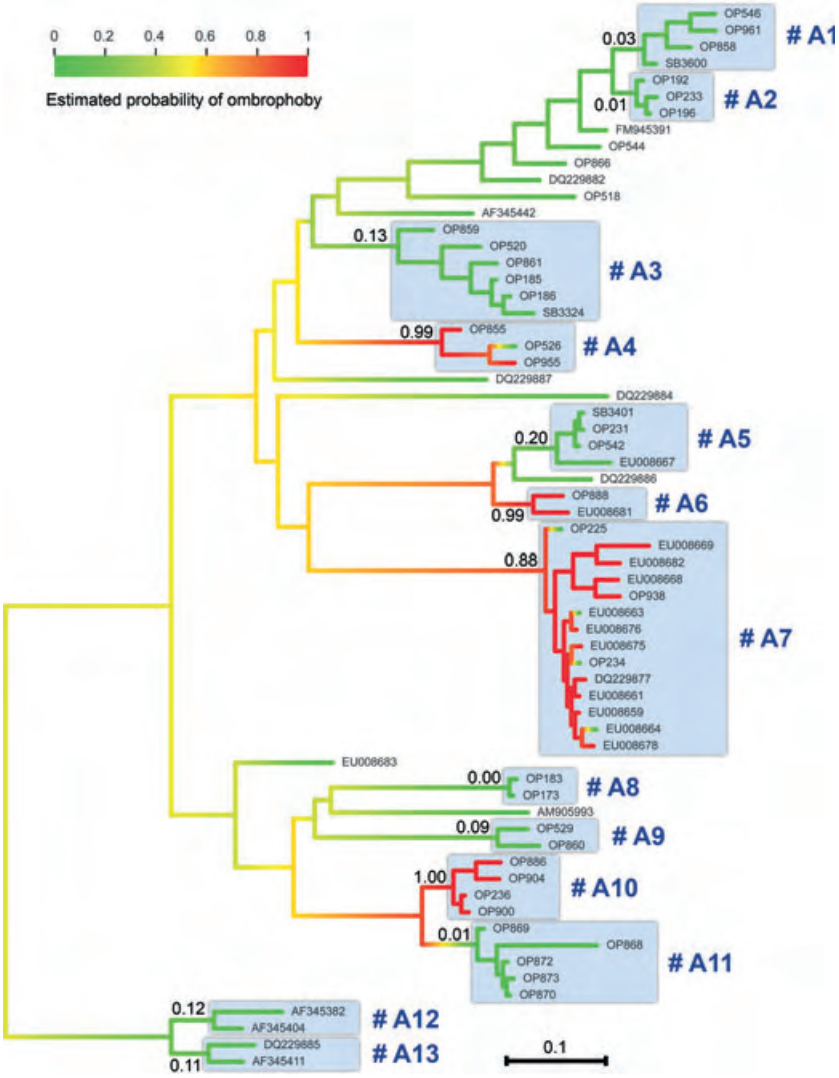

Fig. 2 The evolution of the selected ecological character—the ombrophoby (ombrophily) of lichens—mapped onto the photobiont phylogenetic tree. Colours are used to visualize estimated probabilities of the presence of ombrophoby along the phylogenetic tree. Red indicates a high probability of ombrophoby, whereas green denotes a low probability of ombrophobic preference. The estimated probabilities for ombrophoby are indicated for ancestors of each significantly supported clade (see Fig. 1). The topology of the tree corresponds completely to the topology of the phylogenetic tree in Fig. 1.

such as rock fissures; see Discussion). In total, we had 92 photobiont sequences from ombrophilic lichens and 55 sequences from ombrophobic lichens. The result of character mapping clearly showed the prevailing presence of clades A4, A6, A7 and A10 in ombrophobic *Lepraria* species (with ancestral probabilities for ombrophoby 0.99, 0.99, 0.88 and 1.00, respectively). The other lineages (including all samples outside the supported clades) were completely associated with ombrophilic lichens.

Among all samples, two interesting cases have occurred, indicating the sensitivity of photobiont to the water and light regime in its (micro) habitat. First, the sample OP186 (clade A3) was labelled as ombrophilic (see Fig. 2), although it originated from the common ombrophobic species *L. rigidula*. However, this specimen occurred abnormally on bryophytes covering the rain-exposed edge of a rock. Interestingly, the ombrophilic character of this sample coincided accurately with the nature of all other samples of the clade A3. Second, the sample OP526 from the ombrophilic *L. caesioalba* (clade A4) was collected from the upper surface of a boulder sheltered by deciduous oak tree. The sample was labelled as ombrophilic because the thallus was not fully sheltered against precipitation (especially in winter). However, it clustered with strictly ombrophobic samples in the clade A4. Probably, the specific water and light conditions under the tree-top corresponded

rather to rain/sun-sheltered habitats. In both cases, the photobiont type correlated with the environmental conditions rather than with the mycobiont nature.

In addition to water conditions, some clades were found to be completely dissimilar in their altitude and substrate preferences. The algae of the clade A1 (*A. phycobiontica*) occurred predominantly in regions with altitudes of about 1500 m, whereas members of the clade A5 were found mostly in areas approximately 450 m a.s.l. (Fig. 3). Moreover, both these clades were correlated with different substrates, as shown by the principal component analysis (Fig. 4). Both were found in ombrophilic lichens collected from bryophytes, soil or directly from rock surface, however, photobionts of the clade A1 were rather in lichens growing in habitats of acidic siliceous rocks (rocks and screes from granite or gneiss), whereas lichens housing photobionts of the clade A5 preferred SiO_2-poor rocks (shales or basalts). In addition, the clade A10 was most often associated with ombrophobic lichens growing on the bark of broadleaf trees.

