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Phylogenetic diversity of the lichenized algal genus *Trebouxia* (Trebouxiophyceae, Chlorophyta): a new lineage and novel insights from fungal-algal association patterns of Icelandic cetrarioid lichens (Parmeliaceae, Ascomycota)

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Lichens have high tolerance to harsh environmental conditions, where lichen symbiont interactions (e.g. myco- and photobionts) may play a crucial role. The characterization of fungal-algal association patterns is essential to understand their symbiotic interactions. This study investigated fungal-algal association patterns in Icelandic cetrarioid lichens using a multi-locus phylogenetic framework, including fungal nrITS, MCM7, mtSSU, *RPB1* and *RPB2* and algal nrITS, nrLSU, *rbcL* and *mtCOXII* data. Most Icelandic cetrarioid lichenized fungi were found to be specifically associated to the known *Trebouxia* clade "S" (*Trebouxia simplex/suecica* group), whereas the lichen-forming fungus *Cetrariella delisei* forms a symbiosis with a previously unrecognized lineage of *Trebouxia*, provisionally named as the "D" clade. This new *Trebouxia* lineage is supported by maximum likelihood and Bayesian phylogenetic analyses using all four included algal loci.

ADDITIONAL KEYWORDS: Iceland – lichen – Parmeliaceae – phylogeny – symbiosis – *Trebouxia*.

INTRODUCTION

Lichens are microbial communities, mainly consisting of nutritionally specialized heterotrophic fungi and photosynthetic green algae and/or cyanobacteria (Honegger, 1998). The photosynthetic partners are called photobionts. Lichens exhibit high tolerance to extreme environmental conditions, such as the polar and desert regions, and they are the predominant life forms on *c*. 8% of global land surface (Larson, 1987; Domaschke *et al.*, 2012). Environmental tolerance and dispersal capability of lichens appears to be associated with the synergistic power of symbionts (De Vera, Rettberg & Ott, 2008). Fungal selectivity for photobionts refers to their preferential selection of photobionts when more than one photobiont is present, whereas fungal specificity for photobionts represents specific or exclusive interactions between fungi and one certain type of photobiont (Singh *et al.*, 2016).

The most common lichen photobionts are species in the unicellular green algal genus *Trebouxia* Puymaly (Friedl & Rybalka, 2012). Species diversity of the genus is still poorly understood, as many *Trebouxia* spp. cannot be cultured using standard culture media, in turn leading to an underestimation of species diversity (Grube & Muggia, 2010). Furthermore, morphological characters from cultured *Trebouxia* spp. have limited discriminatory power at the species or even genus level, as exemplified by six green algal species of *Asterochloris* Tschermak-Woess that were mistakenly identified as *Trebouxia* spp. using morphological characters (Skaloud & Peksa, 2010). Therefore, robust identification of those species relied on combining phylogenetic

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analysis with morphological evidence (Skaloud & Peksa, 2010). The algal nuclear ribosomal internal transcribed spacer region (algal nrITS) has been shown to be powerful in resolving species relationships in *Trebouxia* and uncovering the photobiont diversity in lichens (Kroken & Taylor, 2000; Ruprecht, Brunauer & Printzen, 2012), and it has been suggested that the algal nrITS region should be used for DNA barcoding of *Trebouxia* spp. due to its high genetic variability (Grube & Muggia, 2010; Friedl & Rybalka, 2012). Using available algal nrITS sequences in GenBank, phylogenetic analyses have revealed that *Trebouxia* spp. tend to form four well-supported monophyletic clades, including clade "A" [*Trebouxia arboricola* Puymaly/ *Trebouxia gigantea* (Hildreth & Ahmadjian) Gärtner group], clade "I" (*Trebouxia impressa* Ahmadjian/ *Trebouxia gelatinosa* Ahmadjian ex P.A.Archibald group), clade "S" (*Trebouxia simplex* Tschermak-Woess/ *Trebouxia suecica* Beck group) and clade "C" [*Trebouxia corticola* (P.A.Archibald) Gärtner/ *Trebouxia galapagensis* (Hildreth & Ahmadjian) Gärtner group] (Beck, 2002; Helms, 2003; Leavitt *et al.*, 2015).

