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Phylogenetic analysis of LSU and SSU rDNA group I introns of lichen photobionts associated with the genera *Xanthoria* and *Xanthomendoza* (Teloschistaceae, lichenized Ascomycetes)

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Abstract

We studied group I introns in sterile cultures of selected groups of lichen photobionts, focusing on *Trebouxia* species associated with *Xanthoria* s. lat. (including *Xanthomendoza* spp.; lichen-forming ascomycetes). Group I introns were found inserted after position 798 (*Escherichia coli* numbering) in the large subunit (LSU) rRNA in representatives of the green algal genera *Trebouxia* and *Asterochloris*. The 798 intron was found in about 25% of *Xanthoria* photobionts including several reference strains obtained from algal culture collections. An alignment of LSU-encoded rDNA intron sequences revealed high similarity of these sequences allowing their phylogenetic analysis. The 798 group I intron phylogeny was largely congruent with a phylogeny of the Internal Transcribed Spacer Region (ITS), indicating that the insertion of the intron most likely occurred in the common ancestor of the genera *Trebouxia* and *Asterochloris.* The intron was vertically inherited in some taxa, but lost in others. The high sequence similarity of this intron to one found in *Chlorella angustoellipsoidea* suggests that the 798 intron was either present in the common ancestor of Trebouxiophyceae, or that its present distribution results from more recent horizontal transfers, followed by vertical inheritance and loss. Analysis of another group I intron shared by these photobionts at small subunit (SSU) position 1512 supports the hypothesis of repeated lateral transfers of this intron among some taxa, but loss among others. Our data confirm that the history of group I introns is characterized by repeated horizontal transfers, and suggests that some of these introns have ancient origins within Chlorophyta.

Keywords

Lichen; LSU 798 group I intron; Photobiont; rbcL; SSU 1512 group I intron; Trebouxiophyceae; Trebouxia

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INTRODUCTION

Group I introns are a distinctive group of RNA sequences that catalyze their own excision from precursor RNA transcripts with concomitant exon ligation. Group I introns have a random and highly biased distribution owing to the two intron transfer mechanisms of reverse splicing and homing (Hoshina & Imamura 2009). Group I introns have been reported in various genes from a variety of organisms (Michel & Westhof, 1990). About 2,939 group I introns (for details, see http://www.rna.icmb.utexas.edu/SAE/2C/Distributions/g12tpt.php) have been identified in eukaryotic genomes (Haugen et al. 2005a, Nielsen & Johansen 2009, Bachar et al. 2013). Of these, 2,311 are found in the nucleus at dozens of sites in small and large subunit (SSU, LSU) ribosomal DNA. About 235 occur in mitochondrial genes and 393 in plastid DNA. Group I introns are characterized by four conserved core sequences (P, Q, R and S) and ten conserved secondary structures (P1 to P10; Burke et al. 1987). Five major subgroups of group I introns, termed IA, IB, IC, ID, and IE are recognized based on secondary structure (Michel & Westhof, 1990) and are always inserted into highly conserved regions of rRNA genes (Johansen et al. 1996). All group I introns catalyze their excision from primary transcripts using guanosine as a cofactor. Many of these ribozymes are autocatalytic, others rely on host-derived factors to facilitate splicing (Cech 1988, Cech et al. 1994). Among two *Chlorella* photobiont species of the ciliate *Paramecium bursaria* a monophyletic clade of six introns was found, all of them derived from an ancestral intron (Csw.L2449) that had spread into heterologous sites (Hoshina and Imamura 2009). Some of these introns share common internal guide sequences, but in others these sequence fragments are inserted further upstream (Hoshina & Imamura 2009). These *Chlorella* photobionts are proposed to be a model system for studies on how group I introns insert at novel sites.

In eukaryotic microorganisms, horizontal transfer of group I introns appears to be the rule, rather than the exception. Therefore, the mapping and phylogenetic characterization of the widespread, but scattered nuclear group I introns can potentially clarify pathways and mechanisms of intron movement among eukaryotes (Einvik et al. 1998). Various studies have focused on the mechanisms underlying group I intron mobility. Many group I introns in the genomes of plastids, mitochondria, phages and eubacteria contain open reading frames (ORFs) that encode endonucleases. These endonucleases mediate sequence-specific ‘homing’ of group I introns into allelic sites (Dujon 1989). Although group I introns do not normally contain more than one ORF, the intron in the *cox1* gene of the non-photosynthetic, parasitic chlorophyte *Helicosporidium* sp. contains two ORFs (Pombert & Keeling 2010). Intron movement can also occur via reverse splicing, as demonstrated for the *Tetrahymena thermophila* LSU group I intron (Sogin et al. 1986).

Intron loss appears to be common as demonstrated by the ‘optional’ distribution of group I introns within closely related taxa (Bhattacharya et al. 1996a, Bhattacharya et al. 1996b). At the RNA level, intron loss seems to occur by reverse transcription of an intron-less RNA followed by homologous recombination with the intron containing genomic copy of the coding region (Dujon 1989). In some eukaryotic lineages (e.g., plants, including green algae, charophytes, red algae, or ciliates, fungi etc.) mobility conferring - open reading frames (ORFs) have not yet been found in group I introns. This suggests that intron mobility (and loss) is likely to be mediated by reverse-splicing that does not rely on a group I intron-encoded ORF (Bhattacharya et al. 1996b). However in myxomycetes (Amoebozoa) such as *Didymium iridis* and *Physarum polycephalum*, nuclear group I introns spread extensively via homing (e.g., Ruoff et al. 1992, Haugen et al. 2005b). Intron sequences encoding homing endonucleases have also been reported from lichenized ascomycetes (Reeb et al. 2007). Putative group I introns were found in nuclear the rSSU gene of the non-lichenized ascomycetes *Phialophora americana*, *P. europaea* (both saprotrophs) and *P. verrucosa*

(human pathogen; Dothideomycetes), which were found to be monophyletic along with introns of lichen forming ascomycetes *Porpidia crustulata* (Lecanoromycetes) and *Arthonia lapidicola* (Arthoniomycetes; Harris & Rogers 2011). During a large phylogenetic survey of green algae, twenty three distantly related isolates were found to contain at least one group I intron in the chloroplast *rbc*L gene, all of them with distinct evolutionary origins. These introns were inserted either after position 462 or 699 (McManus et al. 2012).