To test the substrate preferences of individual clades, we modelled the evolution of the preference for each substrate and inferred ancestral probabilities, for each of the 13 well-supported clades (Table 2). According to the inferred ancestral probabilities, some lineages exhibited significant substrate preferences towards shale, wood and basalt, whereas for granite-gneiss, serpentine and sandstone no apparent preferences have been detected along the entire phylogenetic tree. The analysis confirmed high affinity of the clades A5 and A10 to shale and wood, respectively. Conversely, negative substrate preferences were detected for the clades A2 (shale and wood), A8 (shale) and A11 (wood).

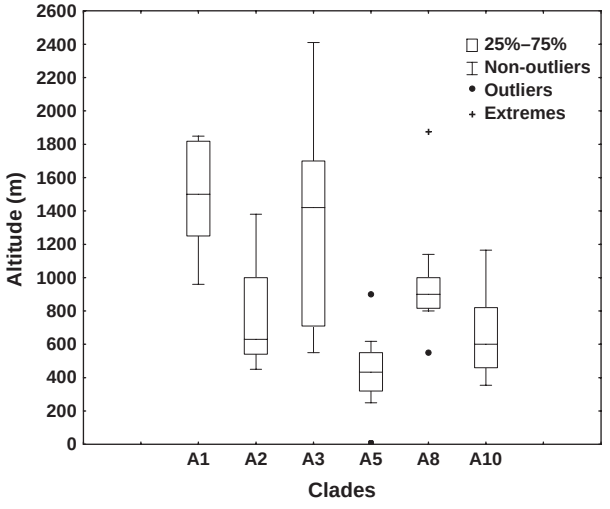

Fig. 3 Differences in the distribution of selected *Asterochloris* clades along the altitudinal gradient. Box and whisker plots are based on altitudinal data from six clades (only those represented by at least eight samples). All samples were collected in similar latitudes in Europe (43–58°N, i.e. somewhere in the temperate belt). The approximate upper borders of vertical vegetation belts for central Europe are as follows: 200 m a.s.l.—lowland, 600 m—colline, 1000 m—submontane, 1400 m—montane, 1800 m—subalpine, 2400 m—alpine.

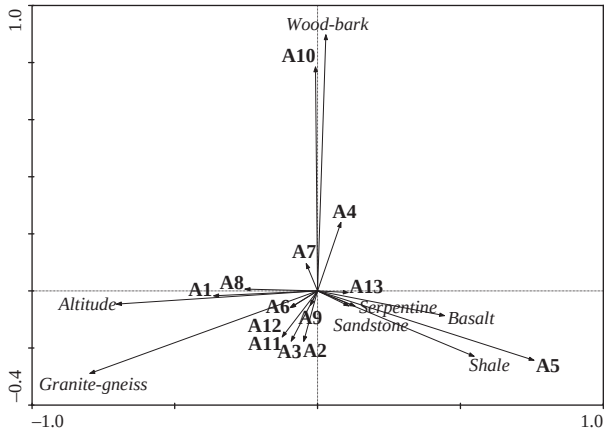

Fig. 4 The distribution of the *Asterochloris* clades (A1–A13) in relation to environmental factors: altitude and type of substrate. Ordination plot of the principal component analysis based on 82 samples of *Lepraria* and *Stereocaulon* with complete environmental data. Note: the substrates with nearly similar character—acidic siliceous rocks *granite* and *gneiss* as well as *wood* and *bark*—were used as combined variables in the analysis.

Discussion

Lepraria and *Stereocaulon* represent two closely related genera of lichen-forming ascomycetes (Ekman & Tønsberg 2002; Myllys *et al.* 2005). We found these fungi to be exclusively specific to the green-algal genus *Asterochloris*. Thus, we could not confirm the findings of Beck (2002) and Engelen *et al.* (2010) who found them to be associated also with *Chloroidium* (*S. nanodes*) and *Trebouxia* (*L. borealis*), respectively.

In ~25 lichen morphospecies, relatively high diversity of photobionts was revealed. In addition to 11 well-supported lineages, three clades (A2, A12 and A13) achieved low levels of statistical support in the current analysis; however, they were statistically supported by the phylogenetic analysis in our previous study of *Asterochloris* algae (Skaloud & Peksa 2010). The low statistical significance in the current analysis was very probably caused by the absence of actin type I sequences in several strains from these three clades. Moreover, some of the other weakly supported lineages represented in the current study by solitary samples (DQ229887 from *S. paschale*, DQ229884 from *Stereocaulon* sp., EU008683 from *L. nylanderiana*) were inferred with

Table 2 Ancestral state probabilities of the substrate preferences for particular photobiont clades. Values with clear positive (*) or negative (†) probabilities are given in bold

Clade	Shale	Wood-bark	Basalt	Granite-gneiss	Serpentine	Sandstone
A1	0.21	0.15	0.38	0.50	0.50	0.50
A2	**0.06†**	**0.02†**	0.57	0.50	0.50	0.50
A3	0.81	0.26	0.49	0.50	0.50	0.50
A4	0.31	0.89	0.44	0.50	0.50	0.50
A5	**0.92***	0.23	0.54	0.50	0.50	0.50
A6	0.40	0.31	0.48	0.50	0.50	0.50
A7	0.19	0.13	0.45	0.50	0.50	0.50
A8	**0.03†**	0.69	0.11	0.50	0.50	0.49
A9	0.35	0.20	0.48	0.50	0.50	0.50
A10	0.17	**1.00***	0.69	0.50	0.50	0.50
A11	0.37	**0.04†**	0.26	0.50	0.50	0.50
A12	0.40	0.30	0.48	0.50	0.50	0.50
A13	0.42	0.33	0.49	0.50	0.50	0.50

The substrates with nearly similar character—the siliceous rocks *granite* and *gneiss* as well as *wood* and *bark*—were used as combined variables in the analysis.

a high statistical support in previous studies by Piercey-Normore & DePriest (2001), Nelsen & Gargas (2008), Bačkor *et al.* (2010) and Skaloud & Peksa (2010).

