RESEARCH ARTICLE

The symbiotic playground of lichen thalli – a highly flexible photobiont association in rock-inhabiting lichens

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Abstract

The development of characteristic thallus structures in lichen-forming fungi requires the association with suitable photoautotrophic partners. Previous work suggests that fungi have a specific range of compatible photobionts and that selected algal strains are also correlated with the habitat conditions. We selected the rock-inhabiting crust lichen *Protoparmeliopsis muralis*, which exhibits high flexibility in algal associations. We present a geographically extended and detailed analysis of algal association patterns including thalli which host superficial algal colonies. We sampled 17 localities in Europe, and investigated the photobiont genotypic diversity within and between thalli and compared the diversity of intrathalline photobionts and externally associate algal communities between washed and unwashed thalli by single-strand conformation polymorphism analyses and ITS sequence data. The results show that (1) photobiont population within the lichen thalli is homogeneous; (2) multiple photobiont genotypes occur within single areoles and lobes of individual lichens; and (3) algal communities which superficially colonize the lichen thalli host taxa known as photobionts in unrelated lichens. Photobiont association patterns are extremely flexible in this ecologically versatile crust-forming lichen. We suggest that lichen surfaces represent a potential temporary niche for free-living stages of lichen photobionts, which could facilitate the establishment of further lichens in the proximal area.

Introduction

Many lichenized fungi produce long-persisting and light-exposed thallus structures, which provide a shelter for the photoautotropic symbionts, the photobionts. Since photobionts do not exhibit sufficient diagnostic characters, they remain to be identified by molecular approaches in the majority of species. Thus far, only little more than 100 algal species have been reported (Lücking *et al.*, 2009). In about 90% of the lichen species, photobionts belong to green algae in the *Trebouxiales* and *Trentepohliales* (whereas *c.* 10% lichen symbioses host cyanobacteria; Friedl & Büdel, 2008). In the largest group of primarily lichenized ascomycetes, *Lecanoromycetes*, the preference for main algal types largely coincides with main fungal lineages (Miadlikowska *et al.*, 2006). At a more detailed level of lichenized fungal taxonomy, the patterns of photobiont associations are less understood, although these may be important for ecological success.

The photobionts required for developing lichen morphologies originate from free-living cells, from co-transmission with their fungal partners (i.e. by vertical transmission of symbionts) or by capture from other lichens. In particular, lichen photobiont representatives of *Trentepohliales* are well known as free-living algae in bark communities (Honegger, 1998; Nelsen *et al.*, 2011), whereas species belonging to the genus *Trebouxia*, which are the most common lichen photobionts, are less frequently encountered in a non-lichenized state. Although their free-living occurrence is well established (e.g. Honegger, 1998, 2012; Sanders, 2001, 2005), they are not dominant in aerophilic algal communities (Ahmadjian, 1988; Mukhtar *et al.*, 1994). Earlier, Ahmadjian (1988) even proposed that *Trebouxia* photobiont is 'so dependent

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of the mycobiont that it cannot live independently'. Algal colonies are commonly observed on the surfaces (and crevices) of lichen thalli, especially in shady conditions, but these epithalline algae have not yet been studied by molecular approaches.

Flexibility in photobiont choice was recognized early in phycosymbiodemes, that is alternative cyanobacterial and green algal morphs of the same mycobiont species (Renner & Galloway, 1982). Their association with either cyanobacteria or green algae was known to correlate with ecological settings (Demmig-Adams *et al.*, 1990). More recent phylogenetic studies have discovered that switching among green algal strains is also a common phenomenon in lichens (Piercey-Normore & DePriest, 2001; Nelsen & Gargas, 2008). The number of selectable algal partners is variable among fungal species (Blaha *et al.*, 2006; Hauck *et al.*, 2007; Muggia *et al.*, 2010a, b). To categorize this variation, Yahr *et al.* (2006) distinguished between photobiont specialists, intermediates and generalists. In the genus *Lecanora*, for example, specialists accept only partners of a single algal lineage in *Trebouxia* (*Lecanora conizaeoides*; Hauck *et al.*, 2007). In contrast, generalists in the same genus can associate with a wide range of *Trebouxia* photobionts (*Lecanora rupicola*; Blaha *et al.*, 2006). For example, alpine samples of *L. rupicola* and *Tephromela atra* associate with *Trebouxia simplex*, whereas lowland and Mediterranean samples are found in symbiosis with other lineages in the same algal genus, which are also common in other lichens in the same habitats (Blaha *et al.*, 2006; Muggia *et al.*, 2010a). Apparently, generalists often have wider ecological distribution and may select their algal strains according to the local conditions.