The lichen symbiosis is particularly interesting with regard to group I intron presence and distribution. In lichen-forming ascomycetes, several group I introns at 13 different insertion positions within the nuclear SSU rDNA have been identified (Bhattacharya et al. 2002). The SSU rDNA intron variation observed by DePriest (1993) in a population of *Cladonia chlorophaea* was due to optional group I introns, which varied in number, position, restriction pattern and size. Some of these insertion positions are unique, whereas others (516, 943, 1046, and 1506) are also found in other organisms including unicellular green algae (Gargas et al. 1995b). Within the monophyletic Parmeliaceae (Lecanorales; more than 60 species investigated) correlations between intron insertion sites and ecological and geographical parameters were observed (Gutierrez et al. 2007). Because the green algal photobionts of lichen-forming fungi contain numerous nuclear-encoded rDNA group I introns, they are a model group to study the origin and phylogeny of these sequences (Bhattacharya et al. 1998, Bhattacharya et al. 1994, Bhattacharya et al. 1996a, Bhattacharya et al. 1996b). Friedl et al. (2000) concluded that the SSU rDNA 1,512 group I intron was present in the common ancestor of the green algal classes Trebouxiophyceae, Chlorophyceae and Ulvophyceae and that it was laterally transferred at least three times among species of *Trebouxia* de Puymaly. They also concluded that intron loss was a common event during chlorophyte evolution. The green algal SSU group I introns at insertion sites 1,056, 1,506, and 1,512 (position relative to the *E. coli* coding region) form distinct phylogenetic lineages based on the insertion site (Bhattacharya et al. 1994, Bhattacharya et al. 1996b). The intron phylogenies were largely congruent with the rDNA (i.e., host cell) phylogeny, suggesting vertical inheritance of the introns rather than lateral transfers during the evolution of green algae, with some exceptions being described by Friedl et al. (2000) and Bhattacharya et al. (2001).

Del Campo et al. (2010) partially sequenced plastid LSU rDNA in fifteen *Trebouxia* and *Asterochloris* species and observed a high diversity of group I introns. The resulting phylograms revealed two main clades, one comprising *A. erici*, *A. glomerata*, *A. irregularis*, the other *T. arboricola*, *T. crenulata*, *T. decolorans*, *T. shomanii* and *T. jamesii*. The authors suggested combining the phylogeneitic analyses of the chloroplast and nuclear (nrITS) for improving phylogenetic accuracy. (del Campo et al. 2010) described different group I introns (at position cL2449 and cL2504) in chloroplast LSU rDNA in *Trebouxia* photobiont within the same thallus of *Ramalina farinacea* (L) Ach. and speculated on presence of different photobiont species within a thallus.

In studies of the genetic diversity among green algal photobionts of *Xanthoria* s. lat. and *Xanthomedoza* spp. (lichen-forming ascomycetes), several *Trebouxia* strains and species were found to contain group I introns (Nyati et al. submitted). Moreover, these lichenized fungi associate with a well-defined, limited number of algal strains. Hence, this group of lichen photobionts represents a model system to study intron gains and losses in *Trebouxia*. The genus *Asterochloris* Tscherm.-Woess forms lichen symbioses with *Cladonia* and other lichenized ascomycetes. We included type strains of *Asterochloris* to infer whether some introns could be ancient, as evidenced by intron sharing among *Trebouxia* and *Asterochloris* lineages. The main aim of this study was to characterize the group I introns and investigate the possibility of intron gains and losses among photobionts associated with lichen-forming ascomycetes of the genera *Xanthoria* s. lat. and *Xanthomendoza*. For this purpose, we: 1)

characterize a newly found nuclear LSU rDNA group I intron at site 798 (L798); 2) determine the distribution of the L798 and nuclear SSU rDNA 1512 (S1512) group I introns; and 3) investigate congruence between nrITS and intron phylogenies among green algal lichen photobionts of the genera *Trebouxia* and *Asterochloris*.

MATERIALS AND METHODS

Lichen collection, storage and photobiont isolation and culture

Photobionts were isolated and cultured as described (Honegger 2003) from several *Xanthoria* spp. including the broadly sampled *X. parietina* (Table 1). Type cultures of algal strains were obtained from the Culture Collection of Algae at the University of Innsbruck, Austria (IB/ASIB) (Table 2). All cultured algal strains included in this study including reference strains of sterile algal cultures, are stored in liquid nitrogen at the Institute of Plant Biology, University of Zürich, Switzerland following the method of Honegger (2003).

DNA extraction, PCR amplification and sequencing

Genomic DNA from algal isolates or whole lichens was extracted using the GFX PCR, DNA and Gel Band Purification Kit (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's protocol. PCR amplification of a partial LSU rDNA fragment (position 660-1100 of *T. asymmetrica*, Z95380) was done with newly designed primers (Table 3). The PCR reactions were performed in 50 μL reaction volumes containing a reaction mix of 0.2 mM of each of four dNTPs, 1.5 μM of each PCR primer, and 1.25 U of Taq DNA polymerase (Sigma Aldrich, Buchs, SG, Switzerland) and 5 μL of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin). Reactions were performed in a PTC 200 DNA engine (MJ Research Inc., Watertown, MA, USA) with the following conditions: initial denaturation for 3 min at 95°C, followed by 30 cycles of (30 sec at 95°C, 30 sec at 60°C, 1 min at 72°C) and a final extension for 10 min at 72°C. Newly designed intron specific primers were used for PCRs (Table 3), which were carried out with genomic DNA isolated from algal strains and from whole lichen DNA extracts. PCR amplification was carried out with the following settings: 3 min at 95°C, 30 cycles of 30 sec at 95°C, 40 sec at 56°C, and 1 min 20 sec at 72°C with final extension at 72°C for 10 min. Sequencing was carried out using ABI Prism BigDye Terminator Mix V3.0 Cycle Sequencing Kit (Life Technologies, Rotkreuz, Switzerland) following the protocol of the manufacturer and capillary electrophoresis of cycle sequencing products was performed on an Applied BioSystems ABI 3730 DNA Analyzer (Life Technologies, Rotkreuz, Switzerland).

