Genetic diversity of sterile cultured *Trebouxia* photobionts associated with the lichen-forming fungus *Xanthoria parietina* visualized with RAPD-PCR fingerprinting techniques

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Abstract: Photobiont diversity within populations of *Xanthoria parietina* was studied at the species level by means of ITS analyses and at the subspecific level with fingerprinting techniques (RAPD-PCR) applied to sterile cultured algal isolates. Populations from coastal, rural and urban sites from NW, SW and central France and from NE Switzerland were investigated. Between 8 and 63 samples per population, altogether 150 isolates, were subjected to phenetic and ordination analyses. Epiphytic samples of *X. parietina* associated with different genotypes of *Trebouxia decolorans* but saxicolous samples contained *T. arboricola*. For comparison the *T. gelatinosa* photobiont of a small population of *Teloschistes chrysophthalmus* (4 samples) was investigated. ITS sequences of *T. decolorans* isolates from different geographic locations were largely similar. In all populations a surprisingly high diversity of genotypes was observed in *Trebouxia* isolated from lichen thalli growing side by side. As *Trebouxia* spp. are assumed to be asexually reproducing haplonts, the genetic background of this diversity is discussed. Fingerprinting techniques are a powerful tool for obtaining valuable insights into the genetic diversity within the algal partner of lichen-forming fungi at the population level, provided that sterile cultured isolates are available.

Key words: Internal Transcribed Spacer (ITS), population genetics, *Teloschistes chrysophthalmus*, *Trebouxia arboricola*, *Trebouxia decolorans*, *Trebouxia gelatinosa*

Introduction

The photoautotrophic inhabitants of lichen thalli are distinctly less intensely studied than the lichen-forming fungi themselves. Before the advent of molecular techniques, photobiont morphospecies were distinguished by light microscopy. In the case of the unicellular representatives of *Trebouxiophyceae*, the most widespread photobionts of lichen-forming fungi, this was a difficult task. The prerequisites were sterile cultured isolates maintained under defined conditions and cultures of type species for comparison (Tschermak-Woess 1988). Later, the photobiont diversity within thalli of conspecific lichen-forming fungi were biochemically analysed, the focus being on isoenzyme patterns (Kilias 1988; Kilias *et al.* 1988; Fahselt 1989). With molecular techniques and algal-specific primers applied to whole lichen DNA, the photobionts can be conveniently identified at the species level without any isolation and culturing (Beck *et al.* 1998; Friedl *et al.* 2000; Dahlkild *et al.* 2001; Helms *et al.* 2001; Beck *et al.* 2002). Thanks to this type of investigation our knowledge about the range of photobiont taxa associated with particular species of lichen-forming fungi has significantly increased in recent years. In most of these studies photobiont diversity was explored at the species level on the basis of ITS phylogenies of samples collected in geographically different locations. In only a

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few studies was photobiont diversity examined within populations. Photobiont diversity was studied in lichen communities growing on heavy-metal rich rock (Beck *et al.* 1998; Beck 1999; Beck *et al.* 2002). Photobiont diversity was explored in the families *Cladoniaceae* (Piercey-Normore & DePriest 2001; Piercey-Normore 2004, Yahr *et al.* 2004), *Parmeliaceae* (Piercey-Normore 2009) and *Physciaceae* (Dahlkild *et al.* 2001; Helms *et al.* 2001, Helms 2003), in the genera *Caloplaca* Th. Fr. (Vargas Castillo & Beck 2012), *Chaenotheca* (Th. Fr.) Th. Fr. (Tibell 2001), *Letharia* (Th. Fr.) Zahlbr. (Kroken & Taylor 2000), and *Umbilicaria* Hoffm. (Romeike *et al.* 2002). At the intraspecific level, genetic diversity of green algal photobionts was studied in populations of a number of lichen-forming fungi (Opanowicz & Grube 2004; Blaha *et al.* 2006; Guzow-Krzeminska 2006; Ohmura *et al.* 2006; Piercey-Normore 2006; Yahr *et al.* 2006; Muggia *et al.* 2010; Werth & Sork 2010; Wornik & Grube 2010; Casano *et al.* 2011; Fernández-Mendoza *et al.* 2011; Domaschke *et al.* 2012; Francisco De Oliveira *et al.* 2012). Overall, these studies revealed that lichen fungi are able to associate with a range of photobionts although some species are more specialized on particular types of photobionts than others. Some species showed large-scale geographic trends in the distribution of photobionts (Yahr *et al.* 2006; Wornik & Grube 2010; Fernández-Mendoza *et al.* 2011), others showed the ecological specialization of photobionts (Werth & Sork 2010; Peksa & Skaloud 2011).

Many of the previous studies have relied on phylogenies of the Internal Transcribed Spacer Region (ITS) to identify photobiont species, based on the assumption that samples grouping with a known species in a well-supported clade are conspecific. ITS phylogenies are based on only one locus, but genetic diversity at the subspecific level is best explored with a multilocus approach. Fingerprinting techniques can provide valuable data on the genetic diversity of populations and these have been successfully used for characterizing lichen-forming fungi at the subspecific level: microsatellite-based fingerprinting (Walser *et al.* 2003; Walser *et al.*

2005; Werth *et al.* 2006; Werth *et al.* 2007; Widmer *et al.* 2010; Mansournia *et al.* 2011; Dal Grande *et al.* 2012), tRNA fingerprinting (Schmitt *et al.* 2002), or randomly amplified polymorphic DNA (RAPD) analysis applied to either pure fungal material such as central strands of *Usnea* sp. (Heibel *et al.* 1999) or apothecial discs (Printzen *et al.* 1999), or to sterile cultured fungal isolates (Murtagh *et al.* 1999; Dyer *et al.* 2001; Honegger *et al.* 2004b; Honegger & Zippler 2007; Itten & Honegger 2010). Recently, microsatellite markers were also applied to investigate the reproductive mode and phylogeography in *Dictyochloropsis reticulata* (Tscherm.-Woess) Tscherm.-Woess photobionts (Dal Grande *et al.* 2012; Widmer *et al.* 2012). RAPD-PCR applied to either randomly selected single spore isolates from one apothecium (Murtagh *et al.* 2000; Seymour *et al.* 2005) or to the single spore isolates derived from a single ascus (i.e. the progeny of one meiosis, Honegger *et al.* 2004a) was used for studying the mating systems of lichen-forming ascomycetes. Fingerprinting techniques such as short (STRR) or long (LTRR) tandemly repeated repetitive sequences were used for characterizing conspecific cyanobacterial symbionts of angiosperms (Rasmussen & Svenning 1998), whilst M13 minisatellite fingerprinting facilitated the distinction of different genotypes among conspecific unicellular or filamentous green algae (Oppermann *et al.* 1997).