Various lichen dispersal modes also trigger their fungal–algal association patterns, as dispersal via fungal spores requires re-association with algae (horizontal transmission) and joint symbiont propagules maintain successful associations over generations (vertical transmission). Tight linkage of algal and fungal genotypes is therefore expected in lichens which propagate mainly by soredia, stratified isidia, and phyllidia. Agreeing with this presumption, vertical transmission explains local symbiont association patterns for isidiate *Lobaria pulmonaria* and *Parmotrema tinctorum* (Dal Grande *et al.*, 2012; Mansournia *et al.*, 2012). In contrast, gene diversity estimates in populations of sorediate *Physconia grisea* suggest that soredia with loose hyphal organization are more susceptible to algal switching (Wornik & Grube, 2010). Additional complexity was discovered both in culture-based analyses as well as PCR-based analyses, which showed that lichen thalli are not always uniform with respect to photobiont association (Friedl, 1989; Bačkor *et al.*, 2010; Casano *et al.*, 2010; Grube & Muggia, 2010). In *Ramalina farinacea*, the presence of two algal strains in the same thallus appears as a consistent character of this species (Casano *et al.*, 2010; Del Campo *et al.*, 2010; Del Campo *et al.*, 2013). The coexistence of physiologically different algal genotypes could be advantageous under fluctuating environmental conditions and may be a more common phenomenon in ecologically adaptive lichens.

Protoparmeliopsis muralis is a crust-forming lichen with a great ecological amplitude and worldwide geographic distribution. This species commonly grows on calcareous and siliceous rocks but it also colonizes anthropomorphic substrata such as walls or road pavement. The thallus is easily recognizable by its radial and rosette-like growth, typically with small, narrowly spaced areoles in the central parts, and margins developing lobes (Fig. 1). The areoles are minute, isodiametric 'islands' of thallus developing from a spongy fungal tissue, which initially does not contain photobiont cells. Areoles are of various sizes in different species and variably spaced from each other (Smith *et al.*, 2009). Alternatively, lobes are areoles which are extended in one direction after initial development; they are present at the margins of the areolate lichen thallus (Honegger, 1993). In a first analysis, Guzow-Krzeminska (2006) identified four different algal species as photobionts of *P. muralis* at the same sampling locality. With this fairly low degree of algal specificity, *P. muralis* represents a generalist among lichens. We interpret here the terms specificity and selectivity as suggested by Yahr *et al.* (2004): specificity denotes the phylogenetic range of associated partners, whereas selectivity denotes the frequency of association among partners.

In this study we intend to investigate the algal communities associated with thalli of *P. muralis*, and we present

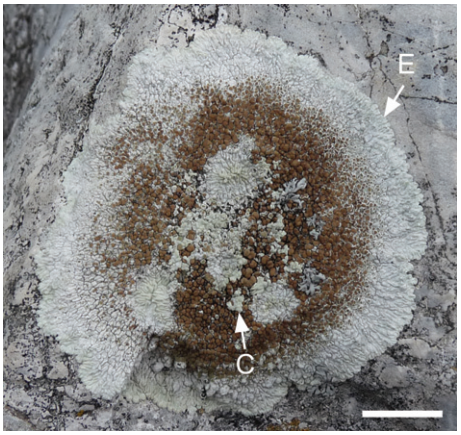

Fig. 1. Thallus of *Protoparmeliopsis muralis*: arrows indicate the central areola (C) located among the apothecia (light-brown circular structures), and external lobes (E) constituting the margin of the thallus.

a geographically extensive and detailed analysis of algal association patterns, including thalli which host superficial algal colonies. We aim to test three hypotheses:
(1) the photobiont population within the lichen thallus is not homogeneous: areoles contain photobionts different from those present in the external lobes;
(2) multiple algal genotypes are found at microscopic scale either in areoles or in external lobes;
(3) substantially higher algal diversity is present on the surface than inside the lichen thalli.

We sampled thalli from different sites in Europe and individual areoles of the central and marginal lobes from the same thalli for photobiont analyses. Further, we focused on one locality where we included the epithalline algal fraction in our community fingerprinting approach. After removal of the epithalline fraction by washing we compared the internal photobiont fraction of the same thalli to characterize the algal community of the thallus surface.

Material and methods

Sampling

In all, 147 thalli of *P. muralis* were collected from 17 localities in five European countries (Table 1): Austria ($n = 4$), Czech Republic ($n = 8$), Germany ($n = 1$), Italy ($n = 2$) and Sweden ($n = 2$). The localities are characterized by different ecological conditions and thalli were collected on different substrata, such as concrete, sandstone, calcareous and siliceous rocks and bark (Table 1). Ten sampling localities were located in anthropogenic environments, that is streets in the surroundings of cities and villages; here the lichen *P. muralis* commonly grows on concrete walls or roof tiles. The other seven localities were natural habitats located in the Austrian Alps above 1000 m altitude on siliceous and calcareous rocks, on the Swedish island Öland on an open limestone pavement area ('alvar'), where *P. muralis* grew on scattered siliceous rocks of moraine origin, and in lowland in Germany.

We performed two analyses based on single-strand conformation polymorphism (SSCP). In a first approach, to test the first two hypotheses, we studied intrathalline variation of photobionts from all sampling localities in different thallus parts. One central areole and one external lobe (without external colonization with algae) were prepared from each of the thalli. We used at least 3–18 thalli from each of the localities (Table 1). The two localities (1 and 5, Table 1) which were sampled more extensively were used for sequence analysis of excised SSCP bands (see below). These two sites are characterized by different ecology. The thalli from Austria were collected on calcareous outcrops in open meadow on the top of a mountain in an unpolluted environment. The thalli from the Czech Republic were collected from old concrete walls along a street in the periphery of Kladno city, a historical birthplace of heavy industry in Bohemia, still a center of steel factories, mining activities and high car traffic. Since the latter population of *P. muralis* also displayed an externally visible population of algae, we used it for a separate SSCP analysis to testing the third hypothesis. In this analysis we used areoles first directly for DNA extraction, before we washed off the superficial algae rigorously. We then used the cleaned thalli to retrieve their internal photobiont fraction. To minimize any possible differences in algal species composition, after the thalli were washed, we took areoles and lobes adjacent to those areoles and lobes removed before the washing. The total and internal algal fractions were then compared on one SSCP gel (Supporting Information, Fig. S1).