Cetrarioid lichens are one of the most studied groups in the lichen-forming fungal family Parmeliaceae (Nelsen *et al.*, 2011). Morphologically they are characterized by erect foliose/subfruticose thalli with marginal apothecia and pycnidia, and chemically they produce *Cetraria* Ach.-type lichenan polysaccharides (Nelsen *et al.*, 2011). Green algae associated with cetrarioid lichen-forming fungi are exclusively from *Trebouxia* (Honegger, 2009; Leavitt *et al.*, 2015) Furthermore, *Cetraria* spp. specifically associate with clade "S" *Trebouxia* (Leavitt *et al.*, 2015). Several studies have focused on the cosmopolitan cetrarioid lichen *Cetraria aculeata* (Schreb.) Fr. (Fernández-Mendoza *et al.*, 2011; Domaschke *et al.*, 2012; Pérez-Ortega *et al.*, 2012; Fernández-Mendoza & Printzen, 2013; Lutsak *et al.*, 2016) and have revealed a higher photobiont diversity in tropical and temperate regions vs. polar regions, suggesting algal switching as an adaptive strategy in *C. aculeata*. The current study focused exclusively on Icelandic cetrarioid lichens, since they display diverse habitat selection (e.g. soil, stone, bark, etc.) and produce a wide array of secondary metabolites (e.g. depsides, depsidones, dibenzofurans, aliphatic lactones, etc.) (Thell & Moberg, 2011; Xu *et al.*, 2016, 2017, 2018). We assessed their fungal-algal association patterns and photobiont diversity. Phylogenetic analyses uncovered a previously undetected *Trebouxia* lineage, here found in association with the lichenized fungus *Cetrariella delisei* (Bory ex Schaer.) Kärnefelt & A.Thell. A phylogenetic framework is used to support this new clade, and an updated phylogenetic analysis of *Trebouxia* is performed.

MATERIAL AND METHODS

Taxon sampling

One hundred and sixty-eight specimens of Icelandic cetrarioid lichens from 13 species in six genera (Fig. 1) were sampled, including terricolous, epiphytic and saxicolous taxa. The sampled terricolous taxa included *Cetraria aculeata* (N = 11), *Cetraria muricata* (Ach.) Eckfeldt (N = 14), *Cetraria ericetorum* Opiz (N = 8) and *Cetraria islandica* (L.) Ach. (N = 54), with wide distributions in Iceland, and rarer species such as *Cetrariella delisei* (N = 26) and *Flavocetraria nivalis* (L.) Kärnefelt & A.Thell (N = 18) from southern Iceland and *Flavocetraria cucullata* (Bellardi) Kärnefelt & A.Thell (N = 4) from near Lake Mývatn in northern Iceland. The sampled epiphytic lichen taxa included *Cetraria sepincola* (Ehrh.) Ach. (N = 6), with a wide distribution, and *Vulpicida pinastri* (L.) J.-E.Mattsson & M.J.Lai (N = 3) and *Tuckermannopsis chlorophylla* (Willd.) Hale (N = 3) that are both most common in eastern Iceland. The sampled saxicolous lichen taxa included all three species of *Melanelia* Essl., *Melanelia hepatizon* (Ach.) A.Thell (N = 10) with a wide distribution, and *Melanelia agnata* (Nyl.) A.Thell (N = 7) and *Melanelia stygia* (L.) Essl. (N = 4) with more restricted distributions. Voucher information and GenBank accession numbers are provided in Supporting Information (Table S1).

For the algal part of the study, the nrITS-based reference operational taxonomic units (OTUs) delimited in Leavitt *et al.* (2015) were used as an initial framework to identify our newly generated *Trebouxia* ITS sequences. The data matrix from Leavitt *et al.* (2015) encompasses the genetic diversity of *Trebouxia* algae from four known clades ("A", "C", "I" and "S"). The matrix was then further enlarged to represent the whole genetic diversity of *Trebouxia* by including additional reference sequences of *Trebouxia* from recognized culture collections, including SAG (https://www.uni-goettingen.de/en/45175.html), UTEX (https://utex.org/) and CCAP (https://www.ccap.ac.uk/). Additionally, four authenticated *Trebouxia* cultures were ordered from National Institute for Environmental Studies (NIES) (https://mcc.nies.go.jp/) and Culture Collection of Algae of Charles University in Prague (CAUP) (https://botany.natur.cuni.cz/algo/caup.html) culture collections and added to our dataset, including *T. anticipata* Ahmadjian ex P.A.Archibald (NIES 1271, "I" clade), *T. corticola* (NIES 1278, "C" clade), *T. crespoana* Barreno, Molins, Moya & Škaloud (CAUP 1019, "C" clade) and *T. higginsiae* (Hildreth & Ahmadjian) Gärtner (NIES 1289, "C" clade).