The present study aims at elucidating photobiont diversity in *Trebouxia* de Puymaly photobionts at the subspecific level in different populations of the yellow wall lichen, *Xanthoria parietina* (L.) Beltr., by means of RAPD-PCR fingerprinting applied to sterile cultured isolates. Thalli were sampled in populations at coastal, rural or urban sites in NW, SW or central France or in NE Switzerland. Some of these populations were old and undisturbed over prolonged periods of time (voucher numbers 120 and 121, 144 and 145), others grew at sites which had been newly built within the last 20 years (voucher numbers 111, 319 and 320). As *Trebouxia* species are assumed to reproduce exclusively asexually (Friedl & Büdel 1996)

and be rare outside lichen thalli (Ahmadjian 1988), we were interested to see whether the photobionts of local populations of *X. parietina* exhibited low diversity, as expected if the algae are reproducing clonally.

Materials and methods

Specimen collection, photobiont isolation and culturing

Lichen thalli from five populations of *Xanthoria parietina* and of one small population of *Teloschistes chrysophthalmus* (L.) Norman ex Tuck. (Table 1; Fig. 1A–B) were stored in a desiccated state at −20°C where they stay viable for prolonged periods (Honegger 2003). Each collecting site and sample was photographically documented. Sample codes included the site number (three-digit Arabic numbers), the substratum number (Arabic numbers), the thallus number (Roman numerals), and the isolate number (letters). Voucher specimens have been deposited in the herbarium of ETH Zürich (Z+ZT). In the Swiss population (319/320) the thalli were left *in situ* with only small fragments being collected.

One, rarely two, mature apothecia per thallus (in the latter case termed a and b) were selected and ascospores were allowed to be ejected for parallel experiments on the genetic diversity within the fungal partner (Honegger & Zippler 2007; Itten & Honegger 2010). In the herbarium sample the outline of each thallus was drawn on a transparent overlay and the site marked where apothecia had been removed. Photobiont cells were scraped out of the bottom (below subhymenial layer) and thalline margin of apothecia with a sterilized platinum needle and spread over the surface of agarized non-nutrient mineral medium (Bold's basal medium [BBM] according to Deason & Bold 1960) with double the amount of nitrogen and 0·005% w/v doxycycline (Sigma) as an antibiotic. Plates were maintained at $15 \pm 1°C$ at a 16h light/8 h dark cycle at approximately 5 μE m^{-2} s^{-1} for 2–3 weeks until cells started to divide. All cultures were screened regularly and fungal contaminants were removed. Dividing algal cells were transferred to *Trebouxia* medium II according to Ahmadjian (1967), with only ¼ amount of glucose and casmino acids (Honegger 2004). Most cultures were multi-cell isolates, with cells originating from a very small area within a single apothecium; however, a few cultures were single cell isolates. A total of 150 photobiont isolates from five *X. parietina* populations were investigated in this study. For comparison four isolates of *Teloschistes chrysopthalmus* growing side by side were analyzed. In parallel experiments the fungal partner was brought into sterile culture.

Genomic DNA isolation

Genomic DNA was isolated using the GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont) with modifications to the manufacturer's protocol as follows. Algal isolates were frozen in liquid nitrogen and ground with a pre-cooled motor-driven micropestle. After addition of 100 μl of capture buffer to ground material, the samples were incubated at 60°C for 10 minutes and subsequently centrifuged. The supernatant was transferred to a GFX column preloaded with 100 μl of capture buffer, incubated for 3 minutes at room temperature, centrifuged and washed with 500 μl of washing buffer. DNA was eluted in 50 μl of elution buffer (10 mM Tris-HCL, pH 8.0) and stored at 4°C.

ITS amplification and sequencing

The nuclear ribosomal ITS region (ITS 1, 5.8S rDNA and ITS 2) was amplified with primer pair ITS 5 and ITS 4 (White *et al.* 1990). Internal primers at 5.8S rDNA were used for sequencing; these were TreSeq1 (fwd): 5′-CAA CTC TCA ACA ACG GAT ATC-3′, TreSeq2 (rev): 5′-GAC GCT GAG GCA GAC ATG CTC-3′, and TreSeq3 (rev): 5′-CCG AAG CCT CGA GCG CAA TTT-3′. For comparison, three ITS sequences were obtained from whole lichen DNA extracts using forward primer AL1500bf situated in the 18S region (Helms *et al.* 2001) and reverse primer LR3 located in the 26S region (Friedl & Rokitta 1997). Amplifications were performed in 50 μl reaction volume containing 2 μl genomic DNA (15–100 ng), 50 nM each dNTP, 5 μl 10 × PCR buffer (100 mM Tris-HCl pH 8·3, 500 mM KCl, 15 mM $MgCl_2$, 0·01% gelatin), 0·6 μM of each primer, 1·5 U Taq DNA polymerase (Sigma) and 35·75 μl autoclaved ddH_2O under the following conditions: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and elongation/extension at 72°C for 60 s and final extension at 72°C for 5 min.

Sequencing was done in a 10 μl reaction mix containing 0·8 μl BigDye Terminator Mix V3.1, 120 nM primer, 1× reaction buffer, and 10–20 ng purified DNA. This reaction mixture was modified from the manufacturer's protocol as follows: initial denaturation at 94°C for 2 min, 60 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 3 min (0·9°C/s ramp). The products were analyzed on an Applied BioSystem/HITACHI ABI 3730 DNA Analyzer (Life Technologies, Rotkreuz, Switzerland).

Phylogenetic analysis of DNA sequences

Sequences were analyzed with Sequencher™ 4.2.2 (Gene Codes Corp. Ann Arbor, USA) and aligned automatically with Clustal X 1.81 (Thompson *et al.* 1997). The resulting alignment was manually aligned on MacClade 4.06 (Maddison & Maddison 2002). Phylogenetic analysis was carried out with PAUP 4.0 b10 (Swofford 1998) by Maximum Parsimony (MP), Maximum Likelihood (data not shown), and Neighbor Joining (data not shown) methods. Bootstrap values for 1000 replicates were calculated. The sequence of *Trebouxia simplex* Tscherm.-Woess was used as an outgroup. This species is not a close relative of *Trebouxia decolorans* Ahmadjian, but we were able to align the ITS sequences. We used the grouping of samples within ITS clades that included known morphospecies of *Trebouxia* to identify the algal species, assuming that unknown samples would group with samples of the known morphospecies within the trees.