The observed variation in lichen associations suggests that the specificity between symbionts is not the only determinant of the composition of individual association (the lichen thallus). It can be further influenced by the availability (occurrence) of compatible partners in relation to the environmental conditions at the locality.

The physiological responses (growth and photosynthesis) to temperature and light conditions may differ between particular photobionts (Casano *et al.* 2011). Thus, an unsuitable light or climatic regime may cause low fitness of the photobiont leading to its very low abundance or even absence in certain habitat. The mycobiont, as an exhabitant, shelters its partner against the harmful UV radiation, it can partly regulate the water content within the thalli (Honegger 2006, 2009) or it can protect the photobiont against the direct influence of substrate pH (Mollenhauer 1997). Nevertheless, the potential of the lichen fungi to protect their photobionts is necessarily limited because the lichen is actually a poikilohydric system strongly dependent on the local climatic regime. Furthermore, the ecology of the algae or cyanobacteria has its own history, preceding the lichenization event. Some photobionts are still recruited from persistent free-living forms (Wirtz *et al.* 2003), necessarily adapted to local conditions. Therefore, we hypothesized that particular photobionts exhibit different environmental preferences, which influence their associations with lichen fungi.

Our research revealed that particular *Asterochloris* lineages were contained in taxonomically different but ecologically similar lichens. Especially, the exposure of the lichen to rain and sun was found to be the crucial factor for distinguishing the *Asterochloris* lineages. The photobionts obtained from the ombrophobic lichens were genetically distinct from those obtained from the ombrophilic lichens. Clades A4, A6 and A10 were contained exclusively in *Lepraria* species growing on rain/sun-sheltered surfaces, such as vertical or overhanging rock walls and tree trunks. Conversely, the majority of the other clades were associated exclusively with the ombrophilic *Lepraria* and *Stereocaulon* species growing in rain/sun-exposed situations. Thus, the dissimilarity in environmental conditions—different water, light and temperature regime—caused the distribution patterns of individual photobiont lineages.

The exceptions to this general scheme were found, especially in the clade A7, containing photobionts from lichens exhibiting both water-seeking and -avoiding types of life strategies (although the ombrophobic preference was predominant here). The dissembling nature of this clade corresponds with its extensive ecological plasticity very well; it was found to associate with a great number of fungal species growing in diverse water and light conditions, in a broad range of altitudes, and on various substrates (from base-rich to acidic substrates). Clade A7 confirms the existence of photobionts exhibiting very wide ecology. Such euryoecious species (i.e. species having a wide range of habitats) were observed also by other authors (e.g. Guzow-Krzemińska 2006; Bačkor *et al.* 2010).

The photobionts from the clade A5 were found to associate with *L. nylanderiana* and very frequently with *L. borealis*. These two lichens grow on rain exposed surfaces; however, their thalli can sometimes grow into shallow rock fissures, partially sheltered from direct

rainfall. The close relationship of the clade A5 to the clades A6 and A7, which are rather ombrophobic (see Fig. 2) may suggest a possible pre-adaptation of this clade to cope with both types of water conditions.

A number of *Asterochloris* clades were markedly tolerant to various climatic conditions and substrates (A2, A3, A10 and A11). However, at least two lineages were revealed to be distinguishable based on these ecological characteristics: the clades A1 and A5 (both from ombrophilic lichens). The photobionts from the clade A1 were detected in lichens growing predominantly on siliceous rocks at altitudes above 1000 m (the lower border of mountain belt with cold and humid climate). Conversely, the clade A5 was characteristic for lichens growing predominantly on naked soils or on bryophytes in fissures of SiO_2-poor rocks (shales, basalts) at low altitudes. Correspondingly to these variations in ecology, the clades A1 and A5 differ in their associated fungi (if we omit the very common but taxonomically questionable species *L. caesioalba*). Clade A1 (*A. phycobiontica*) were detected predominantly in mountain species *L. alpina* and *L. neglecta*. Moreover, *A. phycobiontica* was first isolated and described from the lichen *Anzina carneonivea*, (Tschermak-Woess 1980) which is a typical psychrophilous species (i.e. species growing best at low temperatures; cf. Palice 1999). In contrast, the clade A5 was found in *L. borealis* and *L. nylanderiana*, and additionally in lowland specimens of *Cladonia foliacea*, *C. humilis* and *C. subulata* (Bačkor *et al.* 2010; Skaloud & Peksa 2010). *Cladonia foliacea*, *C. humilis* and *L. nylanderiana* represent species typical for warm localities in lowland, colline and submontane regions.

Our observations imply that the distribution of photobionts is in some respect independent of the particular mycobiont species, being much more accorded to specific conditions and lichen communities. This fact leads us to propose the existence of ecological guilds in lichens containing green algae (a guild of ombrophobic acidophilous lichens, a guild of ombrophilic mountain lichens from siliceous rocks and a guild of ombrophilic lowland lichens from SiO_2-poor substrates). We expect that this hypothesis will be confirmed in future studies examining large photobiont inventories of lichen communities growing in climatically and/or geologically different biotopes.

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O.P. is interested in lichens (currently in diversity and ecology of lichens of pine forests, shrub communities and industrial deposits) and lichen photobionts (specificity and ecology of lichenized green algae, especially of the genus *Asterochloris*). P.Š. is generally interested in genetic and phenotypic diversity in selected groups of algae. Specifically, he focuses on three groups of autotrophic protists: soil and symbiotic green algae (e.g. genera *Asterochloris* and *Klebsormidium*), desmids (the genus *Micrasterias*), and silica-scaled chrysophytes (the genus *Synura*).

Data accessibility

All data, including GenBank Accession nos of all sequences, are included in Table S1, (Supporting Information).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Scheme of the test of phylogenetic structure using the specified genetic distance delimiting closely related strains.

Table S1 List of all samples used in the study, including GenBank Accession nos for photobiont sequences and environmental data.