DNA extractions, PCR and sequence alignment

Total genomic DNA was extracted from lichen thallus tips (*c.* 15–20 mg) using the CTAB method

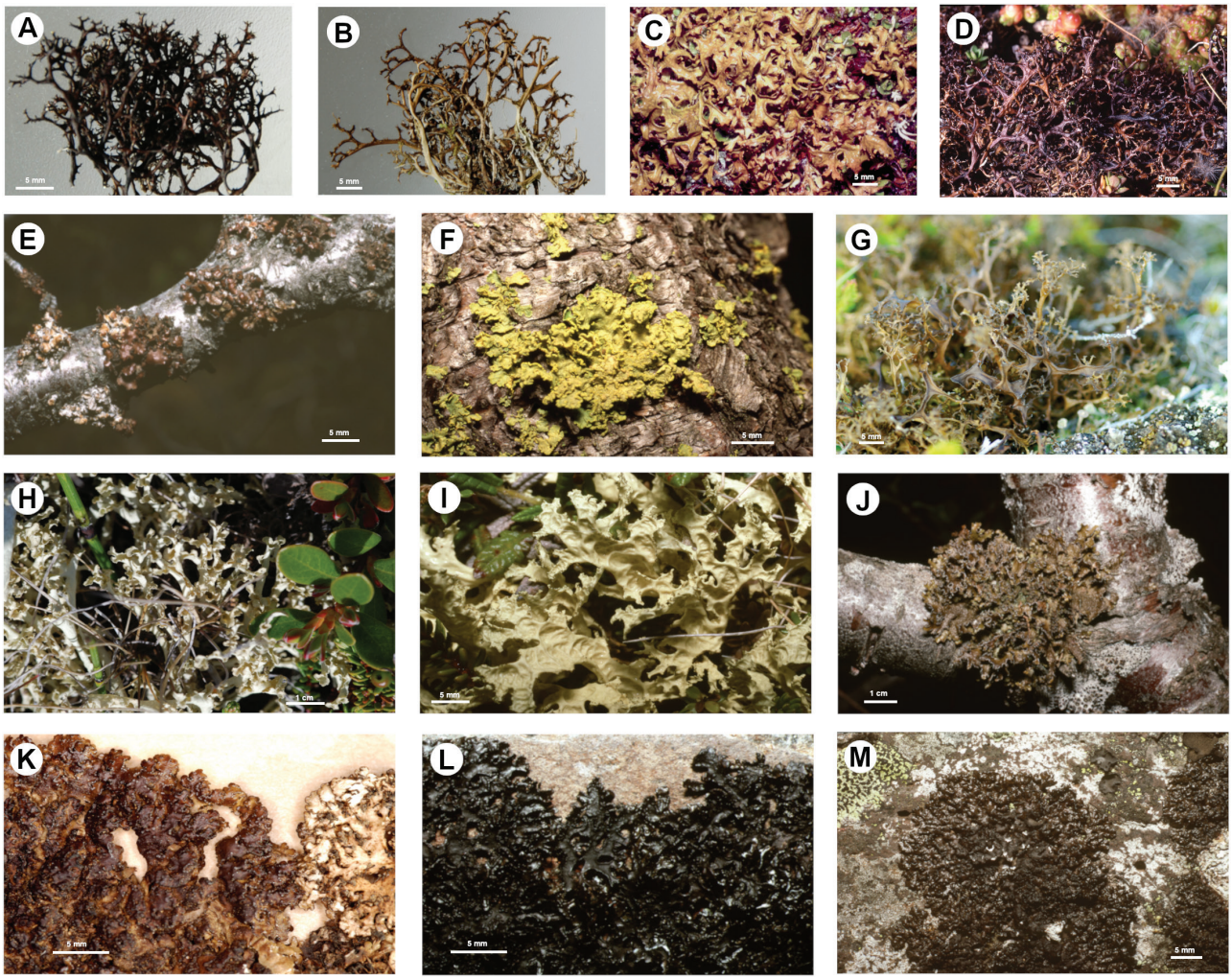

Figure 1. Icelandic cetrarioid lichens: (A) *Cetraria aculeata*, (B) *Cetraria ericetorum*, (C) *Cetraria islandica*, (D) *Cetraria muricata*, (E) *Cetraria sepincola*, (F) *Vulpicida pinastri*, (G) *Cetrariella delisei*, (H) *Flavocetraria cucullata*, (I) *Flavocetraria nivalis*, (J) *Tuckermannopsis chlorophylla*, (K) *Melanelia agnata*, (L) *Melanelia hepatizon* and (M) *Melanelia stygia*. Photographs: Hordur Kristinsson.