DNA extraction and algal fingerprinting by PCR-SSCP

For each thallus we conducted two DNA extractions: one extract each was prepared from the central (C) and external (E) thallus areoles, respectively, under the dissecting stereomicroscope. The dry lichen material was transferred into reaction tubes and pulverized using a TissueLyserII (Retsch). DNA was extracted from the lichen thallus according to Cubero *et al.* (1999).

The comparison between superficial algae and intrathalline photobionts in the second SSCP analysis was performed by washing the selected thalli rigorously to remove the surface fraction. The washing was performed three times with sterile water in a sterile glass tube, followed by a washing step with Tween80 and two final washings with sterile water. During the first washing steps the thalli were carefully cleaned with a clean brush. The washed specimens maintained the original extraction number but the new DNA extractions were further labeled Cw and Ew.

PCR amplifications were prepared using algal-specific primers which amplify the internal transcribe spacer 1 (ITS1) of the nuclear ribosomal RNA gene cluster. The primers used were ITS2N (5′-TCGCTGCGTTCTTC ATC-3′; Beck *et al.*, 1998) and nucSSU-1780-5′ (5′-CTGCGGAAGGATCATTGATTC-3′; Piercey-Normore & DePriest, 2001). The short length of 300 bp is used to allow optimal separation of sequence variants in the subsequent single SSCP analyses. To produce single-strand fragments for SSCP, the phosphorylated primer nucSSU-1780-5′ was used for the exonuclease digestion. PCR amplifications were conducted according standard conditions as in Muggia *et al.* (2010a, b). Amplicons were cleaned using Qiaquick spin columns (Qiagen, Vienna, Austria).

Samples were prepared for SSCP experiments according Schwieger & Tebbe (1998). Fragments were separated using the temperature gradient gel electrophoresis (TGGE) Maxi System (Biometra, Vienna, Austria) but without temperature gradient. The bands were visualized by silver staining before gels were scanned for documentation. For each sample the PCR products obtained from the central and the marginal areole were run in parallel to obtain an immediate comparison of band patterns. Selected bands were cut out from the gel with a sterile razor blade and the DNA was extracted from the bands and cleaned as described in Muggia & Grube (2010). Some samples for which we re-amplified and sequenced successfully all the bands were selected as 'controls' in the SSCP analyses (e.g. L1050, Fig. S1).

Re-amplification of the SSCP fragments

The ITS1 DNA fragments purified from the SSCP bands were re-amplified with the same PCR primers and conditions, but using Illustra Ready-To-Go RT-PCR Beads (GE Healthcare, Amershem Bioscience, 2004). One strand of the cleaned PCR products was sequenced by Macrogen, Inc. (Seoul, South Korea).

Comparison of epithalline algae and intrathalline photobionts

Differences between epithalline and intrathalline fractions were assessed by analysing both washed (Cw, Ew) and unwashed (C, E) specimens by SSCP. The amount of the

Table 1. Geographic origin, growth substratum, number of collected and fingerprint-analysed samples of *Protoparmeliopsis muralis*