Table S2 Length, variability, base composition, selected substitution models and model parameters of different data sets.

Appendix S1 The *R* script for testing the similarity of environmental data in genetically closely related species pairs.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Supporting Information Fig. S1. Scheme of the test of phylogenetic structure using the specified genetic distance delimiting closely related strains. a. Linearized NJ tree inferred on 76 studied strains with known ecological data, ITS data and actin data (not all 76 samples were sequenced for actin, we supposed that the samples with totally identical ITS have identical actin sequences as well). The genetic distance is shown by the scale bar below the tree. b. Histogram of the frequency distribution of the pairwise genetic distances among all strains. The red lines in Fig. a and b represent the selected genetic distance (calculated using Kimura 2 parameter substitution model) delimiting the closely related strains (0.04 in both figures). **c.** Matrices of pairwise genetic distances and distances of the selected environmental variable (both for 76 studied strains used in phylogenetic analysis). The distances are given in colour gradient showed under the histogram in Fig. b. **d.** The set of photobiont pairs whose genetic distances are lower than the selected value delimiting closely related strains is given in blue. The selection is applied to the matrix of the environmental variable **e.** The existence of phylogenetic signal is tested by non-parametric permutation test. The sums of Euclidean distances of environmental data are calculated for each permutation, and compared with the real data (blue regions in Fig. d).

Supporting Information Table S1. List of all samples used in the study, including GenBank accession numbers for photobiont sequences and environmental data. The list is ordered by clade numbers (A1–A13), the samples within clades are in alphabetical order. Our specimens are in bold. Explanatory notes: Chemotype – chemotype of the lichen species according to Leuckert *et al.* (1995); mentioned only for our specimens (the taxa without chemotype were chemically uniform). Collection numbers – collection numbers of lichen specimens or algal strains (UTEX); specimens of O. Peksa were deposited in the herbaria PL (The West

Bohemian Museum in Pilsen; Czech Republic), specimens of Š. Slavíková-Bayerová and Z. Palice in the herbaria PRA (The Institute of Botany of the Academy of Science of the Czech Republic). Culture – sequence was obtained from algal culture deposited in culture collection of O. Peksa and CAUP culture collection: http://botany.natur.cuni.cz/algo/caup (the cultures were obtained within the scope of morphological study of *Asterochloris* algae – Skaloud & Peksa in prep.). Environmental data: exposure to rain (exposed – 1/sheltered – 0); altitude (in metres a.s.l.); substrate (five basic types of bedrock and wood-bark – coded as a set of dummy variables). Complete data on the investigated specimens are available from the first author.

References:

Leuckert C, Kümmerling H, Wirth V (1995) Chemotaxonomy of *Lepraria* Ach. and *Leproloma* Nyl. ex Crombie, with particular reference to Central Europe. *Bibliotheca Lichenologica*, **58**, 245–259.

Skaloud P, Peksa O (2010) Evolutionary inferences based on ITS rDNA and actin sequences reveal extensive diversity of the common lichen alga Asterochloris (Trebouxiophyceae, Chlorophyta), *Molecular Phylogenetics and Evolution*, **54**, 36–46.

Supporting Information Table S2. Length, variability, base composition, selected substitution models, and model parameters of different data sets.

Supporting Information Appendix S1. The R script for testing the similarity of environmental data in genetically closely related species pairs.