TABLE 1. *Collecting sites of lichen thalli and their Trebouxia photobionts isolated and analyzed in the present study*

Population*	Collector	Collecting site	Coordinates	Voucher no.	Substratum	Photobiont species†	ITS clade‡	No. of thalli/No. of photobiont isolates	No. of RAPD markers
Xanthoria parietina									
SW France: Roussillon, Dept. Pyrénées-Orientales	*R. Honegger*	Highway stop Perpignan Sud; mediterranean **urban**	42°41′55″N 02°53′44″E	111	*Celtis australis*	*Trebouxia decolorans*	n.d	5/7	37
SW France: Roussillon, Dept. Pyrénées-Orientales	*R. Honegger*	Villefranche du Conflent; **rural**	42°35′0″N 02°22′0″E	120 121	*Populus* sp. *Prunus spinos.*	*T. decolorans* *T. decolorans*	Ac & others	11/25	44
Central France: Burgundy, Dept. Yonne	*R. Honegger*	Relais fleuri near Avallon; **rural**	47°29′27″N 03°54′33″E	144 145	*Salix* sp. *Cornus alba*	*T. decolorans*	Ac	31/63	52
NW France: Brittany, Dept. Nord-Finistère	*R. Honegger*	Old port of Roscoff, Channel; **coastal**	48°43′08″N 03°85′48″W	164	*Myricaria germanica*	*T. decolorans*	Ac	9/8	25
NE Switzerland: city of Zürich	*S. Nyati & R. Honegger*	University campus, Irchel; **urban**	47°22′0″N 08°33′0″E	319 320/1–/2 320/3	*Parthenocissus tricuspidatus* *Salix* sp. sandstone	*T. decolorans* *T. decolorans* *T. arboricola*	Ac Ac Ab	33/47	57
Teloschistes chrysophthalmus									
Spain: Canary Islands	*R. Gerber*	La Gomera, **rural**	28°06′0″N 17°08′0″W	270	epiphytic	*T. gelatinosa*	Ib	4/4	26

* Collecting sites from the same area are grouped as one population. † Photobiont species identified according to ITS and *rbc*L phylogenies; n.d. not determined.
‡ ITS phylogenies in Fig. 2; "others" indicates *T. decolorans* isolates falling in unresolved part of phylogram between clade A3 and A4.
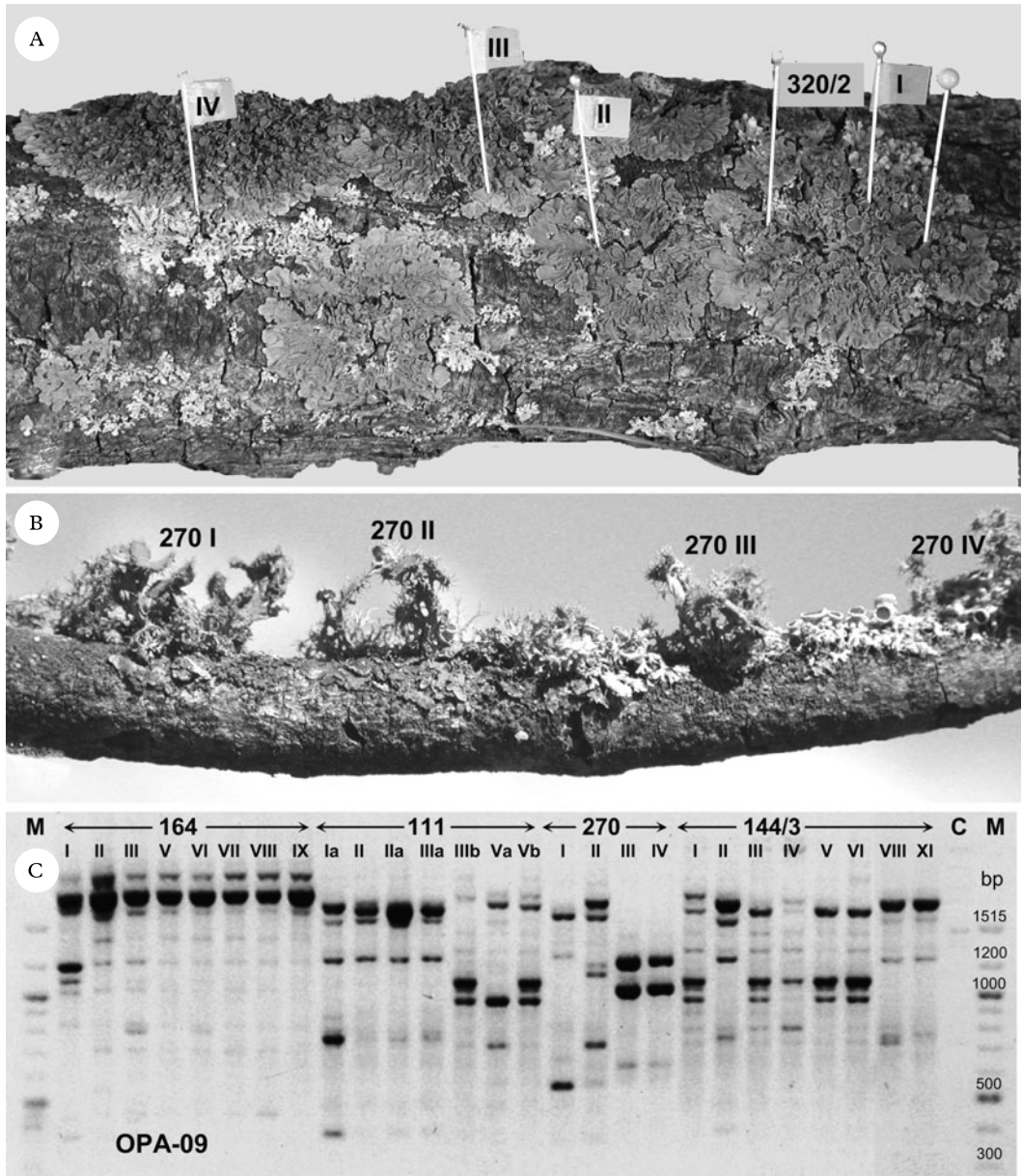
FIG. 1. A, Population 320/2, with *Xanthoria parietina* growing on a vertical branch of *Salix* sp. at the pond in the University park Zürich Irchel; B, Population 270, *Teloschistes chrysophthalmus* growing intermixed with *Physcia* spp. on a vertical branch; C, RAPD-PCR of samples of populations 164, 111 and 144/3 (all *X. parietina*) and 270 (*Telo. chrysophthalmus*). C: control. M: molecular marker. See analysis of this sample set in Fig. 3D.

