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Cryptic diversity and symbiont interactions in rock-posy lichens

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ABSTRACT

Identifying factors that influence species interactions is central to research in symbiotic systems. While lichens represent iconic models of symbiosis and play important roles in understanding the biology of symbiotic interactions, patterns of interactions in lichen symbionts and mechanisms governing these relationships are not well characterized. This is due, in part to the fact that current taxonomic approaches for recognizing diversity in lichen symbionts commonly fail to accurately reflect actual species diversity. In this study, we employed DNA-based approaches to circumscribed candidate species-level lineages in rock-posy lichen symbionts (mycobiont = *Rhizoplaca* s. lat. species; photobiont = *Trebouxia* species). Our results revealed a high degree of cryptic diversity in both the myco- and photobionts in these lichens. Using the candidate species circumscribed here, we investigated the specificity of the symbionts toward their partners and inferred the relative importance of various factors influencing symbiont interactions. Distinct mycobiont species complexes, ecozones, and biomes are significantly correlated with the occurrence of photobiont OTUs, indicating that complex interactions among mycobiont lineages, ecogeography, and microhabitat determine interactions between photobionts and their mycobionts in lichen symbiosis. One-to-one specificity between mycobiont and photobiont species was not found, with the exception of *R. maheui* that associated with a single *Trebouxia* OTU that was not found with other *Rhizoplaca* s. lat. species. We estimated the most recent common ancestor of the core *Rhizoplaca* group at c. 62.5 Ma, similar in age to the diverse parmelioid core group in the well-studied family Parmeliaceae. However, in contrast to Parmeliaceae, species in *Rhizoplaca* were found to associate with a narrow range of photobionts. Our study provides important perspectives into species diversity and interactions in iconic lichen symbiotic systems and establishes a valuable framework for continuing research into rock-posy lichens.

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1. Introduction

Symbiotic interactions are considered a fundamental feature of life (Gilbert et al., 2012; Margulis, 1970; McFall-Ngai, 2008). These intimate, and often obligate, interactions among organisms from evolutionarily distinct lineages play central roles in physiology, reproduction, evolution, and other fundamental biological processes (Godfrey-Smith, 2015; Oliver et al., 2009; Turnbaugh and Gordon, 2009). In spite of the overarching importance of symbioses, teasing apart specific mechanisms structuring interactions among symbionts has historically been stymied due to a variety of factors, including an incomplete perspective of diversity and a general inability to accurately identify microbial partners. Recognition of species-level diversity in symbiotic systems has enabled an improved understanding of several fundamental aspects of symbiotic interactions (Darwell et al., 2014; Franklin et al., 2012; Gazis et al., 2011; Pinzón and LaJeunesse, 2011).

Lichens represent iconic examples of symbioses and consist of a filamentous fungus – the mycobiont – usually obligatorily associated with a photosynthesizing partner – the photobiont. Lichens are commonly referred to as dual or composite organisms, or even miniature ecosystems. The latter two terms may be more appropriate as lichen-forming fungi have been shown to associate with a

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broad range of other microorganisms in addition to their primary photobiont partners (Arnold et al., 2009; Aschenbrenner et al., 2014; Grube et al., 2009; Hodkinson and Lutzoni, 2009; Muggia et al., 2013). Cryptic, or previously unrecognized species-level lineages have been shown to be common in lichen symbionts (Leavitt et al., 2015; Lücking et al., 2014; Lumbsch and Leavitt, 2011; Muggia et al., 2014; Pérez-Ortega et al., 2012; Sadowska-Deś et al., 2014). The increasing ability to accurately delimit and recognize species-level lineages provides opportunities for better understanding symbiosis using these iconic symbiotic systems.

Interactions among potential lichen symbionts have been described in terms of selectivity, specificity, and availability; and each has been shown to have significant impacts on structuring lichen associations (Fernández-Mendoza et al., 2011; Muggia et al., 2013, 2014; Vargas Castillo and Beck, 2012; Werth and Sork, 2010; Wirtz et al., 2003; Yahr et al., 2006). From the perspective of the mycobiont, specificity can be defined as the range of compatible photobiont partners, while selectivity is used to describe the frequency of association with distinct photobiont lineages (Rambold et al., 1998). Distributions of lichen symbionts are known to play important roles in shaping lichen symbiotic interactions (Dal Grande et al., 2012; Fernández-Mendoza et al., 2011; Fernández-Mendoza and Printzen, 2013; Muggia et al., 2014; Peksa and Škaloud, 2011; Werth and Sork, 2014) and evolution in these symbiotic systems (Magain and Sérusiaux, 2014; Rambold et al., 1998; Rikkinen et al., 2002).