(Cubero *et al.*, 1999). For algal symbionts, four loci were amplified: algal nrITS, nuclear ribosomal large subunit (nrLSU), mitochondrial cytochrome c oxidase II (*mtCOXII*) and plastid ribulose-bisphosphate carboxylase (*rbcL*). Sequences of five fungal loci were retrieved from another study, including fungal nuclear ribosomal internal transcribed spacer (fungal nrITS), the DNA replication licensing factor mini-chromosome maintenance complex component 7 (MCM7), the mitochondrial small subunit (mtSSU), and the largest and the second largest subunit of RNA polymerase II gene sequences (*RPB1* and *RPB2*). PCR was carried out in a 25 μL volume, consisting of 1× standard Taq DNA polymerase (New England Biolabs), 2.5 μL 10× reaction buffer, 1 μL DNA template, 0.5 μL 10 mM dNTPs, 0.5 μL for each 10 μM forward and reverse primer, and with PCR-grade water to 25 μL. PCRs were carried out in a thermal cycler (Fisher Scientific, ON, Canada). The primers ITS1T and ITS4T were used for the PCR amplification of algal nrITS (Kroken & Taylor, 2000). Two primer pairs were used for algal *mtCOXII*, including COX2P2fw and COX2P2rev (Fernández-Mendoza *et al.*, 2011), and COX2FOR1 and COXREV1 (Singh *et al.*, 2016). We also designed genus-specific primers for algal nrLSU (i.e. LSU1T and LSU2T) and *rbcL* (i.e. rbcL1T and rbcL2T). All primer sequences are provided in Supporting Information (Table S2). PCR conditions for algal nrITS followed the described touchdown program (Kroken & Taylor, 2000) with minor modifications: initial denaturation for 3 min at 94 °C, five cycles of denaturation at 94 °C for 50s, annealing at 61-57 °C for 40s (decreasing 1 °C per cycle), and an extension at 68 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 50s,

annealing at 57 °C for 40s, and an extension at 68 °C for 1 min; finally, an extension for 7 min and cooling to 10 °C. The same PCR conditions were also used for algal nrLSU and *mtCOXII* using the primer pair COX2P2fw and COX2P2rev. Amplification of algal *rbcL* and *mtCOXII* using COX2FOR1 and COX2REV1 had a modified touchdown gradient from 58 to 54 °C. PCR products were checked using gel electrophoresis on 1.3% agarose gels stained using SYBR Safe (Invitrogen, CA, USA). PCR amplicons were purified using ExoSAP (Fermentas Inc., Hanover, MD, USA) and Macrogen Inc. (Amsterdam, the Netherlands) using the same primers used for the PCR.

Sequence contigs were assembled using PhyDE-Phylogenetic Data Editor v.0.9971, and all trace files were manually checked for ambiguous base calling. Sequence identity from morphologically described specimens was also confirmed using BLAST searches in GenBank. Sequences were aligned using MAFFT (Katoh & Standley, 2013). Aligned sequences were visually inspected and manually adjusted. In our data, there are two algal nrITS sequences having chromatograms with three ambiguous sites. This may indicate the presence of multiple *Trebouxia* photobionts in one lichen thallus, which has been revealed using high-throughput sequencing of algal nrITS (Paul *et al.*, 2018). To avoid overestimation of algal nrITS genetic variation at the scale at which we were working, those sites were manually removed. Other ambiguously aligned sites in algal nrITS matrices were deleted using Gblocks v.0.91b (Talavera & Castresana, 2007). No ambiguous regions were found in other locus data.

Phylogenetic analyses

For the assessment of fungal-algal association pattern, the lichen-forming fungus *Omphalodium pisacomense* Meyen & Flot. was chosen as the outgroup for fungal analyses, and the alga *Asterochloris erici* (Ahmadjian) Skaloud & Peksa was used as the outgroup for algal analyses. For each fungal and algal locus, a maximum likelihood (ML) gene tree was generated using RAxML GUI v.1.3 (Silvestro & Michalak, 2012) using the GTRGAMMA model and 1000 bootstrap pseudoreplicates. Bayesian analyses using MrBayes v.3.2.1 (Ronquist & Huelsenbeck, 2003) were run on four chains for 10 000 000 generations using Markov Chain Monte Carlo (MCMC) sampling every 500 generations and a burn-in set to 25%. Convergence between runs was monitored using TRACER v.1.5. Majority-rule consensus trees were generated from Bayesian analysis. PartitionFinder v.2 (Lanfear *et al.*, 2016) was used to determine the best-fitting partition scheme and to select the evolution models for each matrix under the Akaike information criterion. Individual gene trees using both methods are shown in Supporting Information (Fig. S1). All gene trees were compared within each symbiont to see whether strongly supported topological conflicts exist, where a nodal support with Bayesian posterior probabilities (PP) > 95% and ML bootstrap (BS) values > 70% is considered to be strongly supported. Since no strongly supported conflicts were found, loci from each partner were concatenated for downstream phylogenetic analysis with both ML and Bayesian inference. The resulting multi-locus fungal and algal trees were used to display the fungal-algal association patterns. Phylogenetic trees were visualized in FigTree v.1.4.0.

To identify the *Trebouxia* algae from Icelandic taxa (i.e. to which *Trebouxia* clade they belong), we combined our Icelandic algal nrITS dataset with annotated reference sequences (Leavitt *et al.*, 2015) and performed a phylogenetic analysis using the ML method. The resulting algal nrITS tree is provided in Supporting Information (Fig. S2), with Icelandic taxa marked with a star. Since the algal nrITS tree suggested the existence of a new *Trebouxia* clade [clade "D"; Supporting Information (Fig. S2)] in addition to already existing *Trebouxia* clades, we intended to update the *Trebouxia* algal reference OTU pool with clustered OTUs from the new *Trebouxia* clade (see below). A new algal nrITS matrix was then constructed containing only representative sequences for *Trebouxia* OTUs, and the monophyly of each clade was assessed by performing phylogenetic analyses using both ML and Bayesian methods. The updated pool of all representative *Trebouxia* OTU sequences and their GenBank accession numbers are provided in Supporting Information (Table S3).