RAPD amplification and fingerprinting analysis

RAPD amplification was carried out with decamer primers (Operon Technologies Alameda, CA). Initial primer screening was carried out with 80 primers (Kit A–D) applied to DNA extracted from 2 *Trebouxia* isolates. 11 primers were found to be algal specific, but 5 of them gave only a very weak reaction. 38 primers amplified DNA derived from photobiont and mycobiont isolates. In the current investigation, 4 primers (OPA-05: AGGGGTCTTG; OPA-09: GGGTAACGCC; OPB-10: CTGCTGGGAC, and OPC-06: GAACGGACTC)

were used for amplification. Reactions were performed with the Perkin Elmer Gene Amp PCR system 9600. 25 µl reaction mix was prepared, containing 1 µl genomic DNA, 1 × reaction buffer (Sigma), 0.2 mM dNTPs, 1·25 U Taq polymerase (Sigma), and 0·2 mM single primer. PCR conditions were as follows: initial denaturation for 3 min at 94°C, followed by 40 cycles of 30 s at 93°C, 40 s at 37°C and 80 s at 72°C with final extension of 5 min at 72°C. The ramp was 1°C/s. Negative controls were included in all experiments to detect contamination. 10 µl of each PCR product was loaded onto a 1·2 % agarose gel and run for ~6 hrs for good separation of bands. DNA molecular weight marker VI (Roche Diagnostics, Mannheim, Germany) was used as a fragment size marker (Fig. 1C).

DNA from all algal isolates obtained from the same population was processed in the same run. A few selected isolates were independently amplified to check the reproducibility of band patterns. In three experiments the suitability of RAPD fingerprinting was checked using phenotypically similar single cell and multi-cell isolates from the same apothecia. Five algal isolates were subcloned into 5 isolates each and amplified to test uniformity of isolates. Only clearly visible strong bands were considered, their presence or absence being marked in a binary data matrix per primer and finally in a combined data matrix for each population or dataset. Isolates for which satisfactory amplification was not obtained with all primers were removed from the data matrix. This binary data matrix was then used for phenetic analysis (NJ and UPGMA) on FreeTree (Pavlicek *et al.* 1999; Hampl *et al.* 2001) and for analyses of molecular variance (AMOVA). The robustness of the phenetic trees was tested with bootstrapping and jackknifing methods. Resulting phenograms were depicted with TreeView (Page 1996). Sequential agglomerative hierarchical nested cluster analysis (SAHN clustering) and ordination analysis were carried out on NTSYS-PC (Rohlf 2000). In SAHN clustering, cophenetic-values were used to compute cophenetic correlation as a measure of goodness of fit, and results were plotted in the form of a dendrogram. Principal coordinates analysis (Cox & Cox 2001) was performed in NTSYS-PC to show the relationships between isolates from different populations and substrata. Analysis of molecular variance was carried out with GenAlEx version 6.3 (Peakall & Smouse 2006) grouping isolates by algal species, populations, trees and branches, depending on the hierarchy levels included within a given dataset. AMOVA was performed on four data sets. One dataset included multiple algal lineages and sites, another included two algal species and several substrata from which these species had been collected. Last but not least, two data sets contained isolates collected from multiple branches of several trees. Hence, we were able to study population structure in the photobionts at different scales.

Results

Identity and diversity of photobionts

As shown in the ITS phylogram (Fig. 2) most of the isolates from samples collected in SW France (Roussillon), Burgundy and on our University campus in Zürich, Switzerland belonged to *Trebouxia decolorans* and revealed identical or near identical ITS genotypes. Numerous isolates contained a SSU 1512 group I intron of approx. 460 bases length, whose sequence was removed prior to alignment. For a detailed analysis of the group I introns in *Trebouxia* photobionts of *Teloschistaceae* see Nyati *et al.* (in press). The isolates collected on sandstone from *Xanthoria parietina* belonged to *T. arboricola* and those isolated from *Teloschistes chrysophthalmus* belonged to *Trebouxia gelatinosa* (Fig. 2).

A total of 25 to 57 markers were obtained with four RAPD primers in our fingerprinting experiments (Table 1). The genetic variability of the isolated photobionts was very high, as assessed by RAPD fingerprinting. Reproducibility of the RAPD markers was high when freshly isolated DNA was used and procedures were standardized. RAPD-PCR with genomic DNA isolated from 8 different isolates (voucher 144) was repeated using 4 different RAPD primers which showed very similar band patterns. Nevertheless, as we could not guarantee identical banding patterns among different PCR reactions, we decided against combining the RAPD banding patterns of different PCRs. Hence, several multi-locus RAPD data sets were analyzed separately, each representing the samples that had been included into one PCR reaction.

Population 120/121 from "Roussillon" (Departement Pyrenées Orientales), SW France