The algal genus *Trebouxia* Puymaly associates with half or more of all lichen-forming fungal species, and members of this genus are especially common as photobionts in extra-tropical regions (Ahmadjian, 1993). However, species-level diversity in *Trebouxia* remains poorly characterized. A number of recent studies highlight that the current taxonomy woefully underestimates the actual number of species-level lineages in this important genus of lichen photobionts and the pressing need for a provisional nomenclatural system (Casano et al., 2011; Kroken and Taylor, 2000; Leavitt et al., 2015; Muggia et al., 2014; Sadowska-Deś et al., 2014; Werth and Sork, 2014). Currently, four major clades are known within *Trebouxia*; the '*arboricola*/*gigantea*', '*galapagensis*/*usneae*', '*impressa*/*gelatinosa*', and '*simplex*/*letharii*/*jamesii*' clades (Beck et al., 2002; Helms, 2003). Within each clade, provisional species-level photobiont OTUs have been circumscribed using a barcode gap detection approach for photobionts associating with the diverse mycobiont family Parmeliaceae (Leavitt et al., 2015). Arguably, increasing our ability to recognize species-level lineages in *Trebouxia* and accurately identifying samples will help facilitate research improving our perspective of symbiotic interactions in lichens.

In this study we use rock-posy lichens (Fig. 1) as a model for exploring diversity and interactions among lichen myco- and photobionts. Rock-posy lichens comprise a mycobiont from the genus *Rhizoplaca* s. lat. (Lecanoraceae) – *Rhizoplaca* species and *Protoparmeliopsis peltata* (Ramond) Arup, Zhao Xin & Lumbsch (Zhao et al., 2016) – in association with photobionts from the genus *Trebouxia* (Arup and Grube, 2000). Rock-posies are commonly found in arid, exposed, continental habitats throughout the world. However, *Rhizoplaca* s. lat. species are almost completely absent from Africa, with the exception of Morocco and the Canary Islands, and appear to be completely absent from Australia. The mycobiont genus *Rhizoplaca* s. lat. is comprised of ca. 24 species, with the highest diversity in Central Asia and western North America. Some species are known to occur across broad ecological and intercontinental distributions, including *P. peltata*, *R. chrysoleuca* (Sm.) Zopf, *R. melanophthalma* (DC.) Leuckert & Poelt, and *R. subdiscrepans* (Nyl.) R. Sant. In contrast, other species are only found in geographically and ecologically restricted habitats. For example, *R. macleanii* (C.W. Dodge) Castello occurs strictly in continental Antarctica; *R. maheui* (Hue) Gómez-Bolea & M. Barbero is known exclusively from central Spain; and *R. marginalis* (Hasse) W.A. Weber occurs solely in the southern portion of the Sierra Nevada range in southern California, USA.

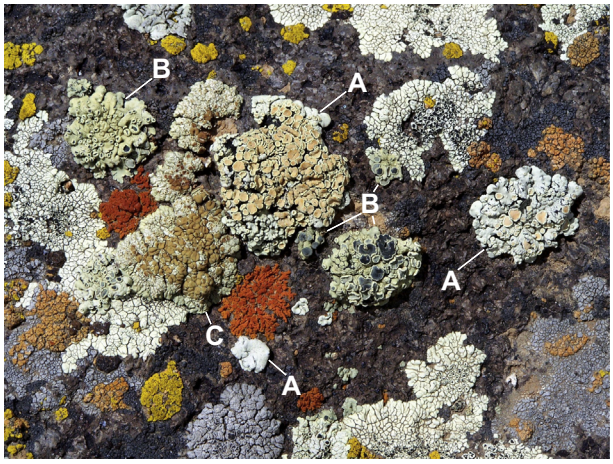


Fig. 1. Three common rock-posy lichens with broad, intercontinental distributions co-occurring on a basalt boulder in southern Utah, USA: A – "orange rock-posy" lichen (mycobiont = *Rhizoplaca chrysoleuca* s. lat.); B – "green rock-posy" lichen (mycobiont = *Rhizoplaca melanophthalma* s. lat.); and C – "scattered rock-posy" lichen (mycobiont = *Rhizoplaca subdiscrepans* s. lat.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Previous studies have revealed cryptic diversity in both the nominal taxa *R. chrysoleuca* and *R. melanophthalma* (Leavitt et al., 2011; Zhou et al., 2006). The center of diversity for the *R. melanophthalma* group is the Great Basin region of western North America, where all known species within this complex occur (Leavitt et al., 2013b). A number of species in this group, including *R. melanophthalma* s. str. and *R. parilis*, occur in geographically disjunct populations worldwide, including documented populations in Central Asia, Europe, the southern Andes in South America, and western North America (Leavitt et al., 2013a). However, the remaining species – *R. haydenii*, *R. idahoensis*, *R. occulta*, *R. polymorpha*, *R. porteri*, and *R. shushanii* – are currently known only from western North America, although *R. haydenii* has been reported from China (Zheng et al., 2007).