No. of localitiy	Origin	Substrate	No. of sample analysed in SSCP (collected)*
1†	Austria, Styria, Graz, Grazer Bergland, Mt. Schöckl, on top in open meadow, 47°12′02″N,5°28′18″E, 1436 m asl, 2010, Muggia L. & Vancurova L. (GZU)	Calcareous rocks	18 (30)
2‡	Austria, Styria, Eisenerz Alpe,Wald am Schoberpass, in open meadow, 47°28′04″N, 14°38′50″E, 850 m asl, 2011, Muggia L. (GZU)	Glay siliceous rocks	5 (8)
3	Austria, Styria, Grazer Bergland, Rote Wand Mt., in open the medow, 47°19′44″N, 15°23′28″E,080 m asl, 2011, Vancurova L. (GZU)	Calcareous rocks	1 (5)
4	Austria, Styria, Eisenerzer Alpe, Trenchtling Massiv, 47°31′56″N,15°00′08″E, 1906 m asl, 2011, Muggia L. (GZU)	Calcareous rocks	4 (10)
5†‡	Czech Repubblic, Central Bohemia, Kladno, wall along street, 50°10′31″N, 14°6′35″E, 350 m asl, 2010, Muggia L., Peksa O. *et al.* (GZU)	Concrete	15 (18)
6	Czech Republic, Dobrany, in Plzenska street, on the roof tile, 49°39′30″N, 13°17′44″E, 330 m asl, 2011, Peksa O. (GZU)	Roof tile (clay)	1 (1)
7	Czech Republic, Kadan, power station Tusimice, on the dam, 50°22′15″N, 13°20′18″E, 330 m asl, 2011, Peksa O. (GZU)	Concrete	1 (1)
8	Czech Republic, North Bohemia, Ruzova, on silage pit near village, 50°50′24″N, 14°18′22″E, 2011, Peksa O. (GZU)	Silage pit (concrete)	4 (5)
9	Czech Republih, West Bohemia, Podborany, on concrete fence, 50°13′38″N, 13°24′22″E, 2011, Peksa O. (GZU)	Concrete	5 (5)
10	Czech Republic, West Bohemia, Podborany, near train sation, on woody fence, 50°13′23″N, 13°24′16″E, 2011, Peksa O. (GZU)	Wood	4 (5)
11	Czech Republic, West Bohemia, Plzen, Belanka, on street wall, 49°44′19″N, 13°22′23″E, 2011, Peksa O. (GZU)	Concrete	2 (4)
12	Czech Republic, South Bohemia, Tyn nad Vltavou, on concrete fence, 49°13′23″N, 14°25′14″E, 2011, Peksa O. (GZU)	Concrete sandstone	3 (5)
13	Germany, Sachsen-Anhalt, Saaledurchbruch bei Rothenburg, exposed slope in dry meadow, Gk R4483358/H5724007, 140 m asl, 2011, Schönbrodt M. (GZU)		5 (15)
14‡	Italy, Calabria, Cosenza Prov., Arcavacata di Rente, Botanical Garden University of Calabria, 2011, Puntillo D. (GZU)	Sandstone, dry concrete	5 (17)
15	Italy, Friuli-Venezia Giulia, Trieste prov., Via Scala Santa, on wall along street, 45°40′14″N, 13°46′13″E, 260 m asl, 2011, Muggia L. (GZU)	Concrete	5 (6)
16	Sweden, Öland, Resmo par., Gynge alvar, on moraine rocks on open alvar, 56°32′14.5″N, 16°28′27″E, 40 m asl, 2012, Wedin M. (GZU)	Siliceous rocks	7 (8)
17	Sweden, Öland, S. Möckleby par., close to Tingsstenen, 56°21′05″N,16°28′01″E, 20 m asl, 2012, Wedin M. (GZU)	Siliceous rocks	3 (4)

SSCP, single-strand conformation polymorphism.

*For each sample, two DNA extractions (C and E) were performed.

Localities selected for the population analyses of intrathalline photobiont diversity (†) and for the comparison of epithalline algae (‡) are indicated.

bands and their intensity was compared between the two set of samples. The absence of certain bands in the washed specimens was taken as evidence of its presence in the epithalline algal fraction. This, in fact, underscores the presence of intrathalline algae on the surface of the lichens.

Sequence analyses

For the best possible assessment of the identity of the sequenced ITS1 fragments we searched the GenBank database. Our newly generated sequences and those retrieved from GenBank were manually aligned using BIOEDIT (Hall, 1999) and, after exclusion of terminal ambiguities, they were subjected to (1) phylogenetic analyses to confirm their relationships; and (2) genotype analyses to study the sequence diversity.

The phylogenetic relationships of the *Trebouxia* spp. were reconstructed on a backbone tree using a Bayesian approach. *Trebouxia* taxa included in the analysis were selected from previous phylogenetic studies (Guzow-Krzeminska, 2006; Muggia *et al.*, 2008). The nucleotide substitution model GTR+I+Γ was determined using JMODELTEST (Posada, 2008). The Bayesian Markov Chain Monte Carlo (B/MCMC) analyses were run in MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2003; Ronquist *et al.*, 2005) with six chains simultaneously, each initiated with a random tree, for 2 million generations, and trees were sampled every 100th generation for a total sample of 200 000 trees. Log-likelihood scores against generation time were plotted using TRACER 1.4 (Rambaut & Drummond, 2007) to determine when the stationarity of likelihood values had been reached as a guide to where to set the burn-in stage (Ronquist *et al.*, 2005). Burn-in was set at 1 million generations and a majority rule consensus tree was calculated from the posterior sample of 20 001 trees. The convergence of the chains was confirmed by the convergent diagnostic PSRF, which approached 1 (Ronquist *et al.*, 2005). The phylogenetic tree was visualized in TREEVIEW (Page, 1996).

The genotype analysis was done using the program TCS (Clement *et al.*, 2000). The analysis included all *Trebouxia* sequences obtained from the two best sampled sites in Austria and in the Czech Republic (Table 1) for construction of a parsimony network focusing on the intrathalline photobiont data. We used the parsimony probability criterion (Templeton *et al.*, 1992) with gaps coded as fifth character state.