Four thalli of *Xanthoria parietina* growing on two fragments (120/1, 120/3) of the same branch of *Salix* sp. and 11 thalli growing on three small branches of *Prunus spinosa* (121/1-121/3) were collected just outside Villefranche-du-Conflent situated in a steep valley running parallel to the Pyrenees in SW France near the Spanish border. These samples are termed "Roussillon" in the ITS phylogram (Fig. 2), the local name of the larger area. All trees and shrubs at this site were completely covered by this golden-yellow lichen, indicating massive eutrophication. Both collecting sites were *c.* 10 m apart
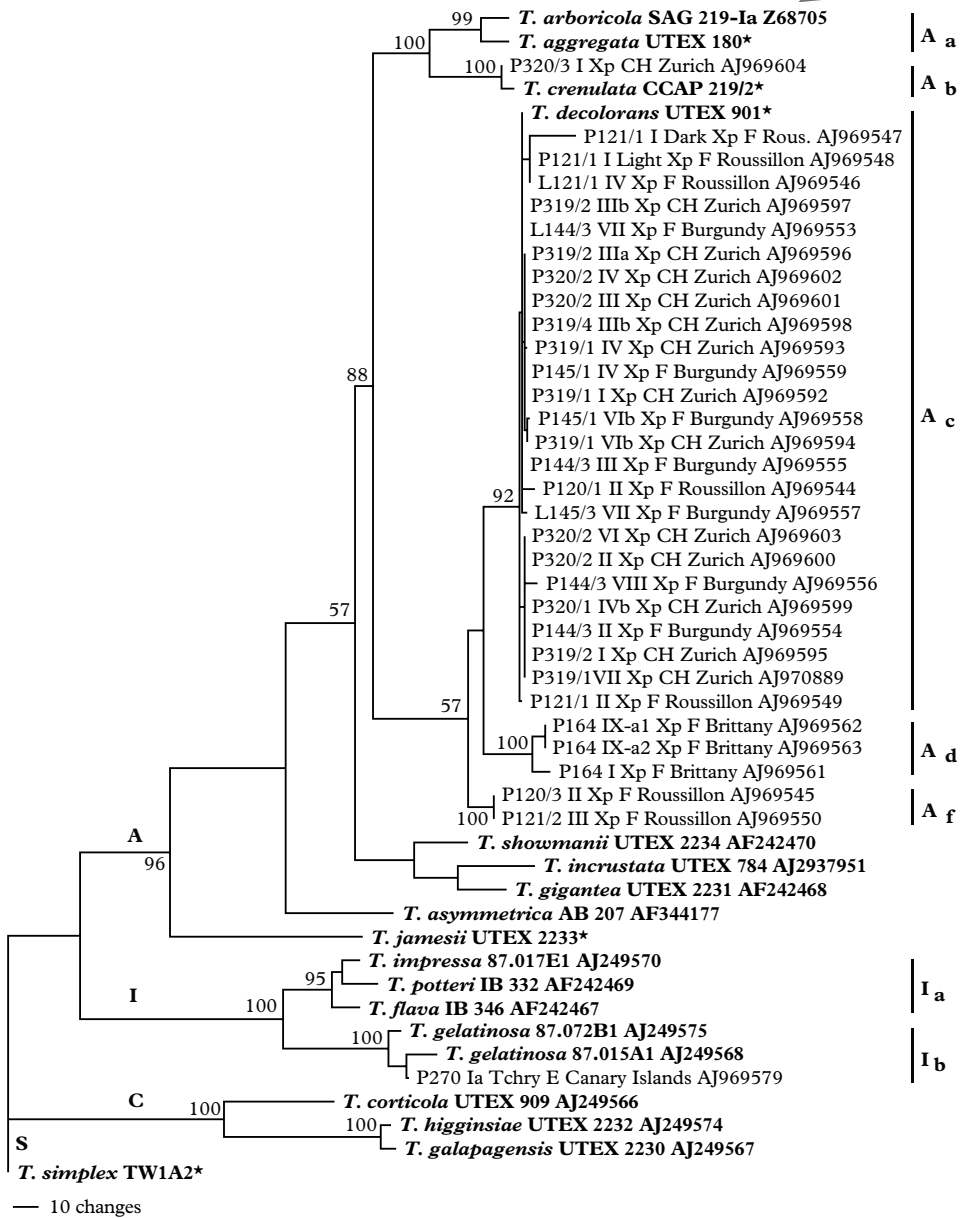
FIG. 2. Maximum parsimony phylogram of internal transcribed spacer regions (ITS1 and ITS2) along with 5.8S rDNA. Bootstrap values for 1000 replicates are given at the nodes. The letters A, I, C, and S indicate 'arboricola', 'impressa', 'corticola' and 'simplex' clades respectively. The tree is outgroup-rooted with a *Trebouxia simplex* sequence. The type species of the genus (*T. arboricola*, SAG 219-Ia) is indicated with arrowhead. Unpublished sequences provided by T. Friedl and G. Helms are marked with an asterisk. Each sample is labelled with voucher number followed by apothecia number, species abbreviation (*Xp: Xanthoria parietina*), country code, city/province and accession number. P denotes sequences obtained from a photobiont isolate while L indicates whole lichen DNA used for PCR amplification.

from each other. All thalli revealed the same phenotype and contained *T. decolorans* as photobiont. From sample 121/1 I (one apothecium) four different algal phenotypes were isolated, which could be distinguished by their colour: brown, green, light green and dark green. Two different ITS genotypes were distinguished (Fig. 2). With fingerprinting techniques three different genotypes were identified among these four different algal phenotypes (Fig. 3A). This was the first and only time that different genotypes from the same algal species were isolated from a very small area, i.e. the thalline margin of an apothecium. In the remaining 14 samples 13 algal genotypes were found (Fig. 3A). SAHN clustering and principal coordinates analysis showed no tendency of samples to group by phorophyte species.

Population 144/145 from Burgundy, (Departement Yonne), Central France

Twenty-two thalli of *Xanthoria parietina* were collected on three fragments of a branch of an old weeping willow (*Salix babylonica*) and 16 thalli on three twigs of a *Cornus albus* shrub in the garden of the mansion Le Relais fleuri near Avallon, situated within large fields adjacent to woods in Burgundy. All thalli of *X. parietina* revealed the same phenotype. ITS genotypes were very similar (Fig. 2). With fingerprinting techniques 36 different genotypes were identified among the 38 isolates of *Trebouxia decolorans* from this site (Fig. 3B). Cluster analysis and principal coordinates of the RAPD data showed a tendency for algal isolates to group by phorophyte species.

Population 164 from Brittany (Departement Nord-Finistère), NW France

Eight thalli of *X. parietina* were collected on a tamarisk shrub at the easternmost mole next to the Chapelle Sainte Barbe in the old port of Roscoff on the Channel in NW France. This small population was unique because it comprised different thalline phenotypes with varying amounts of anthraquinone irrespective of illumination. Thalli growing side by side revealed colours from intense orange-yellow (164 I) to bright intense yellow (164 II) to greenish (164 V), some being greenish with intensely yellow lobe margins (164 IV). Juvenile thalli of *X. parietina* growing side by side on a fully illuminated wooden garden bench next to the *Myricaria* shrubs showed the same varieties of phenotypes (Itten & Honegger 2010). In TLC analyses all thalli of this population comprised parietin as the main anthraquinone (R. Honegger, unpublished data); thus belonging to chemosyndrome A, according to Søchting (1997), a characteristic feature of *X. parietina*. All thalli of *Xanthoria ectaneoides* (Nyl.) Zahlbr. growing on granitic rock at this collecting site revealed chemosyndrome A3 (Søchting 1997), with parietin, teloschistin and fallacinal as the main anthraquinones (R. Honegger, unpublished data), and with *Trebouxia arboricola* de Puymaly as photobiont (Nyati et al. 2014).