To evaluate the evolutionary independence of the newly proposed *Trebouxia* clade further, we performed a multi-locus phylogenetic analysis, including taxa from the new clade and authenticated *Trebouxia* algal cultures from the four known "A", "C", "I" and "S" clades in our dataset. *Asterochloris irregularis* (Hildreth & Ahmadjian) Skaloud & Peksa and *Asterochloris erici* were used as outgroups. Taxon information and GenBank accession numbers for this analysis are provided in Supporting Information (Table S4). Four algal loci (nrITS, nrLSU, *rbcL* and *mtCOXII*) were concatenated, since no strongly supported conflicts were found among individual gene trees (Supporting Information, Fig. S3).

The number of hypothetical species or OTUs within the proposed new *Trebouxia* clade was assessed using phylogenetic analyses and species delimitation methods. Phylogenetic analyses were conducted using ML and Bayesian inference methods as described above. A four-locus concatenated data

matrix including all taxa in the new *Trebouxia* clade was used, with *Trebouxia asymmetrica* Friedl & Gärtner and *Trebouxia incrustata* Ahmadjian ex Gärtner from clade "A" included as outgroups. Two species delimitation methods were used: automatic barcode gap discovery (ABGD) (Puillandre *et al.*, 2012) and an updated Bayesian implementation of the Poisson tree process model (bPTP) (Zhang *et al.*, 2013). ABGD analysis was based on pairwise genetic distances of single-locus data (i.e. the algal nrITS alignment of the proposed new *Trebouxia* clade) to estimate the number of hypothetical species or OTUs. This approach has also been used recently in other studies delimiting *Trebouxia* species (Leavitt *et al.*, 2015; Moya *et al.*, 2017). The settings for the ABGD analysis were: Pmin = 0.001, Pmax = 0.01, steps = 10, X (relative gap width) = 1.5, Nb bins (for distance distribution) = 20 and Jukes-Cantor model (JC69). An alternative species delimitation tool using the bPTP web server (https://species.h-its.org/) was performed, and the algal nrITS tree was used as an input. The concatenated four-locus (algal nrITS, nrLSU, *mtCOXII* and *rbcL*) ML tree was used as an input and results were compared. The implemented default settings for the bPTP analysis were as follows: Number of MCMC generations = 100 000, thinning = 100, burn-in = 0.1 and seed = 123 (Supporting Information, Fig. S4).

RESULTS

Fungal-algal association patterns

The multi-locus fungal and the algal trees constructed with both ML and Bayesian analysis were used to investigate the fungal-algal association patterns in Icelandic cetrarioid lichens (Fig. 2). The topology of the fungal tree is resolved and well-supported. The "*Cetraria*" clade is monophyletic, consisting of *Cetraria islandica*, *Cetraria ericetorum*, *Cetraria muricata*, *Cetraria aculeata*, *Cetraria sepincola*, *Vulpicida pinastri* and *Cetrariella delisei*. The "*Nephromopsis* Müll.Arg." clade includes *Flavocetraria nivalis*, *Flavocetraria cucullata* and *Tuckermannopsis chlorophylla*, which is sister to the "*Cetraria*" clade. *Melanelia* forms a clade and is sister to both the "*Cetraria*" and "*Nephromopsis*" clades. The algal multi-locus tree in Figure 2 represents *Trebouxia* algae associated with lichen-forming fungi in Icelandic cetrarioid lichens, and it contained two major lineages, labelled here as *Trebouxia* lineages 1 and 2