Sequence alignment, secondary structure prediction, and phylogenetic analysis

Sequence contigs were assembled using Sequencher™ 4.2.2 (Gene Codes Corp. Ann Arbor, USA) and ambiguous positions were manually corrected. The final sequences were aligned using ClustalX (Thompson et al. 1997). The resulting alignment was visually checked for any discrepancies and manually corrected on MacClade V5.0 (Maddison & Maddison, 2002). Intron insertion position was determined based on BLAST searches and sequence alignments. The putative secondary structure of the L798 intron was determined using comparisons to existing structures and m-fold (Zuker, 2003) following the guidelines of Cech (1988) and Cech et al. (1994).

Maximum likelihood (ML) analysis was carried out using PAUP 4.0 b10 (Swofford, 2003). Bayesian phylogenetic reconstruction was performed with MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003, Huelsenbeck & Ronquist 2001). To determine the best models according to the AIC criterion (Posada & Buckley 2004, Sakamoto et al. 1986) for ML and Bayesian phylogenetic analyses, MrModeltest version 2.3 (Nylander, 2004) was applied to

each data set. For the LSU and SSU green algal and SSU intron data sets, the General Time Reversible (Tavaré, 1986) (GTR+G) model provided the best fit to the data as determined by AIC in MrModeltest. In contrast, the model best describing the LSU intron data set according to AIC was a version of the General Time Reversible (GTR+I+G, AIC=8411.15) although its AIC was only slightly lower than that of GTR+G model (AIC=8411.28). Using the models determined by AIC in MrModeltest, ML and Bayesian analyses were performed on three data sets: 1) Twenty-eight rDNA sequences of the rDNA region excluding all introns, i.e. the "host" of the intron (data set 'LSU host'), compiled for comparability with the LSU L798 intron sequences. The host cell phylogeny, as presented in Figure 1, is based on alignments of the complete ITS region, including the 5.8S rRNA gene. 2) The LSU intron, comprising 31 sequences ('LSU intron'); and 3) thirty-six DNA sequences of the SSU S1512 intron ('SSU intron'). To assess for statistical support, the ML analysis in PAUP used 100 bootstrap replicates in a heuristic search based on optimality criterion likelihood, with random addition of sequences. Maximum parsimony analysis was performed in PAUP with 100 bootstraps. Tree-bisection-reconnection was used as a branch-swapping algorithm. In MrBayes, analyses were run for 5 Mio generations with four chains sampling the parameter space, sampling trees every 1,000 generations for a total of 5,000 trees, reaching stationarity and receiving effective sample sizes > 3,000 as assessed using Tracer version 1.5 (Rambaut & Drummond 2007). Of the sampled trees, 10% were discarded as burn-in. TreeAnnotator version 1.6.0, part of the Beast software (Drummond et al. 2013), was used to summarize trees and create a maximum clade credibility tree which was visualized using FigTree version 1.4.1 (Rambaut 2008).

To compare whether partitioned rDNA-host and intron data sets were homogeneous, incongruence length difference tests (Farris et al. 1995) were performed on two combined data sets in PAUP, using 1000 replicates and a heuristic search strategy, with parsimony as optimality criterion. The first combined data set contained 23 sequences of the L798 intron with corresponding rDNA host sequences ('Combined LSU intron'). The second combined data set contained 33 DNA sequences of the S1512 intron with their corresponding rDNA host sequences ('Combined SSU intron').

Missing characters, gaps, and ambiguous sites were excluded in all phylogenetic analyses. For the visualization of phylogenetic results, distance trees were constructed by the neighbor-joining algorithm implemented in MEGA version 5 (Tamura et al. 2007) and annotated with support values from Bayesian and ML analyses as well as with support values from 500 bootstraps using the neighbor-joining algorithm in MEGA.

RESULTS AND DISCUSSION

Trebouxia L798 group I intron

In the current investigation 28 LSU rDNA group I introns were found, which are all inserted at position L798 and belong to group IB introns (Fig. 2). In a secondary structure based alignment of *Trebouxia* LSU introns, the sequence of *Chlorella angustoellipsoidea* C-87 was included (Aimi et al. 1994) because this intron was also 445 base pairs (bp) in length and had all the characteristic RNA folding (P1-P10) properties of group IB introns (Burke et al. 1987). The flanking exon regions were also highly conserved.

The L798 group I intron phylogeny (Fig 3) showed a similar grouping of taxa into clades as the ITS host cell tree (Fig. 1), indicating vertical inheritance of the intron. The only exception was the intron of *T. showmanii* (Figs. 1 and 3), which was placed in the 'arboricola' clade, indicating the possibility of lateral transfer. A comparison of the conserved core catalytic sequences (P, Q, R, S) revealed high similarity with multiple nucleotide substitutions between *Trebouxia* subclades and the *Asterochloris* clade (Table 4).

Moreover, an incongruent length difference test rejected the hypothesis of homogeneity among intron and host data sets (p=0.005). Due to the conserved secondary structures among the LSU group I intron of representatives of the genera *Trebouxia* and *Asterochloris* (Microthamniales) and of *Chlorella angustoellipsoidea* (Chlorellales; Table 4), we hypothesize that this intron was present in the common ancestor of the genera *Trebouxia* and *Asterochloris*.

Trebouxia S1512 group I intron

Intron specific primers were used for PCR amplification reactions, which were carried out with genomic DNA isolated from algal strains and from whole lichen DNA extracts. Several different primer combinations were used to exclude PCR bias. These tests were done to infer the frequencies of introns in the investigated algal isolates. Of the 124 nrDNA sequences generated for *Trebouxia* photobionts of *Xanthoria* s. lat and *Xanthomendoza* spp., 32 contained a group I intron. The intron-harboring *Trebouxia* photobionts belonged to the "arboricola" clade A as described by Helms (2003). In all photobiont samples except for one, the intron varied between 434 bp and 530 bp in length. The 460 bp intron of the *T. arboricola* photobiont of the African *Xanthoria turbinata* (accession No. AJ969509) had an additional insertion of 1090 bp at a conserved site, making the entire intron sequence 1550 bp in length. Although this intron is very long, it does encode an ORF for a homing endonuclease as assessed by blast searches (Altschul et al. 1997). No similarities were found between this longer intron and complex nested inserts found in 18S rDNA of ascomycetes, where putative spliceosomal introns were inserted within group I introns (DePriest & Been 1992, DePriest 2004, Myllys et al. 1999)