Together with other *Trebouxia* isolates from *Xanthoria* spp. from maritime collecting sites on the Canary Islands, in the Eastern Mediterranean area and in Southern Australia the algal isolates of this population belong to a unique subclade A_d (Fig. 2) within clade A *sensu* Helms (2003). With fingerprinting techniques, five different genotypes

FIG. 3. Dendrograms obtained by SAHN clustering (left) and principal coordinate analysis with the first two axes shown (right). Each symbol represents an isolate. All except voucher numbers 270 are *Trebouxia* isolates from thalli of *Xanthoria parietina*. Details are given in the legend to the right. A, *Trebouxia decolorans* isolates from 'Roussillon' population (voucher numbers 120 on *Populus* sp., 121 on *Prunus spinosa*). B, *T. decolorans* isolates from Burgundy population (144 on *Salix babylonica*, 145 on *Cornus alba*). C, *T. decolorans* isolates from epiphytic populations (319 on *Parthenocissus tricuspidatus*, 320/1-320/2 on *Salix* sp.) and *T. arboricola* from saxicolous population (320/3) in Zürich. D, *T. decolorans* isolates from populations in Brittany (164 on *Myricaria germanica*), Perpignan (111 on *Celtis australis*), Burgundy (144/3 on *Populus* sp.) and *T. gelatinosa* from *Teloschistes chrysophthalmus* (270) from Canary Islands.
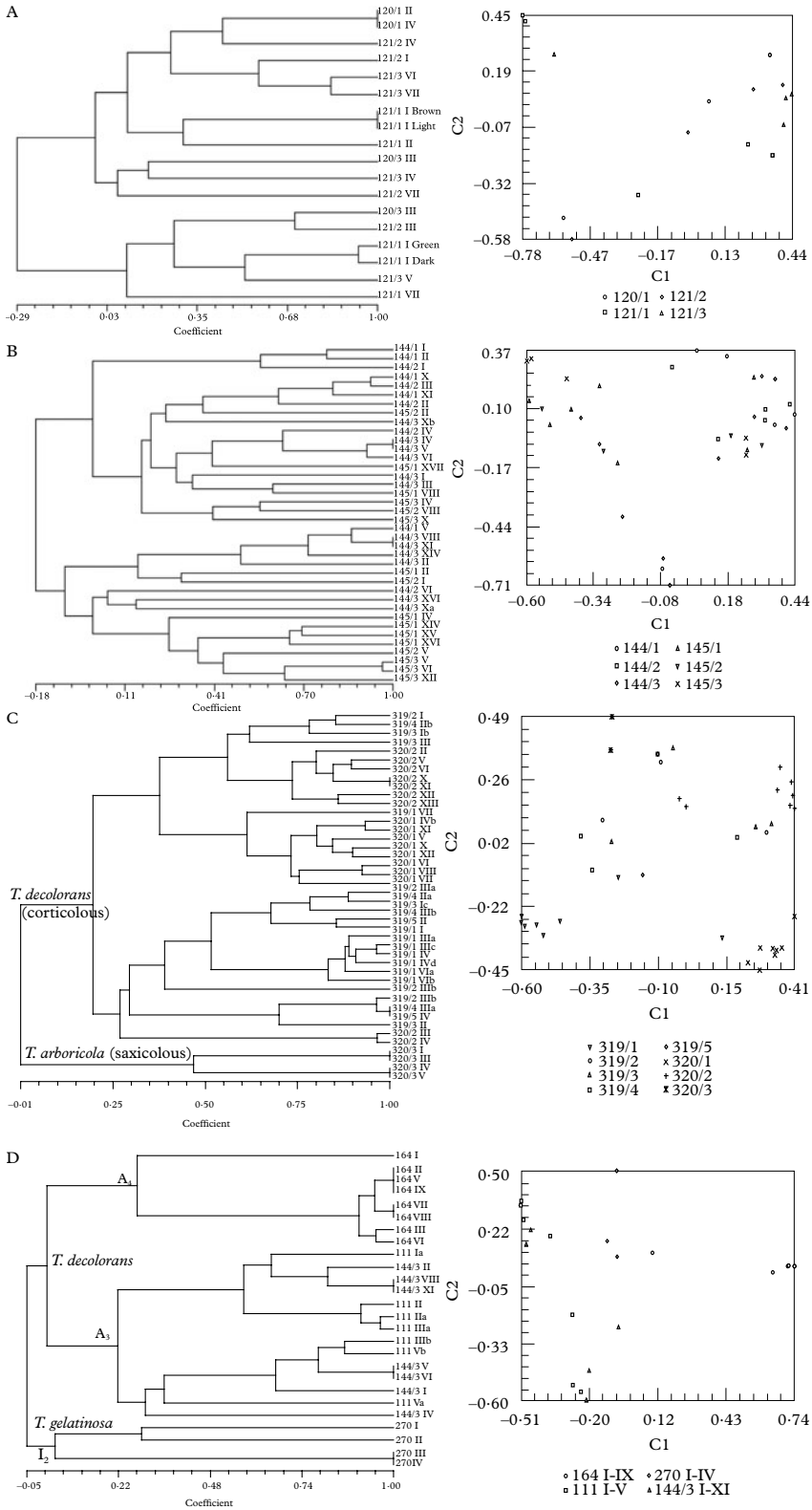
were found among the eight *Trebouxia* isolates from this site (Fig. 3), isolate Nr. 164/1 from the intensely orange coloured thallus, with a different ITS genotype (Fig. 2), being distinctly different from all others (Figs. 1 & 3).