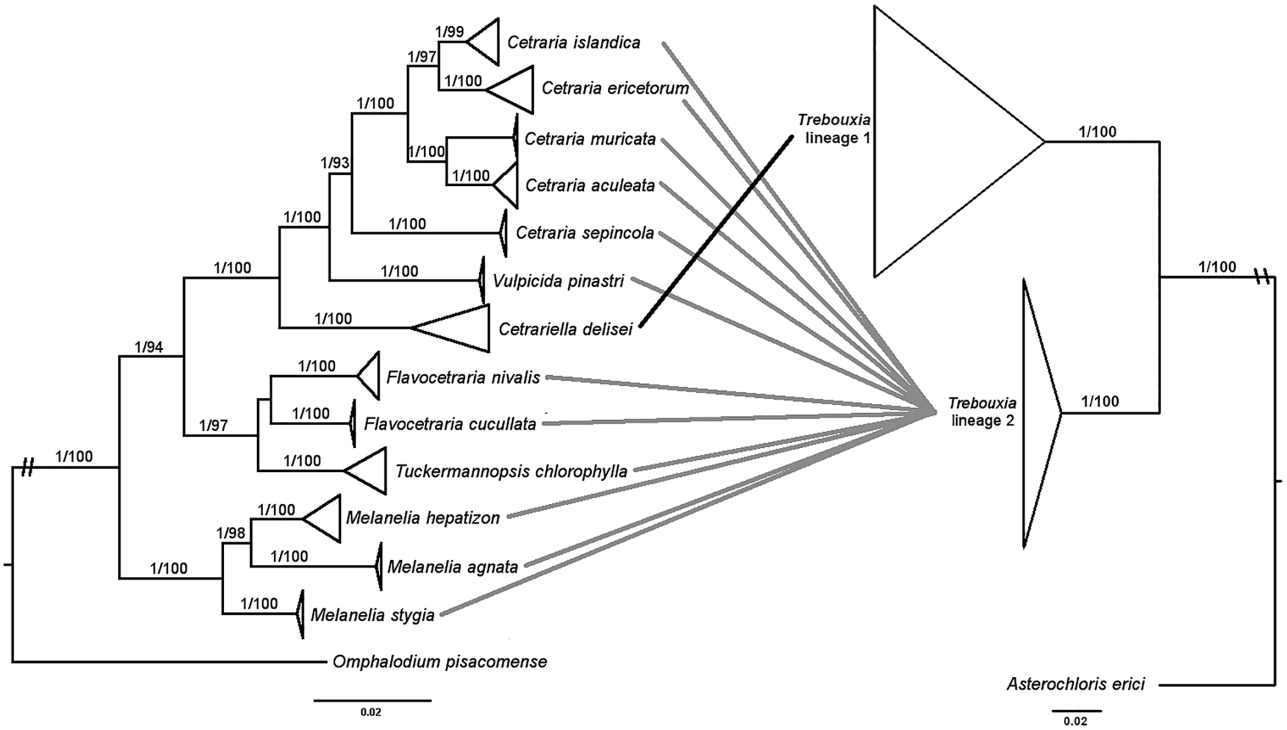

Figure 2. Fungal-algal association patterns in Icelandic cetrarioid lichens. The ML fungal tree (left) was constructed using a concatenated matrix of fungal nrITS, RPB1, RPB2, MCM7 and mtSSU. The ML algal tree (right) was constructed using a concatenated matrix of algal nrITS, nrLSU, *mtCOXII* and *rbcL*, and showed two major clades. Black connectors indicate associations between fungal clades and *Trebouxia* clade 1. Grey connectors indicate associations between fungal clades and *Trebouxia* clade 2. Bayesian posterior probabilities (PP) and bootstrap values (BS) are indicated at each node.

and collapsed in Figure 2. The fungal-algal association pattern in Icelandic cetrarioid lichens is shown by linking fungi to their algal partners belonging to either *Trebouxia* lineage 1 or 2. Most cetrarioid species are specifically associated with algae in *Trebouxia* lineage 2, whereas only *Cetrariella delisei* establishes a symbiosis with algae in *Trebouxia* lineage 1.

Extended phylogenetic diversity of *Trebouxia*

Phylogenetic analysis of the expanded algal nrITS dataset including a large number of Icelandic and reference sequences indicates that algae in *Trebouxia* lineage 2 belong to the formerly recognised *Trebouxia simplex/suecica* group – the "S" clade (Supporting Information, Fig. S2), whereas lineage 1 shown in Fig. 2 does not cluster with any known *Trebouxia* "A", "C", "I" or "S" clades shown in Fig. S2, instead forming a separate lineage. Using the same method as in Leavitt *et al.* (2015) and an only nrITS dataset including exclusively representatives for all delimited *Trebouxia* OTUs, we also confirmed five well-supported clades (Fig. 3A). In addition to the four known clades ("A", "C", "I" and "S"), a fifth clade corresponding to *Trebouxia* lineage 1 in our dataset (Fig. 2) was found. BLAST searches of algal nrITS sequences from the *Trebouxia* lineage 1 retrieved from GenBank an additional 25 homologue sequences with a sequence similarity > 95% that could potentially belong to this newly discovered *Trebouxia* lineage. Among these are 23 algal sequences associated with *Cetrariella delisei* specimens collected in Svalbard, Norway (Zhang *et al.*, 2015), and two from the lichen *Porpidia navarina* U.Rupr. & Türk (Ruprecht, Søchting & Türk, 2016). Voucher information for these 25 homologue sequences is provided in Supporting Information (Table S5).

The monophyly of the newly proposed *Trebouxia* clade was further supported when sequences of the newly revealed clade were embedded in a multi-locus phylogenetic analysis including only nuclear, mitochondrial and plastid loci from known authenticated *Trebouxia* algae cultures (Fig. 3B). In this case the new clade appears sister to clades "A", "C" and "S". The *Trebouxia* "I" clade (BS: 98%; PP: 100%) is reconstructed as sister to the rest. We predicted a preliminary number of six OTUs in this clade using different species delimitation methods (Supporting Information, Fig. S4).