In comparison with published data (Bhattacharya et al. 1996b) all insertions at position 1512 were identified as group I introns. Twenty-nine newly generated intron sequences together with available data were analyzed using phylogenetic methods (Figs. 3 and 4) and compared with the ITS phylogeny (Fig. 1) to identify potential cases of lateral transfer (Bhattacharya et al. 1996b, Friedl et al. 2000). The ITS and S1512 group I intron phylogenies showed a similar grouping of clades (Figs. 1 and 4), indicating overall vertical inheritance. However, incongruence length difference tests suggested that the data sets were not homogeneous (p=0.001). Indeed, three interesting incongruities were found among host and intron phylogenies (marked with arrowheads in Fig. 4): 1) the intron sequence of *Trebouxia corticola* (UTEX 909, a putatively free-living alga from tree bark in Webster, MA, USA isolated by V. Ahmadjian in 1959), a representative of the "corticola" clade C according to (Helms, 2003), falls near three intron sequences from clade A. These *Trebouxia decolorans* photobionts were identified from *X. parietina* thalli from Barossa valley (AUS) and Sonoma County (California, USA) and from an unnamed *Xanthoria* species from Tasmania (AUS). 2) The intron sequence of *Trebouxia impressa* (Friedl et al. 2000), a representative of the "impressa" clade I according to Helms (2003), falls near two *T. decolorans* intron sequences from clade A. These *Trebouxia decolorans* photobionts were found in thalli of *X. parietina* and *Xanthomendoza hasseana* in California. 3) Intron sequences of *T. arboricola* isolates from *X. ectaneoides* (Sicily, Italy), *X. parietina* (Avenches, Switzerland), *X. polycarpa* (Otago, New Zealand), and *X. turbinata* (Port Nolloth, South Africa) formed a separate group outside the group of samples belonging to *T. decolorans*, receiving high support in all three analyses (Fig 4). All these incongruities are likely to have resulted from intron lateral transfers.

LSU and SSU group I intron distribution in *Trebouxia* and *Asterochloris* spp

Introns in both SSU and LSU rDNA were found in only nine *Trebouxia* isolates. The distribution of these taxa in the photobionts of *Xanthoria* s. lat. (Table 1) and in the host tree (Table 2) follows neither a geographic nor a taxonomic pattern. *Trebouxia* isolates from

thalli of *Xanthoria parietina* from the Northern Hemisphere are however over-represented in this analysis, and only one sample was studied from some of the other species. In a survey of the genetic diversity among *Trebouxia* photobionts of Teloschistaceae (n = 124) the S1512 group I intron was found in 50% of *T. arboricola*, but in only 23% of *T. decolorans* samples (Nyati et al. submitted). Friedl et al. (2000) found a S1512 intron in 28% of 85 algal strains that were tested. In a PCR-based screening of sterile cultured isolates of *T. arboricola* or *T. decolorans*, respectively, from populations of *Xanthoria parietina* and *X. ectaneoides* from maritime, coastal, rural, and urban sites (for details see Itten & Honegger 2010) a very unequal distribution of S1512 group I introns was observed among populations, ranging from none to 87% (Table 5). The percentage distribution of introns was assessed by combining sequences obtained from several photobiont isolates from within a single lichen thallus as well as from a number of different lichen thalli within a population. This result may reflect the dynamic nature of intron gain and loss. Very high genetic diversity by RADP-PCR fingerprinting has been observed from the axenically cultured mycobiont isolates from the same populations (Itten & Honegger 2010). The *Trebouxia* photobionts of *Xanthoria ectaneoides* or *X. parietina* populations with the highest and lowest percentages of S1512 group I introns were collected less than 1 km apart from each other. The first was found on granite rock in the supralittoral fringe of a tiny island (Île verte), the other on shrubs in the old port of Roscoff in Brittany (NW France; Table 5). However, we cannot exclude that recombination could redistribute introns among individual algal cells in free-living populations of *Trebouxia*, followed by gene flow, which would lead to the same pattern of intron distribution in populations.

Introns of lichen photobionts and mycobionts

The origin of S1512 group I introns in chlorophytes is still a matter of debate. Based on the observation of a close phylogenetic relationship of the S1512 group I intron lineage in chlorophytes to viral introns found in *Chlorella* spp., the viruses were hypothesized to be either the source or at least the vector, which facilitates the spread of group I introns among eukaryotes (Bhattacharya et al. 1996b, Aimi et al. 1994).

Horizontal transfer of group I introns is known from many biological systems. Examples of interkingdom transfers are: 1) plant parasitic fungus to host plant (Nishida & Sugiyama 1995, Vaughn et al. 1995, Sanchez-Puerta et al. 2008); 2) fungus to green alga (Lindstrom & Pistolic 2005); 3) fungus to red alga (Müller et al. 2005); 4) red alga to brown alga (Bhattacharya *et al.* 2001); 5) algae to amoebae (Turmel et al. 1995); 6) between eubacteria and chloroplasts (Kuhsel et al. 1990). Inter-organellar transfer of group I introns has been shown from mitochondria to the nucleus (Curtis & Archibald 2010), and from mitochondria to the chloroplast (Pombert et al. 2005). Interfamilial transfers among lichen-forming ascomycetes within the Physciaceae were described by Simon et al. (2005). Based on the close relationship of introns in green algal lichen photobionts with bacterial and fungal introns, del Campo et al. (2009) proposed lichen thalli to be potential sites of horizontal transfer of introns. Indeed, already in the Lower Devonian (approx. 415 Myr ago) were the thalli of lichen-forming fungi microcosms, built up by the quantitatively predominant lichen-forming fungus and its photoautotrophic green algal or cyanobacterial partner, but comprising also large numbers of epi- and endolichenic bacteria and non-lichenized fungi, all in close physical contact with the lichen mycobiont and partly also with the photobiont (Honegger et al. 2013), as is the case in extant lichens (Honegger 2012), However, no direct evidence has yet been reported demonstrating SSU rDNA group I intron movement between the fungal and algal partners in the lichen symbiosis.