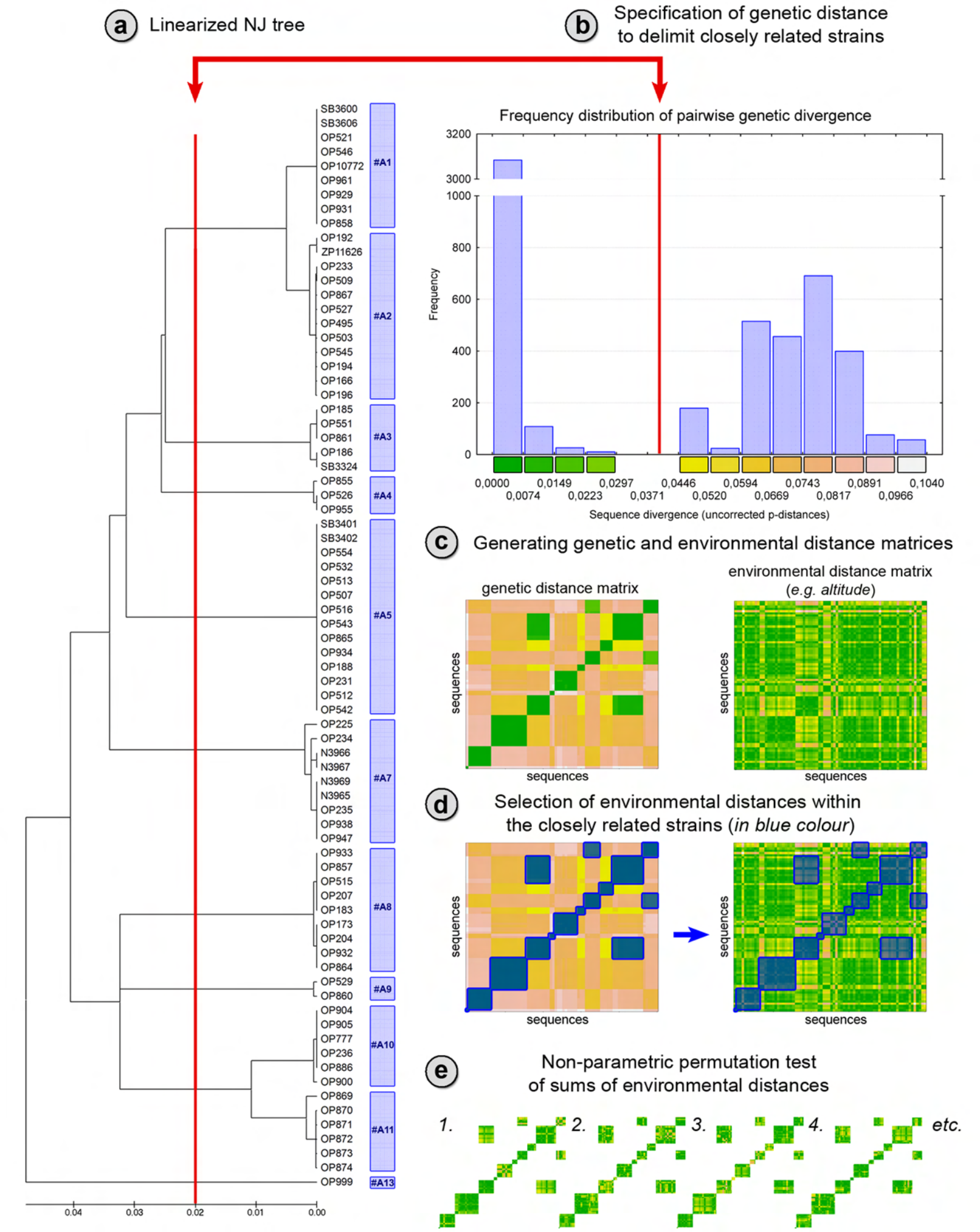
a Linearized NJ tree
b Specification of genetic distance to delimit closely related strains
SB3600
SB3606
OP521
OP546
OP10772
OP961
OP929
OP931
OP858
#A1
OP192
ZP11626
OP233
OP509
OP867
OP527
OP495
OP503
OP545
OP194
OP166
OP196
#A2
OP185
OP551
OP861
OP186
SB3324
#A3
OP855
OP526
OP955
#A4
SB3401
SB3402
OP554
OP532
OP513
OP507
OP516
OP543
OP865
OP934
OP188
OP231
OP512
OP542
#A5
OP225
OP234
N3966
N3967
N3969
N3965
OP235
OP938
OP947
#A7
OP933
OP857
OP515
OP207
OP183
OP173
OP204
OP932
OP864
#A8
OP529
OP860
#A9
OP904
OP905
OP777
OP236
OP886
OP900
#A10
OP869
OP870
OP871
OP872
OP873
OP874
#A11
OP999
#A13
0.04
0.03
0.02
0.01
0.00
Frequency distribution of pairwise genetic divergence
Frequency
3200
3000
1000
800
600
400
200
0
0,0000
0,0074
0,0149
0,0223
0,0297
0,0371
0,0446
0,0520
0,0594
0,0669
0,0743
0,0817
0,0891
0,0966
0,1040
Sequence divergence (uncorrected p-distances)
c Generating genetic and environmental distance matrices
genetic distance matrix
environmental distance matrix
(e.g. altitude)
sequences
sequences
sequences
sequences
d Selection of environmental distances within the closely related strains (in blue colour)
sequences
sequences
sequences
sequences
e Non-parametric permutation test of sums of environmental distances
1.
2.
3.
4.
etc.

Table S1, Supporting Information

Fungal taxon	Chemotype	Origin	Collection number	Culture	GenBank accession number for photobiont sequence		Clade labelling sensu Skaloud & Peksa (2010)	Exposure to rain	Altitude	Substrate					
					ITS	actin				basalt	shale	granite-gneiss	sandstone	serpentine	wood-bark
clade A1 (*Asterochloris phycobiontica*)							clade 15								
Lepraria alpina (de Lesd.) Tretiach & Baruffo		Czech Republic	Peksa 546	*	FN556023	-		1	960	0	0	1	0	0	0
Lepraria alpina (de Lesd.) Tretiach & Baruffo		Norway	Palice 10772	*	as FN556023	-		1	160	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 961	*	FN556024	-		1	1200	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 929		as FN556024	-		1	1300	0	0	0	0	0	1
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 931		as FN556024	-		1	1400	0	0	1	0	0	0
Lepraria neglecta (Nyl.) Lettau		Ukraine	Bayerová 3600	*	AM906013	AM906044		1	1849	0	0	1	0	0	0
Lepraria neglecta (Nyl.) Lettau		Ukraine	Bayerová 3606	*	AM900941	AM906043		1	1835	0	0	1	0	0	0
Lepraria neglecta (Nyl.) Lettau		Slovakia	Peksa 521	*	as AM900941	-		1	1600	0	0	1	0	0	0
Lepraria sp.*		Austria	Peksa 858		FN556025	-		1	1800	0	0	1	0	0	0
clade A2							clade 16								
Lepraria alpina (de Lesd.) Tretiach & Baruffo		Czech Republic	Peksa 192	*	AM906010	AM906039		1	1000	0	0	1	0	0	0
Lepraria alpina (de Lesd.) Tretiach & Baruffo		Czech Republic	Palice 11626		as AM906010	-		1	1380	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 233	*	AM906006	AM906035		1	690	1	0	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 509	*	as AM906006	-		1	450	1	0	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 3	Czech Republic	Peksa 196	*	AM906007	AM906036		1	465	1	0	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 166	*	AM906008	AM906037		1	900	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 194	*	AM906009	AM906038		1	630	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 545	*	as AM906009	-		1	555	0	0	0	1	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 503	*	as AM906009	-		1	1370	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 495	*	as AM906009	-		1	540	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 527	*	as AM906009	-		1	560	1	0	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 3	USA, California	Peksa 867		as AM906009	-		1	2000	0	0	1	0	0	0
clade A3							clade 14								
Lepraria alpina		Spain	Peksa 861		FN556026	-		1	1700	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 185	*	FM955666	FM955670		1	620	1	0	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 551	*	FM955667	FM955671		1	550	1	0	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Slovakia	Peksa 520	*	FN556027	-		1	1690	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Palice 11603		as FN556027	-		1	1420	0	0	1	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Spain	Peksa 859		FN556028	-		1	1700	0	1	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 930		as FN556028	-		1	1300	0	0	1	0	0	0
Lepraria granulata Slavíková-Bayerová		Bulgaria	Bayerová 3324		FN556029	-		1	2410	0	0	1	0	0	0
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 186	*	AM905992	AM906017		1	710	1	0	0	0	0	0
clade A4							clade 17								
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 526	*	FN556030	-		1	250	1	0	0	0	0	0
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 855		FN556031	FN556047		0	730	0	0	0	0	0	1
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 955		FN556032	-		0	790	0	0	1	0	0	0
clade A5							clade 10								
Lepraria borealis Lohtander & Tønsberg		Bulgaria	Bayerová 3401	*	AM900492	AM906045		1	618	0	1	0	0	0	0
Lepraria borealis Lohtander & Tønsberg		Bulgaria	Bayerová 3402	*	AM906015	AM906048		1	618	0	1	0	0	0	0
Lepraria borealis Lohtander & Tønsberg		Sweden	Peksa 554	*	as AM906015	-		1	10	0	0	1	0	0	0
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 532	*	as AM906015	-		1	500	1	0	0	0	0	0
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 513	*	as AM906015	-		1	450	1	0	0	0	0	0
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 507	*	as AM906015	-		1	400	1	0	0	0	0	0
Lepraria borealis Lohtander & Tønsberg		Slovakia	Peksa 516	*	as AM906015	-		1	900	0	1	0	0	0	0
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 543	*	as AM906015	-		1	250	0	1	0	0	0	0
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 865		as AM906015	-		1	450	0	0	1	0	0	0
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 934		as AM906015	-		1	550	0	0	0	0	1	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 231	*	AM906014	AM906047		1	320	0	1	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 188	*	as AM906014	-		1	400	0	1	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 512	*	as AM906014	-		1	417	1	0	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon		Spain	Sipman 45330		EU008667	-		1	-	-	-	-	-	-	-
Lepraria nylanderiana Kümmerl. & Leuckert		Czech Republic	Peksa 542	*	AM900493	AM906046		1	280	0	1	0	0	0	0