DISCUSSION

The topology of the fungal tree in Figure 2 is in agreement with recent studies (Nelsen *et al.*, 2011; Divakar *et al.*, 2015, 2017), and in our study all lichen-forming fungi relationships are resolved using five loci. BLAST searches confirmed that all the algal nrITS sequences belong to *Trebouxia*. This further supports the exclusive association of the lichen-forming fungi family Parmeliaceae with green algae in the genus *Trebouxia* (Honegger, 2009). The exclusive specificity of Icelandic cetrarioid fungi to the *Trebouxia* "S" clade also supports former findings of a high specificity of terricolous *Cetraria* spp. to algae in the *T. simplex/suecica* group (Fernández-Mendoza *et al.*, 2011; Leavitt *et al.*, 2015).

In light of the symbiotic pattern in Icelandic cetrarioid lichens, we argue that the phylogenetic signal of fungal specificity for algal partners is strong in our dataset, since most lichen-forming fungi, excluding *Cetrariella delisei*, associate with *Trebouxia* algae in the "S" clade. The special association pattern for the lichenized fungus *Cetrariella delisei* may explain its ecological niche (high-elevation and humid substrates with prolonged snow cover). In addition, we also found that *Cetrariella delisei* has a unique characteristic secondary metabolite profile among cetrarioid lichens, dominated by depsides instead of depsidones, dibenzofurans and aliphatic lactones as in other cetrarioid taxa (Thell & Moberg, 2011). It would be interesting to investigate if and how the fungal-algal association pattern and chemotypic features of *Cetrariella delisei* are related.

The *Trebouxia* algae associated with *Cetrariella delisei* in our dataset appears to be a previously unrecognized clade, at the same level as the known "A", "C", "I" and "S" clades. This is supported by the algal nrITS and multi-locus phylogenetic trees (Figs S2, 3). The topology of the algal nrITS tree in Fig. 3A does not have strong support for the inter-relationship between different *Trebouxia* clades, and only the monophyly of each clade is strongly supported and labelled. The phylogenetic relationship between *Trebouxia* clades is only reflected in the multi-locus phylogenetic tree in Fig. 3B. Due to an incomplete species-level taxonomic framework of *Trebouxia*, it has been suggested that a practical approach should be adopted using phylogenetic analyses (Leavitt *et al.*, 2015). Here, we used the suggested approach and detected a new *Trebouxia* clade with data from multiple nuclear, mitochondrial and plastid loci. We provisionally name the new clade as the "*Trebouxia delisei*" clade, abbreviated as "D". A formal species-level taxonomic treatment of the existing OTUs in the proposed new *Trebouxia* clade awaits future investigations, probably including ultrastructural anatomical characters, after successful algal isolation and cultivation.