Only few insertion positions (S516, S943, S1046, and S1506) for group I introns are found in both green algae and ascomycetes (Gargas et al. 1995b). The SSU intron at position

S1512, which is present on the surface of the mature ribosome in tertiary structure, is restricted to green algae (Gargas et al. 1995a, Gargas et al. 1995b); it has never been found in fungi or other organisms. DePriest and Been (1992) concluded that group I introns of lichen algae do not originate from their respective fungal partners or vice versa. Bhattacharya et al. (2002) demonstrated that there is no evolutionary relationship between group I introns of lichen-forming ascomycetes and their green algal partners; thus horizontal transfer can be excluded. One process that might lead to a similar pattern of intron distribution as observed in our study is photobiont switching (Piercey-Normore & DePriest 2001, Nelsen & Gargas 2008, Nelsen & Gargas 2009). Photobiont switches could occur if a developing germling incorporates either a free-living alga, or an alga that has been released from a thallus e.g. after being digested by invertebrates. Algal cells have been shown to survive the gut passage of mites (Meier et al. 2002) and snails (Boch et al. 2011), providing an opportunity for algal uptake by developing germlings. Friedl et al. (2000) suggested that lichenization might facilitate the spread of 1512 introns among algal strains that coexist in fungal thalli. However, it is difficult to imagine how intron gain might proceed within the lichen thallus. In morphologically advanced, foliose, or fruticose lichens the algal cells have physical contact only with sister cells derived from the same mother cell, but each of them is in direct contact with the fungal partner via the appressorial or haustorial complex (Honegger, 1991). A mycobiont-derived, water-repellent wall surface layer composed of hydrophobins and/or other hydrophobic wall surface components (Scherrer et al. 2000), ensheaths the algal and fungal surfaces in the thalline interior (Honegger 1991, Honegger 2001). During the regular wetting and drying cycles solutes are passively exchanged underneath this hydrophobic sealing via the apoplastic continuum of both partners, but it seems unlikely that mobile genetic elements would be transmitted through this process. It is conceivable that intron gain occurs while lichen photobionts are not symbiotic, but free-living and in contact with cells of other species or strains. The example of the S1512 group I intron of the type strain of *Trebouxia corticola*, as reported in this study, is consistent with this idea. This strain was isolated from putatively free-living cells, not from a lichen, and its intron is similar to that in *Trebouxia decolorans* isolates from a different clade, the “arboricola” clade A (i.e., Helms 2003). According to the literature, *Trebouxia arboricola*, the type species of the genus and photobiont of numerous lichen-forming ascomycetes, including Teloschistaceae, is also a common and widespread free-living alga (Rindi & Guiry 2003, John et al. 2002, Ettl & Gärtner 1995, Beck et al. 1998), despite contradictory claims (Ahmadjian 1988). The mechanisms underlying intron gain in lichen photobionts merit therefore a thorough investigation in future studies.

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Abbreviations

LSU	large subunit
SSU	small subunit
ITS	internal transcribed spacer

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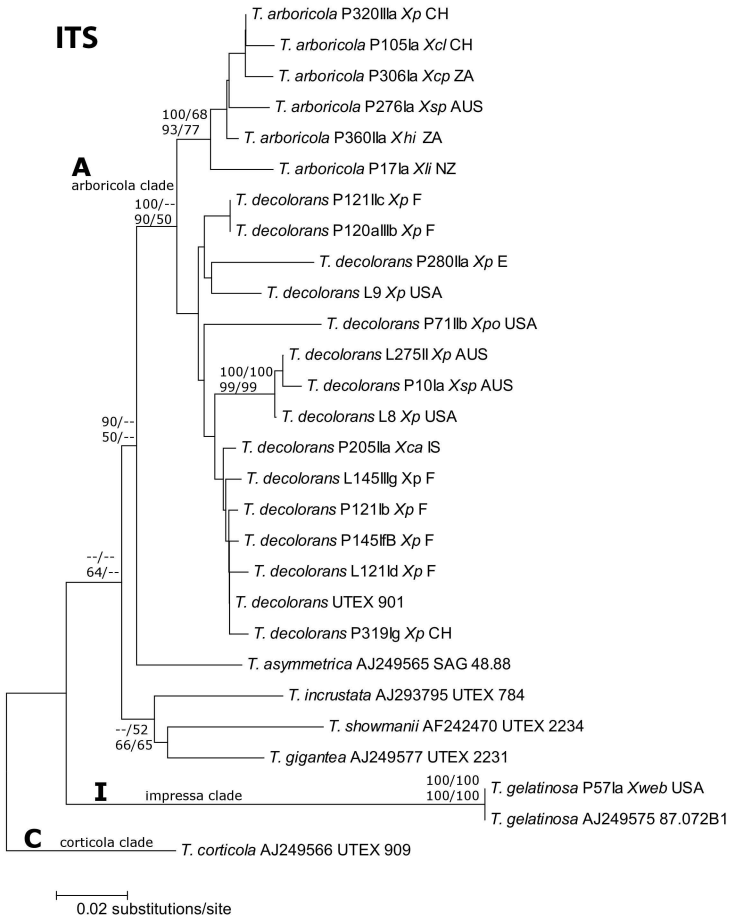

Fig. 1.

Phylogeny of *Trebouxia* s. lat.. Sequences obtained from GenBank are indicated in Table 3. Support values shown at a respective node are posterior probabilities of nodes from Bayesian analysis in MrBayes (first row, first number), values from 100 bootstrap replicates in a maximum likelihood analysis in Paup (first row, second number), values from 500 bootstrap replicates in a neighbor-joining analysis in MEGA (second row, first number) and maximum parsimony support values from 100 bootstraps generated with PAUP (second row, second number). Clades A, I and C represent ‘arboricola’, ‘impressa’ and ‘corticola’, respectively, as proposed by Helms (2003). The identity of photobionts was inferred on the basis of ITS and *rbc*L phylogenetic analyses including reference strains (Nyati, Scherrer, Werth and Honegger, in press). Abbreviations used: Xca: Xanthoria candelaria, Xcl: X. calcicola, Xcp: X. capensis, Xec: X. ectaneoides, Xh: X. hirsuta, Xli: X. ligulata, Xp: X. parietina, Xpo: X. polycarpa, Xtu: X. turbinata, Xsp: Xanthoria sp., *Xweb: Xanthomendoza weberi*.**.** Phylogeny of the nuclear ribosomal internal transcribed spacer region (complete ITS region, including the 5.8S rRNA gene) as inferred using Maximum Likelihood (ML) analysis. *Asterochloris* sequences not used in analyses due to very high sequence divergence.