clade A6							clade 18									
Lepraria crassissima **(Hue) Lettau**		**Czech Republic**	**Peksa 888**		**FN556033**	**-**		**0**	**800**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria yunnaniana (Hue) Zahlbr.		Costa Rica, San José	Nelsen 3637b		EU008681	EU008710		0	-	-	-	-	-	-	-
clade A7							clade 11									
Lepraria caesiella R.C. Harris		USA, Massachusetts	Nelsen 3962		EU008661	EU008694		0	-	-	-	-	-	-	-
Lepraria caesiella R.C. Harris		USA, Wisconsin	Nelsen 3953		EU008660	-		0	-	-	-	-	-	-	-
Lepraria caesiella R.C. Harris		USA, Pennsylvania	Nelsen 3968		EU008662	EU008695		0	-	-	-	-	-	-	-
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**Romania**	**Peksa 225**	*	**AM905996**	**AM906022**		**1**	**1900**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 3**	**Slovakia**	**Peksa 234**	*	**AM905994**	**AM906020**		**1**	**1000**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 2**	**Slovakia**	**Peksa 235**	*	**AM905995**	**AM906021**		**1**	**1330**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba (de Lesd.) J.R.Laundon		USA, Pennsylvania	Nelsen 3965		EU008663	EU008696		1	-	1	0	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon		USA, Pennsylvania	Nelsen 3969		EU008666	EU008699		1	-	0	0	0	0	0	1
Lepraria caesioalba (de Lesd.) J.R.Laundon		USA, Pennsylvania	Nelsen 3966		EU008664	EU008697		1	-	1	0	0	0	0	0
Lepraria caesioalba (de Lesd.) J.R.Laundon		USA, Pennsylvania	Nelsen 3967		EU008665	EU008698		1	-	0	0	0	0	0	1
Lepraria cupressicola (Hue) J.R.Laundon		China, Yunnan	Nelsen 2598		EU008659	EU008693		0	-	-	-	-	-	-	-
Lepraria incana (L.) Ach.		USA, Virginia	Nelsen 3972		EU008669	-		0	-	-	-	-	-	-	-
Lepraria incana (L.) Ach.		USA, Maryland	Nelsen 3971		EU008668	-		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3974		DQ229877	DQ229898		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Wisconsin	Neslen 3960		EU008675	EU008704		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Massachusetts	Nelsen 3961		EU008676	EU008705		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3950		EU008670	EU008700		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3956		EU008672	-		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Tennessee	Nelsen 3958		EU008673	EU008702		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Indiana	Nelsen 3957		EU008674	EU008703		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, West Virgina	Nelsen 3970		EU008677	EU008706		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Michigan	Nelsen 3976		EU008679	EU008708		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		China, Yunnan	Nelsen 2590		EU008680	EU008709		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3973		EU008678	EU008707		0	-	-	-	-	-	-	-
Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3951		EU008671	EU008701		0	-	-	-	-	-	-	-
Lepraria nivalis J.R.Laundon		Canada, Quebec	Olszewski 5409o		EU008682	-		0	-	-	-	-	-	-	-
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 938**		**FN556034**	**-**		**0**	**850**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Slovakia**	**Peksa 947**		**as FN556034**	**-**		**0**	**600**	**0**	**0**	**1**	**0**	**0**	**0**
clade A8							clade 6									
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**Czech Republic**	**Peksa 173**	*	**AM906003**	**AM906032**		**1**	**800**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**Czech Republic**	**Peksa 204**	*	**AM906004**	**AM906033**		**1**	**900**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**Czech Republic**	**Peksa 932**		**as AM906004**	**-**		**1**	**1140**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**Czech Republic**	**Peksa 864**		**as AM906004**	**-**		**1**	**980**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria neglecta **(Nyl.) Lettau**		**Czech Republic**	**Peksa 183**	*	**AM906002**	**AM906031**		**1**	**1000**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria neglecta **(Nyl.) Lettau**		**Czech Republic**	**Peksa 207**	*	**AM906005**	**AM906034**		**1**	**900**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria neglecta **(Nyl.) Lettau**		**Slovakia**	**Peksa 515**	*	**as AM906005**	**-**		**1**	**1875**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria neglecta **(Nyl.) Lettau**		**Czech Republic**	**Peksa 933**		**as AM906005**	**-**		**1**	**550**	**0**	**0**	**0**	**0**	**1**	**0**
Lepraria neglecta **(Nyl.) Lettau**		**Czech Republic**	**Peksa 857**		**as AM906005**	**-**		**1**	**817**	**0**	**0**	**0**	**0**	**0**	**1**
clade A9							clade 19									
Lepraria alpina **(de Lesd.) Tretiach & Baruffo**		**Spain**	**Peksa 860**		**FN556035**	**FN556048**		**1**	**1450**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**Czech Republic**	**Peksa 529**	*	**FN556036**	**-**		**1**	**450**	**0**	**0**	**0**	**1**	**0**	**0**
clade A10							clade 7									
Lepraria incana **(L.) Ach.**		**Czech Republic**	**Peksa 886**		**FN556037**	**-**		**0**	**400**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria membranacea **(Dicks.) Vain.**		**Czech Republic**	**Peksa 904**		**FN556038**	**-**		**0**	**530**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria membranacea **(Dicks.) Vain.**		**Czech Republic**	**Peksa 905**		**as FN556038**	**-**		**0**	**800**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 236**	*	**AM905997**	**AM906023**		**0**	**1030**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 877**		**FM955668**	**FM955672**		**0**	**800**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 851**		**as FM955668**			**0**	**600**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 852**		**as FM955668**			**0**	**355**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 853**		**as FM955668**			**0**	**460**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 854**		**as FM955668**			**0**	**460**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 856**		**as FM955668**			**0**	**827**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 875**		**as FM955668**			**0**	**570**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 878**		**as FM955668**			**0**	**1150**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 880**		**as FM955668**			**0**	**450**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 881**		**as FM955668**			**0**	**450**	**0**	**0**	**0**	**0**	**0**	**1**

Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 883**		**as FM955668**			**0**	**600**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 885**		**as FM955668**			**0**	**820**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 887**		**as FM955668**			**0**	**530**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 889**		**as FM955668**			**0**	**730**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 901**		**as FM955668**			**0**	**950**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 902**		**as FM955668**			**0**	**860**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 937**		**as FM955668**			**0**	**560**	**0**	**0**	**0**	**0**	**1**	**0**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 940**		**as FM955668**			**0**	**600**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 941**		**as FM955668**			**0**	**550**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Germany**	**Peksa 943**		**as FM955668**			**0**	**400**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Germany**	**Peksa 944**		**as FM955668**			**0**	**450**	**0**	**0**	**0**	**1**	**0**	**0**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 945**		**as FM955668**			**0**	**1165**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Slovakia**	**Peksa 946**		**as FM955668**			**0**	**700**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Slovakia**	**Peksa 948**		**as FM955668**			**0**	**750**	**0**	**0**	**0**	**0**	**0**	**1**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 956**		**as FM955668**			**0**	**380**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria rigidula **(de Lesd.) Tønsberg**		**Czech Republic**	**Peksa 900**		**FM955669**	**FM955673**		**0**	**920**	**0**	**0**	**0**	**0**	**0**	**1**
clade A11							**clade 20**								
Lepraria borealis **Lohtander & Tønsberg**		**USA, California**	**Peksa 869**		**FN556039**	**FN556049**		**1**	**2000**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**USA, California**	**Peksa 868**		**FN556040**	**-**		**1**	**900**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**USA, California**	**Peksa 872**		**FN556041**	**FN556050**		**1**	**1000**	**0**	**1**	**0**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**USA, California**	**Peksa 874**		**as FN556041**	**-**		**1**	**1000**	**0**	**0**	**0**	**1**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**USA, California**	**Peksa 873**		**FN556042**	**FN556051**		**1**	**460**	**0**	**0**	**0**	**0**	**1**	**0**
Lepraria **sp.***		**USA, California**	**Peksa 870**		**FN556043**	**FN556052**		**1**	**2050**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria **sp.***		**USA, California**	**Peksa 871**		**as FN556043**	**-**		**1**	**2440**	**0**	**0**	**1**	**0**	**0**	**0**
clade A12 (*Asterochloris glomerata*)							**clade 1**								
Stereocaulon **sp.**		**Slovakia**	**Peksa 801**		**FM945392**	**-**		**1**	**780**	**0**	**0**	**1**	**0**	**0**	**0**
Stereocaulon pileatum Ach.		USA, Massachusetts	UTEX 896		AF345404	-		1	-	-	-	-	-	-	-
Stereocaulon pileatum Ach.		USA, Massachusetts	UTEX 897		AF345405	-		1	-	-	-	-	-	-	-
Stereocaulon pileatum Ach.		USA, Massachusetts	UTEX 1713		AF345407	-		1	-	-	-	-	-	-	-
Stereocaulon saxatile H.Magn.		USA, Massachusetts	UTEX 895		AF345382	AM906024		1	-	-	-	-	-	-	-
clade A13 (*Asterochloris irregularis*)							**clade 2**								
Stereocaulon pileatum **Ach.**		**Czech Republic**	**Peksa 999**	*	**AM905999**	**AM906028**		**1**	**420**	**0**	**0**	**0**	**1**	**0**	**0**
Stereocaulon botryosum Ach.		USA, Alaska	Talbot 153		DQ229880	DQ229889		1	-	-	-	-	-	-	-
Stereocaulon sp.		Iceland	UTEX 2236		AF345411	AM906027		1	-	-	-	-	-	-	-
Stereocaulon subcoralloides (Nyl.) Nyl.		USA, Alaska	Talbot 167		DQ229881	DQ229890		1	-	-	-	-	-	-	-
Stereocaulon vesuvianum Pers.		USA, Alaska	Talbot 281		DQ229885	DQ229888		1	-	-	-	-	-	-	-
others							**others**								
Lepraria borealis **Lohtander & Tønsberg**		**Czech Republic**	**Peksa 866**		**FN556044**	**-**		**1**	**850**	**0**	**0**	**1**	**0**	**0**	**0**
Lepraria caesioalba **(de Lesd.) J.R.Laundon**	**chem. 1**	**Czech Republic**	**Peksa 544**	*	**FN556045**	**-**		**1**	**600**	**1**	**0**	**0**	**0**	**0**	**0**
Lepraria nylanderiana Kümmerl. & Leuckert		Italy, Sardinia	Nöske 246		EU008683	-		1	-	-	-	-	-	-	-
Lepraria **sp.***		**Slovakia**	**Peksa 518**	*	**FN556046**	**-**		**1**	**1900**	**0**	**0**	**1**	**0**	**0**	**0**
Stereocaulon dactylophyllum Flörke		USA, Vermont	UTEX 1714		AM905993	AM906019	clade 5 (*Asterochloris excentrica*)	1	-	-	-	-	-	-	-
Stereocaulon dactylophyllum Flörke		Canada, NS	Normore 375		AF345442	-		1	-	-	-	-	-	-	-
Stereocaulon paschale (L.) Hoffm.		USA, Alaska	Talbot 101		DQ229887	DQ229891	clade 12	1	-	-	-	-	-	-	-
Stereocaulon saxatile H.Magn.		USA, Alaska	Talbot KIS 187		DQ229886	DQ229897		1	-	-	-	-	-	-	-
Stereocaulon sp.		Costa Rica, San José	Nelsen 2181b		DQ229884	DQ229896	clade 9	1	-	-	-	-	-	-	-
Stereocaulon tomentosum **Fr.**		**Slovakia**	**Peksa 786**		**FM945391**	**-**		**1**	**500**	**0**	**0**	**1**	**0**	**0**	**0**
Stereocaulon tomentosum Fr.		USA, Alaska	Talbot 400		DQ229882	DQ229893		1	-	-	-	-	-	-	-