Results

Sequence analysis

We excised, purified and re-amplified 104 SSCP bands selected from 26 thalli, which came from the two most thoroughly sampled populations in Austria and the Czech Republic (Table 1): 21 bands were excided from 11 specimens from the Austrian locality N. 1 and 83 bands were excised from 15 specimens of the Czech locality N. 5. Of the excised bands, a total of 78 were successfully amplified and sequenced. The amplification of DNA purified from 26 bands was not successful. The sequences had an average length of 240 nucleotides, which agrees with the expected fragment size. Comparisons with public data assigned the majority of the sequences (61) to *Trebouxia* photobionts. One sequence was identified as *Trebouxia decolorans*, two as *Trebouxia asymmetrica*, 19 sequences were identified as *Trebouxia incrustata* and 39 as an uncultured *Trebouxia* sp. Apart from *Trebouxia*, high similarity was found with sequences of *Asterochloris* (five bands), *Chloridium* spp. (two bands), *Chlorella* (five bands), *Dictyosphaerium* sp. (two bands) and *Stichococcus* sp. (three bands). The sequences matched in the NCBI-BLAST analyses with *Asterochloris* sp. sequences obtained from different species of *Cladonia*, the *Chloridium* sequences matched with *Chloridium angusto-ellipsoideum*, the sequences of one *Chlorella* sp. was similar to the photobiont of the lichen *Schaereria dolodes*, whereas the remaining *Chlorella* sp., *Dictyospherium* sp. and *Stichococcus* sp. sequences were all highly similar to algae from environmental samples.

The phylogenetic analysis (Fig. 3) performed with the *Trebouxia* sequences was used primarily to assign taxon names. The analysis justifies the classification of the two *T. asymmetrica* and the 21 *T. incrustata* sequences, but placed the *Trebouxia* sp. sequences in two different clades. Two sequences were assigned to the *T. incrustata* clade (L975EA, L980CC), 17 sequences were in the lineage *Trebouxia* 'muralis I', as originally named by Guzow-Krzeminska (2006), whereas the last 20 sequences formed their own lineage, here called *Trebouxia* sp. We used the names *T. incrustata*, *T.* 'muralis I' and *Trebouxia* sp. to identify the genotype groups in the network analysis.

In all the analysed samples, the sequences identified as *T. incrustata*, *T.* 'muralis I' and *Trebouxia* sp. correspond to the bands which have the strongest intensity in the SSCP gels, suggesting their dominance as photobionts in both central and external lobes of the thalli.

Genotype analysis of intrathalline photobiont diversity

For this analysis we focused on the most frequent lineages found in the Austrian and Czech populations: *T. incrustata*, *T.* 'muralis I' and *Trebouxia* sp., from which we recovered 30 genotypes (Fig. 2). Three groups can be distinguished, corresponding to *T. incrustata*, *T.* 'muralis I' and *Trebouxia* sp., respectively. The genotypes generally present a high degree of diversity, some being separated by up to 22

mutational steps, which suggests a high genetic variation within a single algal species. The majority of the genotypes ($n = 25$) are represented by only one sequenced band; only two genotypes, respectively of *T. incrustata* from Czech the Czech locality and *Trebouxia* sp. from the Austria locality, are shared by seven samples. In the Czech locality, all the three *Trebouxia* lineages are found, whereas the Austrian locality hosts only the lineage *Trebouxia* sp. (Fig. 2). No genotypes are unique to either central or external lobes of *P. muralis* thalli. The same genotypes, either of *T. incrustata* or of *T.* 'muralis I', have indeed been found both in central and external lobes, for example for samples 968 (CC and EA), 972 (CA and EA), 974 (CA and EC), 965 (CA and EA), 976 (CB and EA), 978 (CA and EA) and 1042 (CA and EA). However, within a single lobe more than one genotype could be found. This intrathalline multiplicity was found with genotypes of *T. incrustata* for the samples 968 (EA, EB), 970 (CA, CB, CC), 974 (EC, ED), 975 (EA, EB), 980 (EA, EB), and genotypes of *T.* 'muralis I' for the samples 967 (CwA, CwB, CA; EA, EC), 1027 (CA, CC) and 1029 (CB, CC). The presence of the same genotypes as different SSCP bands, as detected for the sample 1052 (CA, CC), could be attributed to alternative tertiary structures of sequence in the SSCP gel.

Presence of epithalline algae

Washed samples resulted in a reduced number of bands in the SSCP analyses (Fig. S1). Those sequences identified as the taxa *Asterochloris*, *Chlorella* sp., *C. angusto-ellipsoideum*, *Dictyosphaerium* sp., and *Stichococcus* sp. were recovered only from the unwashed samples. Their corresponding bands disappeared in the SSCP analyses after washing and surface sterilization of the thalli (Fig. S1). These bands are clearly distinguishable from those corresponding to *T. incrustata*, *T.* 'muralis I' and *Trebouxia* sp., because the latter have always different run-length patterns, much higher intensity, and are present also in the washed samples. We therefore concluded that *T. incrustata*, *T.* 'muralis I' and *Trebouxia* sp. represent the main symbiotic partners of *P. muralis*. The presence of bands that can be assigned to the epithalline photobiont fraction was detected also in the SSCP analyses extended to the other localities (Table 1), for example in Czech localities N. 6-12, German locality N. 13, Italian localities N. 14 and 15, in two samples in the Austrian locality N. 1 and in Swedish localities N. 16 and N. 17.

The intensity of the bands representing the intrathalline fraction differs between the washed and the unwashed samples, unwashed samples having higher intensities.