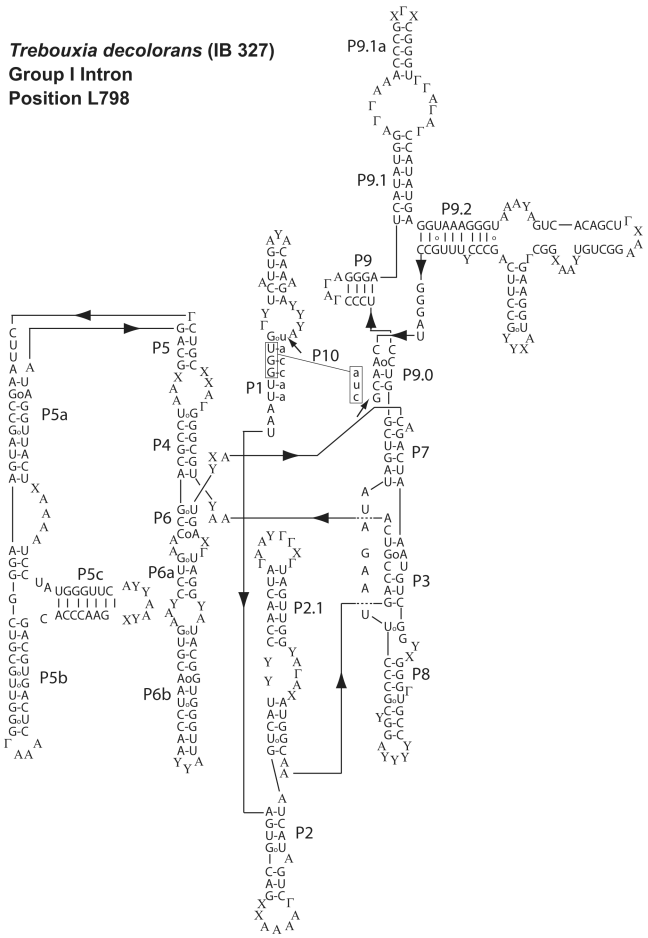

Fig. 2.

Putative secondary structure of the group I intron within the large subunit rRNA precursor of *Trebouxia decolorans* UTEX 901 drawn according to the conventions of Cech et al. (1994). Arrows point to the 5′ and 3′ splice junctions of this L798 group I intron. Also shown are the locations of the pairing segments P1-P10. The solid lines are used to position secondary structure elements that are believed to interact in close proximity.

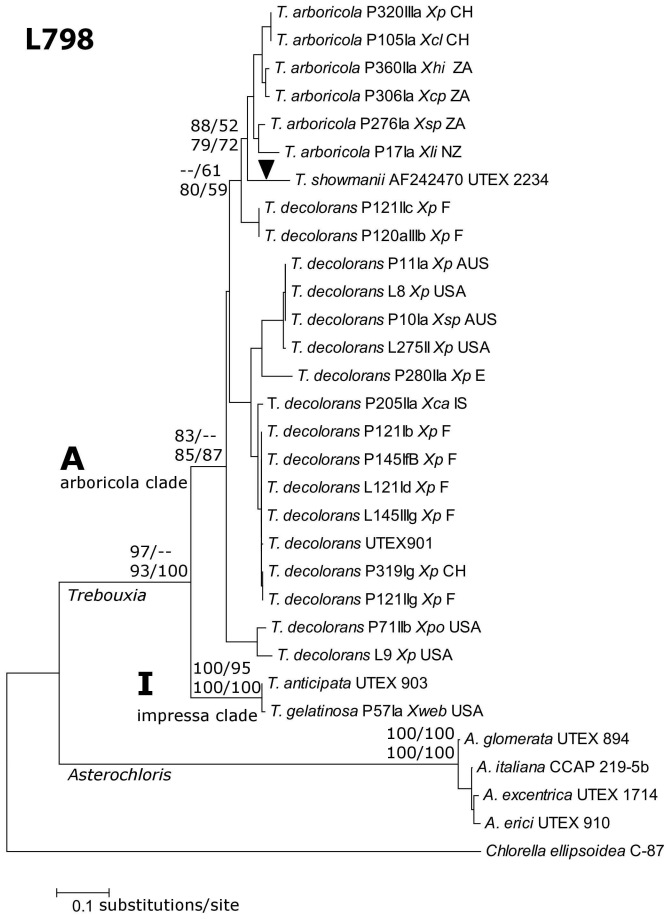

Fig. 3.

Phylogeny of the LSU 798 group I intron in representatives of *Trebouxia* s. str. and *Asterochloris* Tscherm.-Woess. An intron sequence of *Chlorella angustoellipsoidea* strain C-87 was obtained from GenBank (accession D17810) and used as outgroup taxon. Arrowhead indicates potential site for lateral intron transfer. All the support values were generated as in Figure 2.

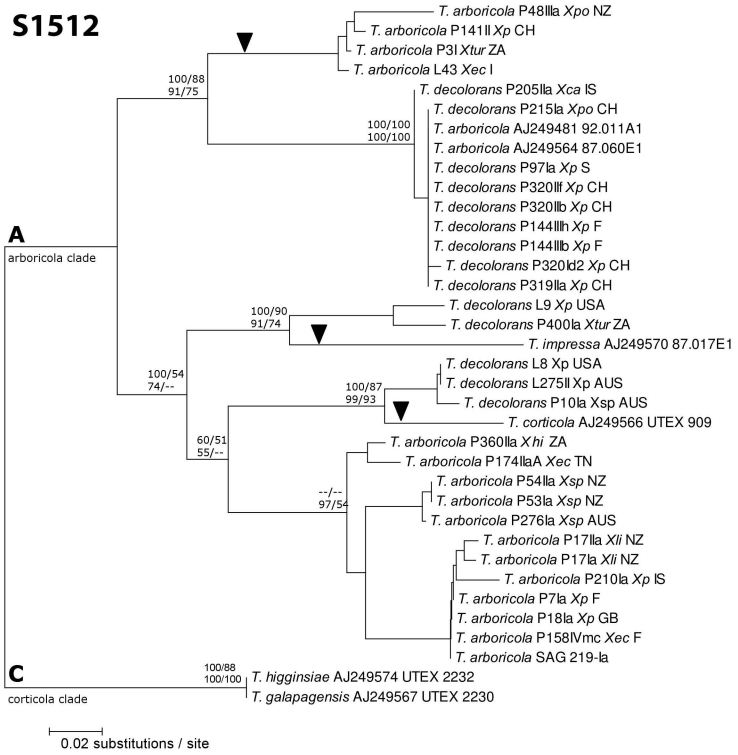

Fig. 4.

Phylogeny of the SSU 1512 group I intron in representatives of *Trebouxia* s. str. All the support values were generated as in Figure 2.