Table S2, Supporting information.

	ITS			actin		
	ITS1	ITS2	5.8 rRNA	intron 1	intron 2	exon
Alignment length	152	196	166	227	307	125
Variable sites/parsimony informative sites (in %)	40/26 (26.3/17.1)	32/21 (16.3/10.7)	6/2 (3.6/1.2)	161/122 (70.9/53.7)	212/162 (69.1/52.8)	19/13 (15.2/10.4)
A	16.8	18.2	27.7	14.1	19.1	26.8
C	33.6	27.8	28.3	29.5	28.4	22.1
G	28.1	26.9	23.5	28.7	31.8	32.2
T	21.4	27.1	20.5	27.7	20.7	18.9
Model estimated[a]	HKY + I	HKY + I	F81	HKY + Γ	GTR + Γ	K80 + I
I, Γ values[b]	0.5241/-	0.7741/-	0/-	0/1.6922	0/2.7044	5.4595/-

[a]Estimated by the the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b.

[b]Proportion of invariable sites (I) and gamma distribution shape parameter (Γ) as estimated by PAUP/MrModeltest 1.0b.

```
#############################################################################
#               Analysis of ecological pattern in phylogenetic tree         #
#############################################################################
#                                                                           #
# Peksa, O. & Skaloud, P.                                                   #
#                                                                           #
# Script to test for possible similarity of environmental data in genetically    #
# closely related species pairs. The similarity is evaluated as the sum of       #
# distances of environmental data. The presence of ecological pattern is         #
# tested by the permutation test.                                           #
# For graphic illustration of the procedure, see Fig. S1 (Supporting information)#
#                                                                           #
# Requires two distance matrices                                            #
# Requires uploading of the "ecodist" package                               #
#                                                                           #
# Script is written for R (www.r-project.org) by Pavel Skaloud, January, 2011    #
# Department of Botany, Charles University in Prague, Benatska 2,                #
# 12801 Praha 2, Czech Republic, email: skaloud@natur.cuni.cz               #
#                                                                           #
#############################################################################
#                         Parameters to be specified                        #
#############################################################################
# nperm             = number of permutations                                #
# nrow              = number of rows in matrices                            #
# matrix1           = name of a full matrix of genetic distances            #
# matrix2           = name of a full matrix of environmental distances      #
# selected.distance = selected genetic distance delimiting the closely related   #
#                     strains                                               #
#############################################################################

library(ecodist)

#specification of required parameters
nperm             =VALUE   #e.g. 10000
nrow              =VALUE   #e.g. 76
matrix1           =NAME    #e.g. "matrix1.txt"
matrix2           =NAME    #e.g. "matrix2.txt"
selected.distance =VALUE   #e.g. 400

#import of genetic and environmental distance matrices
m1 <- matrix(scan(matrix1, n = nrow*nrow), nrow, nrow, byrow = TRUE)
   g.distance = lower(m1)
m2 <- matrix(scan(matrix2, n = nrow*nrow), nrow, nrow, byrow = TRUE)
   m.distance = lower(m2)

#counting sum of environmental distances within lineages
tested.distances <- which (g.distance<selected.distance)
morpho.dist <- m.distance [tested.distances]
suma <- sum(morpho.dist)

#permutation test
results <- vector(mode="numeric", length=nperm)
  for (xx in 1:nperm)
    {
    results [xx] <- sum(sample(m.distance, size=length(tested.distances),
replace=FALSE))
    }

# counting p-value
p.results <- c(suma, results)
p.results.sort <- sort(p.results)
p.rank <- which(p.results.sort==suma)
p.value <- p.rank/nperm
p.value
```