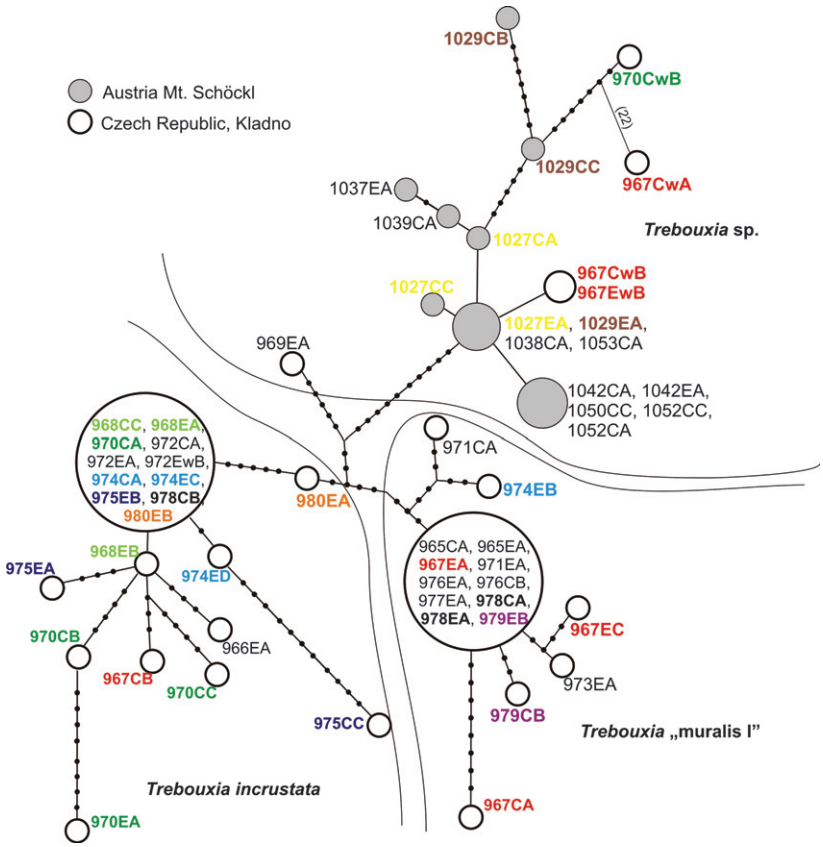

Fig. 2. Genotype analyses of ITS1 of the intrathalline photobionts of *Protoparmeliopsis muralis*. Genotypes, identified according to BLAST similarity search and phylogenetic analysis (Fig. S1), correspond to the lineages *Trebouxia incrustata*, *Trebouxia* 'muralis I' and *Trebouxia* sp. The sizes of the circles are proportional to the number of sampled sequences of the genotype and samples numbers are reported on the side. Genotypes which are not present in the sampling are represented by small dots; a line between two genotypes (or dots) represents one mutational step. Samples for which multiple genotypes of *T. incrustata*, *Trebouxia* 'muralis I' and *Trebouxia* sp. were found are colour-coded.