Table 1

List of samples analyzed in this study for LSU and SSU introns.

Trebouxia species[b]	Isolated from *Xanthoria* sp.	Country	Voucher No[a]	ITS accession no.	LSU accession no.
T. arboricola de Puymaly	*X. calcicola* Oxner	CH	P-105-I-a	AJ969542	AM261263
T. arboricola	*X. calcicola*	CH	P-141-II	AJ969552	
T. arboricola	*X. capensis* Kärnefelt, Arup & L. Lindblom	ZA	P-306-I-a	AJ969591	AM261277
T. arboricola	*X. ectaneoides* (Nyl.) Zahlbr.	F	P-158-IV-mc	AJ969560	
T. arboricola	*X. ectaneoides*	I	L-43	AJ969523	
T. arboricola	*X. ectaneoides*	TN	P-174-II-aA	AJ969565	
T. arboricola	*X. ligulata* (Körb.) P. James	NZ	P-17-I-a[c]	AJ969518	AM261260
T. arboricola	*X. ligulata*	NZ	P-17-II-a	AJ969519	
T. arboricola	*X. ligulata*	NZ	P-53-I-a	AJ969528	
T. arboricola	*X. ligulata*	NZ	P-54-II-a	AJ969530	
T. arboricola	*X. parietina* (L.) Beltr.	GB	P-18-I-a	AJ969520	
T. arboricola	*X. parietina*	CY	P-5-I-a-A	AJ969510	
T. arboricola	*X. parietina*	IS	P-198-II-a	AJ969568	
T. arboricola	*X. parietina*	IS	P-210-I-a	AJ969570	
T. arboricola	*X. parietina*	F	P-7-I-a	AJ969512	
T. arboricola	*X. parietina*	CH	P-320-III-a	AJ969604	AM261279
T. arboricola	*X. polycarpa* (Hoffm.) Th.Fr. ex Rieber[1]	NZ	P-48-III-a	AJ969526	
T. arboricola	*X. sp.*	CY	P-5-I-a	AJ969510	
T. arboricola	*X. sp.*	AUS	P-276-I-a[c]	AJ969581	AM261275
T. arboricola	*X. hirsuta* Eichenberger, Aptroot & Honegger	ZA	P-360-II-a[c]	AJ969609	AM261281
T. arboricola	*X. turbinata* Vain.[2]	ZA	P-3-I[d]	AJ969509	
T. decolorans Ahmadjian	*X. candelaria* (L.) Th. Fr.[3]	IS	P-205-II-a[c]	AJ969569	AM261272
T. decolorans	*Xanthomendoza hasseana* (Räsänen) Søchting, Kärnefelt & S.Y. Kondr.[4]	USA	P-400-Ia	AM159210	
T. decolorans	*X. parietina*	AUS	P-10-I-a[c]	AJ969515	AM261258
T. decolorans	*X. parietina*	AUS	P-11-Ia	---	AM261259
T. decolorans	*X. parietina*	AUS	L-275-II[c]	AJ969580	AM261274

Trebouxia species[b]	Isolated from *Xanthoria* sp.	Country	Voucher No[a]	ITS accession no.	LSU accession no.
T. decolorans	*X. parietina*	USA	L-8[c]	AJ969513	AM261256
T. decolorans	*X. parietina*	USA	L-9[c]	AJ969514	AM261257
T. decolorans	*X. parietina*	S	P-97-I-a	AJ969539	
T. decolorans	*X. parietina*	F	P-121a-IIIb	AJ969545	AM261264
T. decolorans	*X. parietina*	F	P-121-I-b	AJ969549	AM261266
T. decolorans	*X. parietina*	F	L-121-I-d	AJ969546	AM261265
T. decolorans	*X. parietina*	F	P-121-II-c	AJ969550	AM261267
T. decolorans	*X. parietina*	F	P-121-II-g	---	AM261268
T. decolorans	*X. parietina*	F	P-144-III-b	AJ969554	
T. decolorans	*X. parietina*	F	P-144-III-h	AJ969556	
T. decolorans	*X. parietina*	F	L-145-III-g	AJ969557	AM261270
T. decolorans	*X. parietina*	F	P-145-I-f-B	AJ969558	AM261271
T. decolorans	*X. parietina*	E	P-280-IIa	AJ969583	AM261276
T. decolorans	*X. parietina*	CH	P-319-I-g[c]	AJ970889	AM261278
T. decolorans	*X. parietina*	CH	P-319-II-a	AJ969595	
T. decolorans	*X. parietina*	CH	P-320-I-d2	AJ969599	
T. decolorans	*X. parietina*	CH	P-320-II-b	AJ969600	
T. decolorans	*X. parietina*	CH	P-320-II-f	AJ969603	
T. decolorans	*X. polycarpa* (Hoffm.) Rieber[1]	USA	P-71-II-b	AJ969535	AM261262
T. decolorans	*X. polycarpa*	CH	P-215-I-a	AJ969572	
T. gelatinosa Ahmadjian ex Archibald	*Xanthomendoza weberi* (S.Y. Kondr. & Kärnefelt) L. Lindblom[5]	USA	P-57-I-a	AJ969532	AM261261

[b] *Trebouxia* species identified according to ITS and *rbc*L phylogenetic analysis (Nyati et al. submitted);

[a] P indicates photobiont isolated, L indicates whole lichen DNA used for PCR amplification;

[c] both introns present;

[d] *T. arboricola* isolated from *X. turbinata* which had an insert of 1090 bases within 1512 group I intron.

[1] *Xanthoria polycarpa* (syn. *Massjukiella polycarpa* Fedorenko et al.);

[2] *X. turbinata* (syn. *Xanthodactylon turbinatum* (Vain.) C.W. Dodge);

[3] *X. candelaria* (syn. *Massjukiella candelaria* Fedorenko et al.);

[4] *Xanthomendoza hasseana* (syn. *Gallowayella hassiana* Fedorenko et al.); *Xanthomendoza weberi* (syn. *Honeggeria rosmariae* Fedorenko et al.).