Population 111 from Perpignan (Departement Pyrenées Orientales), SW France

A small population comprising seven thalli of *X. parietina* was collected on the stem of a *Celtis australis* tree at the stop of highway A9 (Narbonne to Barcelona) at Perpignan Sud in SW France. This site was newly constructed and trees planted less than 20 years ago. All thalli revealed an unusual phenotype with greenish grey colour and a slightly knobbly surface, but in a phylogenetic analysis with multi-locus approach they all turned out to be *X. parietina* (C. Eichenberger, unpublished data). In mediterranean and also in continental climates, the very smooth and hard bark of *Celtis australis* does not normally suppport lichen growth, probably due to its very poor water holding capacity.

Transplantation experiments would be needed to show whether these *X. parietina* thalli might differentiate a more typical phenotype on a different substratum. With fingerprinting techniques seven genotypes were found among the seven *Trebouxia* isolates from this site. None of them was sequenced, but as all isolates clustered with *T. decolorans* isolates from the Burgundy population 144, partly showing identical fingerprints (Fig. 3D), we conclude that they belong to the same species.

Population 319/320 from Zürich University campus, NW Switzerland

Twenty-one thalli of *X. parietina* were collected on the bottom part of five stems of a wild vine (*Parthenocissus tricuspidatus*; 319/1-319/5), nine on the stem (320/1), eight on a branch (320/2) of a willow tree (*Salix* sp.) by a pond and four on the sandstone underneath this willow tree (320/3) on our University campus at Zürich Irchel. All epiphytic samples comprised *Trebouxia decolorans*, all saxicolous samples *T. arboricola* as photobiont, as determined from sequences of the ITS region (Figs. 2, 3C). With fingerprinting techniques 36 genotypes were identified among the 38 epiphytic and two genotypes among the four saxicolous samples. Principal coordinates and cluster analysis showed a clear trend for isolates to group according to substratum.

Population 270: *Teloschistes chrysophthalmus* from La Gomera (Canary Islands)

The small population of *Teloschistes chrysophthalmus* from La Gomera, Canary Islands, was comprised of only four thalli growing on the same branch (Fig. 1B). With fingerprinting techniques, three distinctly different genotypes were found among the four isolates of *Trebouxia gelatinosa* Ahmadjian ex Archibald (Fig. 1C). *Teloschistes chrysophthalmus* is usually richly fertile but can also disperse vegetatively by means of the stiff marginal hairs at the tip of the fruticose thalli and at the apothecial margin, which break off very easily. They are predominantly fungal but contain algal cells in their basal part.

Analysis of molecular variance and cluster analysis

As expected, results from analysis of molecular variance indicated subdivision by algal lineages (Table 2). However, within algal lineages there was significant subdivision between populations or growth substrata, indicating that populations of the studied lichen fungi are structured. All studied sites except one (Roussillon) exhibited significant differentiation between lichen photobionts isolated from different phorophyte trees. Genetic differentiation among lineages of *Trebouxia* was high ($\Phi_{RT} = 0.28–0.46$). Lower but significant differentiation was found between different sites ($\Phi_{PR} = 0.16$). Isolates grouped by algal lineage and within algal lineages by sampling sites (Fig. 3D) or substrata (Fig. 3C) were in agreement with the cluster analysis as well as the analysis of molecular variance. The site which exhibited no differentiation between trees or branches in AMOVA (Roussillon) did not show any such structure

TABLE 2. *Analysis of molecular variance based on RAPD data sets. The table gives the source of variance, the degrees of freedom (df), the sums of squares (Sumsq), the type and value of the Φ-statistic, and its P-value based on 1000 permutations.*

Source	df	Sumsq	Statistic	Value	P-value
Population Roussillon (120–121)					
Among trees	1	8·4	Φ_{RT}	−0·008	0·448
Among branches within trees	2	17·8	Φ_{PR}	0·022	0·324
Among branches	14	113·0	Φ_{PT}	0·014	0·319
Population Burgundy (144–145)					
Among trees	1	27·0	Φ_{RT}	0·128	0·002
Among branches within trees	4	29·6	Φ_{PR}	0·006	0·414
Among branches	32	228·3	Φ_{PT}	0·133	0·004
Population Zürich (319–320)					
Among algal species	1	52·4	Φ_{RT}	0·288	0·001
Among substrata within species	5	158·1	Φ_{PR}	0·432	0·001
Within substrata	35	194·9	Φ_{PT}	0·595	0·001
Populations Perpignan (111)*, Burgundy (144)*, Roscoff (164)†, Gomera (270)‡					
Among algal lineages	3	118·0	Φ_{RT}	0·460	0·001
Among sites within lineages	1	13·1	Φ_{PR}	0·166	0·017
Among sites	21	115·4	Φ_{PT}	0·549	0·001

* *Trebouxia* clade A_c.
† *Trebouxia* clade A_d.
‡ *Teloschistes chrysophthalmus* associated with *Trebouxia gelatinosa* and *Trebouxia* clade I2.

in the cluster analysis either (Fig. 3A) but all other data sets showed some degree of population structure.

Discussion

The sexual lichen-forming ascomycete *Xanthoria parietina* associated with a diverse pool of *T. decolorans* strains in the sampling sites studied; saxicolous specimens associated with *T. arboricola*. *Teloschistes chrysophthalmus* had *T. gelatinosa* as a photobiont. As shown in the present study, RAPD-PCR is suitable for gaining valuable insights into the genetic diversity at the subspecific level in the green algal photobionts of lichen-forming fungi, provided that sterile cultured isolates are available. As it was technically not possible to combine all data from the present study into one large data set, the different populations were analysed separately.

A combined analysis was carried out on 28 photobiont isolates from 3 different *X. parietina* populations (photobiont *T. decolorans*) and a single *T. chrysophthalmus* population

(photobiont *T. gelatinosa*). The combined analysis was conducted to test the suitability of the RAPD technique in differentiating algal genotypes originating from different *X. parietina* populations and differentiating *Trebouxia* species (Fig. 1). SAHN clustering and principal coordinates analysis (Fig. 3) clearly grouped isolates into separate clades (clades Ac, Ad and Ib), as was the case in ITS phylogenetic analysis. A combined analysis was also carried out to infer population subdivision in *Trebouxia* photobionts. AMOVA results indicated that there was little population subdivision at the local scale (among trees and among branches within a site, Table 2), a pattern similar to that found for other photobionts (Werth & Scheidegger 2012). For *T. decolorans* photobionts associated with the epiphytic lichen *Ramalina menziesii*, differentiation was found between photobionts sampled from different phorophyte species, implying a possible ecological specialization of photobiont strains (Werth & Sork 2010). In the present RAPD data, significant genetic differentiation between

photobionts collected from different phorophytes was found in one site (Avallon) but a second site showed no such trend even though the lichens had been collected from multiple phorophyte species (Roussillon).