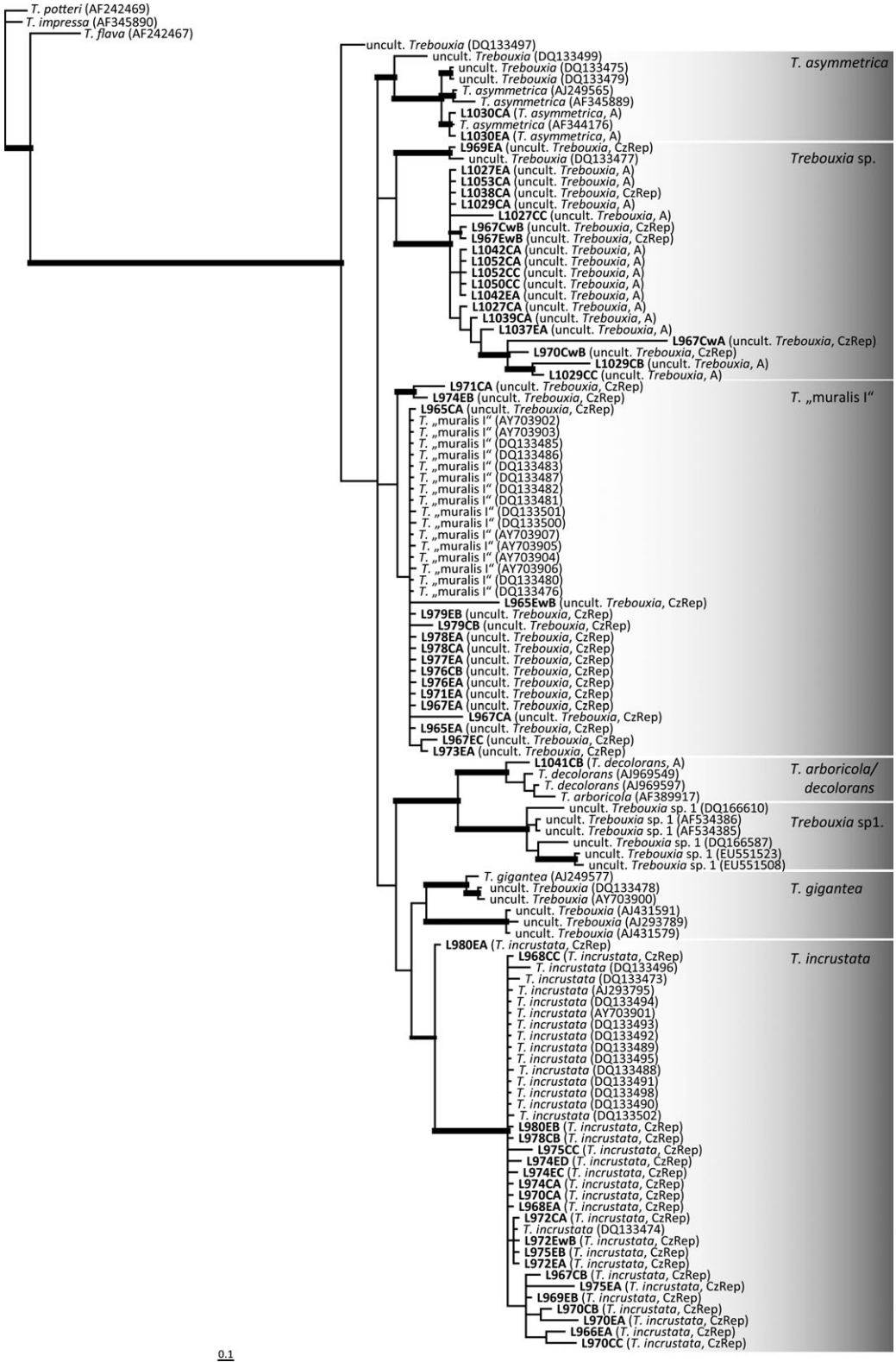

Fig. 3. Phylogenetic hypothesis of *Trebouxia* species: majority rule consensus tree of a B/MCMC analysis; branches in bold denote Bayesian possterior probabilities > 95%. *Trebouxia* clades are named according the previous phylogenetic studies of Guzow-Krzeminska (2006) and Muggia *et al.* (2010b). Taxa retrieved from GenBank are reported with their accession numbers. ITS1 sequences obtained in this study are in bold.

Although we are aware that SSCP cannot be regarded as a quantitative method, we assume that intrathalline photobionts are also present on the lichen thallus surface. In some cases, we also noticed new bands in washed thallus parts, which were not present in unwashed parts of the same thallus. This phenomenon might be a result of intrathalline photobiont variation.

Discussion

Our community fingerprinting approach using SSCP reveals complex patterns of algal variation in lichens. Patterns of multiple samples are conveniently compared with a single gel, and complementary to culture-dependent procedures and direct ITS sequencing. SSCP fingerprints and sequencing revealed that *T. incrustata*, the recently recognized lineage named *Trebouxia* 'muralis I' (Guzow-Krzeminska, 2006), and an unnamed *Trebouxia* sp. represent the principal photobionts in *P. muralis*. *Trebouxia incrustata* and the lineage *T.* 'muralis I' were detected by Guzow-Krzeminska (2006), whereas we found *Trebouxia* sp. in *P. muralis* for the first time. Similar to Guzow-Krzeminska (2006), we found *T.* 'muralis I' also in thalli from different substrata. The three identified *Trebouxia* lineages are not correlated with any specific part of the thallus. The presence of same algal genotypes both in central areoles and external lobes rejects our hypothesis 1, showing that different genotypes are homogeneously distributed in the thalli.

Sequencing of SSCP bands revealed the presence of more than one genotype belonging to either *T. incrustata* or *T.* 'muralis I' in individual areoles and lobes. Recovering multiple algal genotypes in the same thallus parts supports our hypothesis 2 and further confirms previous results with other lichens. The presence of multiple algal genotypes has been noticed previously but this phenomenon has hardly been studied with a larger number of samples, for example *Evernia* (Piercey-Normore, 2009) or *Rinodina* (Helms *et al.*, 2001), and has not been studied for its distribution within thalli, except by Casano *et al.* (2010). There, the screening of numerous thalli for intrathalline variation in *R. farinacea* revealed consistent co-occurrence of two types of physiologically different symbiotic algae throughout the entire thalli.