In contrast to the *R. melanophthalma* group, the potential for cryptic species-level diversity and their associated distribution patterns are not well characterized for other nominal *Rhizoplaca* s. lat. species. Zhou et al. (2006) reported that samples of *R. chrysoleuca* from montane regions in China occur in two distinct phylogenetic clades with corresponding phenotypic variation. Similarly, DNA sequence data revealed the potential for cryptic species in the Antarctic endemic *R. macleanii* (Pérez-Ortega et al., 2012). However, phylogenetic inferences and species delimitation analyses for both *R. chrysoleuca* and *R. macleanii* were limited to a single genetic region, the internal transcribed spacer region (ITS). While a limited number of specimens representing other *Rhizoplaca* species have occasionally been included in phylogenetic studies (Arup and Grube, 2000; Cansaran et al., 2006; Kondratyuk et al., 2014; Zheng et al., 2007), the potential for cryptic species-level lineages within species complexes and evolutionary relationships among species remain largely untested. Furthermore, estimating the timing of diversification of cryptic species-level level lineages can aid in identifying factors that give rise to commonly overlooked biodiversity.

The number of mycobiont species greatly exceeds the number of available photobiont partners, and reciprocal one-to-one specificity is generally not expected (Ahmadjian, 1960; Otálora et al., 2010). However, reciprocal symbiont specificity has been observed

between some lichen-forming fungi and cyanobacterial photobionts (Otálora et al., 2010; Pérez-Ortega et al., 2010). In other cases, lichen-forming fungal lineages exploit a common pool of photobiont partners that may differ from other closely related mycobiont lineages (Leavitt et al., 2015; Rikkinen et al., 2002). Photobiont switches have been shown to play a major role in expanding the geographic and ecological amplitude for some mycobiont taxa (Fernández-Mendoza et al., 2011; Nelsen and Gargas, 2009; Yahr et al., 2006). However, the roles of reciprocal symbiont specificity or photobiont switches among mycobiont species in closely related species complexes have not been adequately investigated. Photobiont diversity in rock-posy lichens has only been investigated for the Antarctic endemic mycobiont *R. macleanii*, where it was shown to have relatively low specificity towards available photobiont partners (Pérez-Ortega et al., 2012). Similarly, high levels of symbiont flexibility were inferred for the cosmopolitan mycobiont species *Protoparmeliopsis muralis* (Rabenh.) M. Choisy (Guzow-Krzemińska, 2006), which is closely related to the umbilicate taxon *P. peltata*, formerly included in *Rhizoplaca* (Zhao et al., 2016).

Given the prevalence of cryptic species-level diversity in rock-posy lichen symbionts and contrasting distribution patterns in *Rhizoplaca* s. lat. mycobiont species, we aim to explore patterns of symbiont interactions to better understand how symbiotic interactions may influence diversification and distributions in this group of lichens. Our overall objective is to characterize species-level diversity in rock-posy lichen symbionts – e.g., nominal *Rhizoplaca* s. lat. species and associated *Trebouxia* photobionts – in order to better understand the roles of symbiotic interactions in shaping lichen distributions and evolutionary histories. Specifically, we ask:

- Similar to the *R. melanophthalma* complex, do previously unrecognized species-level lineages also occur in other traditionally circumscribed *Rhizoplaca* s. lat. species?
- Do closely related mycobiont species-level lineages associate with distinct photobiont OTUs?
- Which factors – e.g., ecogeography, mycobiont evolutionary history, etc. – are associated with patterns of symbiont interactions in *Rhizoplaca*?

To address these questions we used sequence data collected from over 800 rock-posy and other closely related lichens. From these molecular sequence data we delimited candidate species for rock-posy symbionts, assessed species' distributions, and characterize the range of symbiont interactions in rock-posy lichens. We also provide the most comprehensive phylogeny to-date for the mycobiont genus *Rhizoplaca* and closely related taxa.

2. Materials and methods

2.1. Taxon sampling

Our sampling focused on the mycobiont genus *Rhizoplaca* in Lecanoraceae, supplemented with three *Protoparmeliopsis* (Lecanoraceae) species, *P. garovaglii*, *P. muralis*, and the umbilicate taxon *P. peltata*. Although *P. peltata* has previously been shown to be more closely related to *Protoparmeliopsis* species than those in the genus *Rhizoplaca* s. str. (Arup and Grube, 2000; Zhao et al., 2016), this taxon has traditionally been considered a member of the latter due to gross morphological similarities. A total of 816 specimens were sampled across broad geographic and ecological distributions worldwide, with a particular emphasis on the centers of *Rhizoplaca* s. lat. species-level diversity: arid habitats in Central Asia and western North America (Table 1; Supplementary Table S1).