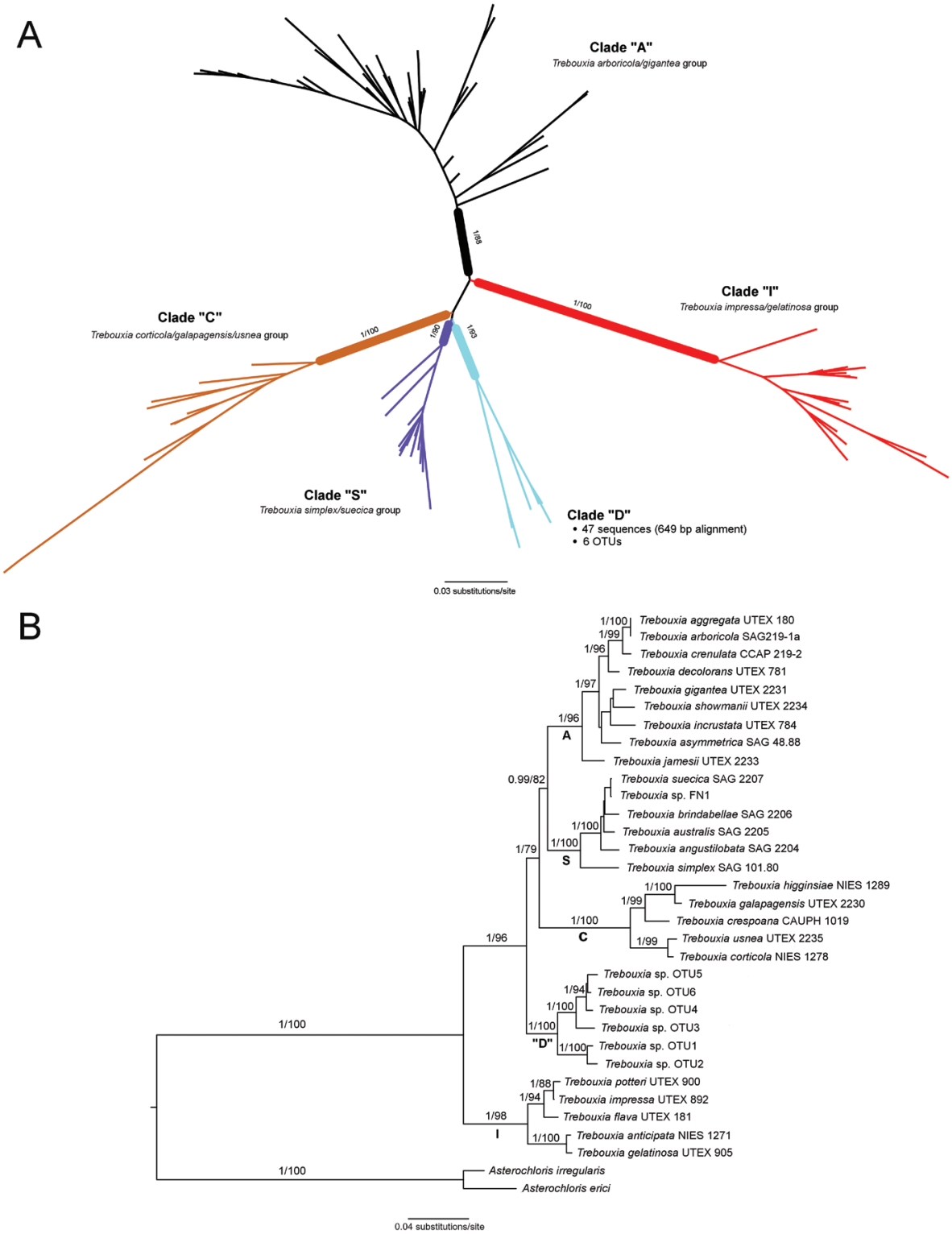

Figure 3. Phylogenetic analyses of the green algal genus *Trebouxia*. A, radial ML tree based on algal nrITS sequences containing all representative *Trebouxia* OTUs. Five clades were resolved, including four known clades ("A", "C", "I" and "S") and one previously unrecognized new *Trebouxia* clade (the "D" clade). B, ML phylogenetic tree of the lichenized algal genus *Trebouxia* using a concatenated data matrix including algal nrITS, nrLSU, *mtCOXII* and *rbcL* loci. Branches with posterior probabilities (PP) > 0.95 and bootstrap values (BS) > 70% are considered as well-supported and indicated as PP/BS in trees.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Fungal and algal gene trees of Icelandic cetrarioid lichens. Four fungal loci (i.e. nrITS, MCM7, mtSSU, *RPB1* and *RPB2*) and four algal loci (nrITS, nrLSU, *mtCOXII* and *rbcL*) were used for phylogenetic inferences. Bayesian PP > 0.95 and BS > 70% are indicated on each node.

Figure S2. ML algal nrITS gene tree for identification of Icelandic *Trebouxia* taxa in cetrarioid lichens. The data matrix contains algal nrITS sequences from Icelandic taxa and 69 representative OTUs from previously known major *Trebouxia* clades (i.e. "A", "C", "I" and "S" clades). Branches with BS > 70% are labelled. *Trebouxia* algae associated with the fungus *Cetrariella delisei* form a distinct monophyletic clade labelled as "D" clade.

Figure S3. Algal gene trees showing the phylogenetic relationships of major *Trebouxia* clades. Four algal loci (nrITS, nrLSU, *mtCOXII* and *rbcL*) were used for single-locus phylogenetic reconstructions using ML and Bayesian methods. Bayesian PP > 0.95 and BS > 70% are indicated above branches.

Figure S4. ML tree of the proposed new *Trebouxia* clade, which is estimated from a concatenated data matrix containing algal nrITS, nrLSU, *mtCOXII* and *rbcL*. Branches with PP > 0.95 and BS > 70% are considered as well-supported and labelled with PP/BS. ABGD and bPTP analysis used ML algal nrITS topology; bPTP* used the ML tree topology obtained from the concatenated data matrix.

Table S1. Voucher information and GenBank accession numbers of Icelandic cetrarioid lichens.

Table S2. PCR primers and annealing temperatures (TA).

Table S3. Taxon information and GenBank accession numbers for the algal nrITS sequences used in Figure 3A, containing 75 algal nrITS-based representative OTUs from five *Trebouxia* clades (i.e. "A", "C", "D", "I" and "S" clades).

Table S4. Taxon information and GenBank accession numbers for the sequences used in the multi-locus phylogenetic analysis of *Trebouxia* in Figure 3B.

Table S5. Taxon information and their GenBank accession numbers for the sequences included in the proposed *Trebouxia* "D" clade.