Here, we found additional complexity of algal association patterns in *P. muralis*. Our results support the hypothesis that the fungus *P. muralis* shows a low level of photobiont specificity (Guzow-Krzeminska, 2006). In our study, *P. muralis* is associated with three lineages of *Trebouxia*. These photobionts are widespread, and at least *T. incrustata* could be globally distributed, but whether the rather pronounced intraspecific genetic diversity (genotype diversity) is linked to ecophysiological differences is not yet understood. The close relationship of *T. incrustata* and *T.* 'muralis I' in our phylogenetic analysis (Fig. 3) supports the previous results of Guzow-Krzeminska (2006). Guzow-Krzeminska (2006) also found other *Trebouxia* species in her study, such as *T. decolorans* and *T. asymmetrica*. In the light of our results, we suggest that these *Trebouxia* species, unrelated to *T. incrustata*, *T.* 'muralis I' and *Trebouxia* sp., are rather part of epithalline algae communities and thus are not involved directly in the symbiosis of *P. muralis*. Notwithstanding, Guzow-Krzeminska & Stocker-Wörgötter (2013) recently proved with *in vitro* culture resynthesis experiments that the mycobiont *P. muralis* is able to interact also with those *Trebouxia* species we identified in the epithalline fraction, but it was not clear with this approach whether these interactions are sufficient to develop proper thallus morphologies. Our data from washed and unwashed parts of the same thallus additionally suggest that the intrathalline *Trebouxia* species are also present as epibionts on the thallus surface. In accordance with our hypothesis 3, the epithalline algae communities host numerous algal species and, if not separately considered, might lead to overestimates of photobiont diversity in *P. muralis*, and lichens in general. The epithalline fraction of algae found on *P. muralis* thalli varied among samples. The majority of the epithalline taxa belong to genera and strains which are also known from other lichens as photobionts: *Asterochloris*, *Chlorella*, *Chloridium*, *Stichococcus*, and other species of *Trebouxia*. The species with such photobionts could be growing adjacent to our samples of *P. muralis* but can also be found in habitats with different ecological settings. As epibionts of *P. muralis* these algae seem to occur both in polluted urban habitats (as identified by sequencing the SSCP bands of the thalli collected in Kladno town, locality 5 in Table 1) and in unpolluted mountain localities, and on different substrata. *Asterochloris* is the main photobiont in *Cladoniinae*, whereas *Stichococcus* associates to calicioid lichen-forming fungi of the genus *Chaenotheca* (Tibell, 2001) and, similar to *Chlorella*, it is also found in many crustose and foliose species of the family *Verrucariaceae* (e.g. *Dermatocarpon*, *Verrucaria*, Thüs *et al.*, 2011). Further, the genus *Chloridium* (Darienko *et al.*, 2010), with *C. angustoellipsoideum*, has been reported as a symbiont in thalli of the genera *Trapelia*, *Bacidia* and *Stereocaulon* (Tschermak-Woess, 1948, 1988; Beck *et al.*, 2002). *Trebouxia asymmetrica* and *T. decolorans* are common photobionts of a very wide range of lichens (mostly *Lecanoromycetes*). These algae, which can live on thalli of *P. muralis*, may originate from various sources, for example free-living algal diaspores, mitotic propagules of other lichens, or even snowflakes (e.g. Handa *et al.*, 2007). Algae found in the epithalline fraction may also occur in sheltered crevices

between thallus areoles, where algal colonies are frequently observed in microscopic analyses (M. Grube, unpublished data). Such micro- environments are apparently important reservoirs of a wider range of lichen photobionts and other algae. It has not been experimentally tested under which circumstances algae from these epithalline niches could be mobilized and translocated. We hypothesize that extended high humidity leads to an increase in the epithalline algal biomass, whereas dry conditions might increase the likelihood of algal detachment.

The genotypic diversity of algae which externally associate with lichen thalli could still be underestimated in our study. We could not sequence SSCP bands, due to technical problems, and SSCP generally offers a resolution of biodiversity which is limited to abundant strains. Moreover, our approach with specific primers was based on previous observations that trebouxioid algae are the only photobionts in *P. muralis*, which were the main focus of our study. As the analysis is restricted here mostly to trebouxioid algae (and few more distantly related genera), future studies may reveal the full extent of algal diversity on lichen surfaces. This requires a broader sampling and more general approach, which should also include samples from surrounding substrate and neighbouring organisms.

To successfully colonize their habitats, lichenized fungi use different and mixed strategies to fine-tune their associations with algae (Grube & Spribille, 2012). The community adaptation hypothesis suggests that lichens can vary the ratio of producers (algae) and consumers (fungi) according to local climatic conditions (Sun & Friedman, 2005). Further, lichenized fungi select locally optimal algal strains for thallus formation (Blaha *et al.*, 2006; Fernandéz-Mendoza *et al.*, 2011), which supports the hypothesis of habitat-adapted symbiosis (Rodriguez *et al.*, 2008). By their reproduction with fungal spores, lichens may readily re-associate with suitable algae in a habitat-adaptive manner ('horizontal transmission', in contrast to 'vertical transmission' with propagules of joined symbionts). Finally, the coexistence of physiologically differing algal partners within thalli recently added another strategy of symbiotic adaptivity (Casano *et al.*, 2010). Our results suggest that lichens may mix and vary these strategies, even within a single thallus and with various strains of algae (compare also Muggia *et al.*, 2010a). We thus suggest that the areole-forming *P. muralis* thalli act as 'symbiotic playgrounds' where different partnerships can be tested at low risk for the entire thallus structure. In our samples, no partnership seemed to clearly succeed over others. We suggest that a higher variety of symbiotic associations could be helpful when changing habitat conditions impose unpredictable selective constraints. Then some of these combinations might turn out to be more successful than others.

The presence of surface-attached algal colonies on *P. muralis*, including photobionts of other fungal species, also adds a new perspective to understand lichen community establishment. Lichens often form dense communities, where each of the species has genuine preferences for symbiotic partners. We suggest that the development and stability of such communities is facilitated by local reservoirs of algal photobionts, either in crevices of the substratum or on lichen surfaces. Although such a community facilitation hypothesis has yet to be studied in greater detail, we argue that epithalline algal diversity needs to be included in metacommunity studies of lichens.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Comparison of epithalline and intrathalline photobiont fractions: SSCP analyses of the five representative washed (Cw and Ew) and unwashed (C and E) samples of locality N. 5.
Table S1. NCBI accession numbers for the newly generated ITS1 algal sequences used in the analyses of Figs 2 and 3.