Table 2

ITS and partial LSU accession numbers of reference algal strains used in this study

Algal species	Strain[a]	LSU accession No.	ITS accession No.	Reference[b]
Asterochloris erici (Ahmadjian) Skaloud & Peksa	UTEX 912		AF345441	Piercey-Normore et al. (2001)
A. erici	UTEX 910/IB 342	AM261248		This study
A. excentrica (Archibald) Skaloud & Peksa	UTEX 1714/IB 345	AM261249		This study
A. glomerata (Warén) Skaloud & Peksa	UTEX 897		AF345405	Piercey-Normore et al. (2001)
A. glomerata	UTEX 894/IB 349	AM261252		This study
A. italiana (Archibald) Skaloud & Peksa	CCAP 219-5b/IB 358	AM261253		This study
Chlorella angustoellipsoidea N.Hanagata & M.Chihara	C-87	D17180		Aimi et al. (1994)
Trebouxia anticipata Ahmadjian ex Archibald	UTEX 903/IB 340	AM261247		This study
T. arboricola	92.011A1		AJ249481	Friedl et al. (2000)
T. arboricola	92.011C3		Z68703	Bhattacharya et al. (1996)
T. arboricola [c]	SAG 219-Ia		Z 68705	Bhattacharya et al. (1996)
T. asymmetrica Friedl & Gärtner	B207		AF344177	Beck et al. (2002)
T. corticola (P.A.Archibald) Gärtner	UTEX 909		AJ249566	Friedl et al. (2000)
T. decolorans	UTEX 901/IB 327	AM261243	Unpubl.	Unpublished
T. galapagensis (Hildreth & Ahmadjian) Gärtner	UTEX 2230		AJ249567	Friedl et al. (2000)
T. gelatinosa	87.072B1		AJ249575	Friedl et al. (2000)
T. higginsiae (Hildreth & Ahmadjian) Gärtner	UTEX 2232		AJ249574	Friedl et al. (2000)
T. impressa Ahmadjian	87.017E1		AJ249570	Friedl et al. (2000)
T. showmanii (Hildreth & Ahmadjian) Gärtner	UTEX 2234/IB 337	AM261246	AF242470	Kroken et al. (2000)

[a] IB: Culture collection of algae at the University of Innsbruck; SAG: Algal culture collection, University of Göttingen; UTEX: algal culture collection, University of Texas

[b] All LSU sequences generated in this study; unpublished ITS sequence kindly provided by T. Friedl and G. Helms

[c] Type strain of the genus *Trebouxia*

Table 3

List of newly designed primers used in this study

Primer	Target	Sequence (5′→3′)	Position	Relative to Accession
Intron 1 (fwd)	SSU intron	CTGTCACTAGACTGAGTGC	208	AM159503
Intron 2 (rev)	SSU intron	CCAGTTTAGAGGCTCGAATC	601	AM159503
Intron 3 (rev)	SSU intron	GGWCCGACTATATCTTAAGC	1716	AJ969509
Intron 4 (fwd)	SSU intron	TTGTTGTAAAGGGCTCCACT	709	AJ969509
Intron 5 (rev)	SSU intron	ACTCCTGTAAGCTCTCCTTCC	1756	AJ969509
LSU fwd	LSU	TTTAACACCCATGAGACGCAAGTAAC	684	D17810
LSU rev	LSU	GCCTTAACTCAGCTTTCGGTTCA	1607	D17810

Table 4

Comparison of catalytic core (PQRS) sequences of LSU 798 group I introns in representatives of the genera *Trebouxia* s. str. and *Asterochloris*. The identity of photobionts of the investigated *Xanthoria* and *Xanthomendoza* species was inferred on the basis of ITS and *rbcL* analyses. The positions of P, Q, R, S regions are based on the reference sequence of the *Chlorella angustoellipsoidea* strain C-87 intron (accession number D17180).

	P	Q	R	S
T. decolorans	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
L145IIIg Xp F	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
L121Id Xp F	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P121IIg Xp F	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P145IdfB Xp F	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P319Ig Xp CH	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P120aIIIb Xp F	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P121II cd Xp F	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P280IIa Xp E	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P205IIa Xca IS	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P121Ib Xp F	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P71IIb Xpo USA	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAGA	AAGAUAUAGUCG
L9 Xp USA	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUGUAGUCG
L8 Xp USA	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAAAUAUAGUCG
L275II Xp USA	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAAAUAUAGUCG
P11Ia Xp AUS	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAAAUAUAGUCG
P10Ia Xsp AUS	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAAAUAUAGUCG
P306Ia Xcp ZA	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P360IIa Xhi ZA	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P105Ia Xcl CH	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P276Ia Xsp ZA	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P320IIIa Xp CH	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P17Ia Xli NZ	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
T. showmanii	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUACA	AAGAUAUAGUCG
T. anticipata	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
P 57 Ia Xweb U	AAUUGCGGGGAC	AAUCCGCAGC	GUUCACAGACUAAA	AAGAUAUAGUCG
T. glomerata	AAUUGCCGGGAC	AAUCGGCAGC	GUUCACAGACUAGA	AAGGUAUAGUCG

	P	Q	R	S
T. italiana	AAUUGCCGGGAC	AAUCGGCAGC	GUUCACAGACUAGA	AAGAUAUAGUCG
T. excentrica	AAUUGCCGGGAC	AAUCGGCAGC	GUUCACAGACUAGA	AAGAUAUAGUCG
T. erici	AAUUGCCGGGAC	AAUCGGCAGC	GUUCACAGACUAGA	AAGAUAUAGUCG
C. angustoellipsoidea	AAUUGCGGGAAA	AAUCCGCAGC	GUUCACAGACUAGA	AAGGUAUAGUCG

Table 5

Presence of SSU1512 group I intron in axenically cultured Trebouxia isolates from five *Xanthoria* populations collected in Europe

Photobiont	Voucher No.	Mycobiont	Collecting site	Substrate	Intron[a]/ isolates, %
Trebouxia arboricola	158	*X. ectaneoides*	maritime, F	saxicolous	20/23, 87%
T. decolorans	120-121	*X. parietina*	rural, F	epiphytic	1/25, 4%
T. decolorans	144-145	*X. parietina*	rural, F	epiphytic	6/63, 9.5%
T. decolorans	164	*X. parietina*	coastal, F	epiphytic	0/12, 0%
T. arboricola, *T. decolorans*	319-320	*X. parietina*	urban, CH[b]	epiphytic, saxicolous	18/47, 38%

[a] Presence of intron tested with PCR assays;

[b] All *T. decolorans* isolates were photobiont of epiphytic samples, while *T. arboricola* was identified from saxicolous specimens. F, France. CH, Switzerland. Details about these *Xanthoria* populations are found in Itten & Honegger (2010).