As expected, there was substantial genetic differentiation between algal lineages (i.e. species of *Trebouxia* and clades of *T. decolorans*) and within a given algal lineage, and there was substantial differentiation between geographically distant sampling sites. Similar large-scale geographic trends have been found in various photobionts of lichen-forming ascomycetes (Yahr *et al.* 2006; Wornik & Grube 2010; Fernández-Mendoza *et al.* 2011; Widmer *et al.* 2012).

Each population of *Xanthoria parietina* and even the small population of *Teloschistes chrysophthalmus* included in this study, turned out to consist of many different algal genotypes. In only a few of the 114 samples were identical fingerprints found. Some of them grew side by side (two isolates from each of six thalli), the others slightly apart from each other (3 × 2 and 1 × 3 isolates). The lowest diversity was found in the maritime epiphytic population 164 with five *Trebouxia* genotypes among eight samples of *Xanthoria parietina*.

How can the surprisingly high genetic diversity among *Trebouxia* isolates within the different populations be explained? One possible explanation for this pattern is recombination, leading to an increased number of algal multilocus genotypes. Even if they occurred in low frequency, recombination events would have a pronounced effect on the number of multilocus genotypes in a population. Based on DNA sequencing data, recombination has been inferred for *Trebouxia jamesii* photobionts of *Letharia* spp. in western North America (Kroken & Taylor 2000). In contrast, in an analysis based on microsatellite genotypes, no recombination was found for the green alga *Dictyochloropsis reticulata*, the photobiont of *Lobaria pulmonaria* (Dal Grande *et al.* 2012). Sexual reproduction has neither been demonstrated in the genus *Trebouxia* nor in other unicellular lichen photobionts of the genera of *Asterochloris* Tscherm.-Woess, *Coccomyxa* Schmidle, *Dictyochloropsis*, *Myrmecia* Printz etc. among the *Trebouxiophyceae*, which are all assumed to be asexual haplonts (Friedl & Büdel 1996). In the absence of sexual reproduction and recombination, genetic diversity results either from parasexual events, of which we have no knowledge in the algae concerned, or from mutations. Asexually reproducing organisms may accumulate a large number of mutations over time, as long as their genome is stable and no 'house-keeping' genes are negatively affected by mutations. Examples of ancient asexual haplonts are the arbuscular mycorrhizal fungi, which have accumulated numerous mutations over time so that their multinucleate mycelia are heterokaryotic, i.e. harbour nuclei belonging to a wide range of different genotypes (Hijri & Sanders 2004). Moreover, an example for the accumulation of mutations in an asexual haplont exists in lichen photobionts: the analysis of microsatellite data from many populations of *L. pulmonaria* demonstrated that the clonal green algal photobiont *Dictyochloropsis reticulata* has accumulated a substantial amount of somatic mutations (Dal Grande *et al.* 2012).

As *X. parietina* does not form symbiotic vegetative propagules but ascospores, it is assumed to re-lichenize at each reproductive cycle. The present findings suggest that there is not a pool of one to few compatible algal genotypes present at each site, which might be accepted by ascospore-derived germ tubes, but many different genotypes. Alternatively, the populations of *X. parietina* and their *Trebouxia* photobiont, as investigated in the present study, might be not true populations as seen, for example in flowering plants, but an assembly of many different combinations of the fungal and algal partners, which were brought to the site from different external sources. *Xanthoria parietina* forms neither soredia nor isidia but it does have options for vegetative dispersal in the symbiotic state. Thallus fragments are one possibility (Honegger 1996), lichenivorous invertebrates and their faecal pellets another one. Faecal pellets of the ever present oribatid mites, which feed on apothecia and thalli of *X. parietina*, grazing them down to the medullary layer, were shown to contain viable ascospores and *Trebouxia* cells (Meier *et al.* 2002).

Migratory and other birds, passively carrying faecal pellets with them, were assumed to play a largely underestimated role in short and long distance dispersal of lichens (Meier *et al.* 2002).

A study using a single cell manipulator to isolate algae from lichen thalli pointed towards the homogeneity of algal strains within the thalli of two lichen species (Beck & Koop 2001). Here, we found evidence for multiple algal strains in a thallus of *X. parietina*. The same result has also been reported for some other species, including *Evernia mesomorpha*, which frequently associates with different photobiont strains in the same thallus (Piercey-Normore 2006) and *Parmotrema tinctorum* (Mansournia *et al.* 2011). Helms *et al.* (2001) found two different photobionts within single thalli of crustose *Physciaceae* (*Rinodina astrocinerea*, *R. tunicata* and *Rinodinella controversa*). According to the findings of Casano and co-workers (2011), *Ramalina farinacea* always associates with two species of *Trebouxia* but such a pattern may be rare. The co-occurrence of multiple photobiont strains in the same thallus could be due to photobiont switching or to the fusion of thalli during development (Mansournia *et al.* 2011). However, it cannot be excluded with certainty that in some cases, the multiple strains may represent epibionts. In particular PCR-based studies based on whole thallus DNA extracts are prone to amplify algal epibionts. We isolated the algae from within the lichen thallus picking algal cells from the apothecial margin. Hence it is not likely that the two photobiont strains found in one apothecium represent the thallus photobiont and an epibiont.

An analysis of the genetic diversity of the fungal partner might show whether the *X. parietina* populations investigated in this study are mixtures of genotypes from different sources. *Xanthoria parietina* was shown to be a self-fertile species, in which the progeny of meiosis is genetically identical with the mycelium of the mother thallus (Honegger *et al.* 2004a; Scherrer *et al.* 2005). RAPD-fingerprinting of ascospore-derived sterile cultured isolates of some of the respective fungal partners has been conducted previously (Itten & Honegger 2010).

To conclude, this is the first study of its type where axenically isolated *Trebouxia* photobionts from several populations of *Teloschistaceae* lichens have been analyzed by the RAPD fingerprinting technique. We detected extensive genetic variation within and between algal lineages (e.g. *T. decolorans* clades), between substratum, and between geographically distant sampling sites. It will be stimulating to combine data sets on the fungal exhabitant and the algal inhabitant of lichen thalli to gain invaluable insights into population structure.

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