Earlier studies have revealed cryptic species-level diversity in the *R. melanophthalma* group, and species within this complex commonly co-occur in some similar habitats in central Asia, Europe, western North America, and southern South America (Leavitt et al., 2011, 2013a, 2013b). To evaluate if closely related mycobiont species-level lineages associate with distinct photobiont OTUs our sampling focused on this complex and included a total of 444 *R. melanophthalma* s. lat. specimens. To investigate local patterns of photobiont sharing among co-occurring mycobiont species in the *R. melanophthalma* complex, we sampled 25 thalli in 10×10 m plots at four localities. Two sites – Mosquito Creek and Barley Creek – in the Table Mountain Wilderness Area in the Great Basin, Nevada, USA; a third site was located near Birch Creek in the Lemhi Valley in east-central Idaho, USA; and the fourth site was located along Nutter's Ridge in the Uintah Basin in eastern Utah, USA (Supplementary Table S1). We also included *Rhizoplaca melanophthalma* s. lat. specimens collected at ten sites along an altitudinal transect on the Aquarius Plateau in southern Utah, USA, ranging from arid sage-steppe communities (ca. 2100 m.a.s.l.) to subalpine meadowlands (ca. 3300 m.a.s.l.) (Leavitt et al., 2011).

In addition to the *R. melanophthalma* complex, three other species groups in *Rhizoplaca* s. lat. with broad, intercontinental distributions were sampled: *P. peltata* ($n = 35$), *R. chrysoleuca* ($n = 128$), and *R. subdiscrepans* ($n = 41$). Three taxa with more restricted geographic distributions were also included: *R. maheui* (Hue) Gómez-Bolea & M. Barbero ($n = 5$), known only from central Spain; *R. marginalis* ($n = 2$) from southern California; and *R. macleanii* from Antarctica ($n = 39$). In addition to *P. peltata*, the genus *Protoparmeliopsis* was also represented by 18 specimens of *P. garovaglii* (Körb.) Arup, Zhao Xin & Lumbsch and 55 of *P. muralis*. *Lecanora saligna* has previously been shown to be closely related to some *Rhizoplaca* lineages, and a single specimen representing *L. saligna* was included. Two species from the '*Lecanora dispersa* group' (Śliwa et al., 2012), now recognized as members of the genus *Myriolecis* Clements (Zhao et al., 2016), were used as outgroups (Miadlikowska et al., 2014).

The ecogeographic affinity of each sample was derived using ecoregion data from the World Wildlife Fund (WWF) boundaries of terrestrial ecoregions of the world available from the Harvard WorldMap platform (http://worldmap.harvard.edu/data/geonode:wwf_terr_ecos_oRn). We include ecogeographical assignments at three hierarchically nested levels: ecozones (three sampled categories – 'Antarctica', 'Nearctic', and 'Palearctic'), biomes (seven categories), and ecoregions (30 categories) (Supplementary Table S1). In a number of cases where latitude and longitude coordinates were not available, sample assignment to a specific ecoregion was inferred based on the description of the collection sites.

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted as described previously (Leavitt et al., 2013b), and mycobiont DNA sequence data were generated for a total of seven loci: two markers from the nuclear ribosomal cistron and five low-copy nuclear protein-coding genes. Markers from the nuclear ribosomal cistron included the ITS and intergenic spacer region (IGS). Nuclear protein-coding genes included the β-tubulin, EF1-alpha, MCM7, RPB1, and RPB2. All markers were amplified, sequenced, and assembled as described previously (Leavitt et al., 2013b). Sequences with more than two ambiguous nucleotide characters were excluded from downstream analyses.

2.3. Multiple sequence alignments

Multiple sequence alignments for each fungal marker were performed using the program MAFFT v7 (Katoh et al., 2005; Katoh and Toh, 2008) as described previously (Leavitt et al.,

Table 1
Summary of sampled *Rhizoplaca* s. lat. species complexes, including candidate species-level lineages within each complex, the geographic distribution of sampled specimens, and the associating *Trebouxia* OTU. Photobiont OTUs 'A01', 'A02', 'A03', 'A08', and 'A13' belong to the *Trebouxia* 'arboricola/gigantea' clade; OTUs 'I01', 'I02' and 'I03' to the *T.* 'impressa/gelatinosa' group; and OTU 'S02' to the "simplex/letharii/jamesii" clade (Leavitt et al., 2015). Photobiont OTUs that were identified in <5% of the samples are indicated with an '*'.

Species complex	Species-level lineages (number of specimens)	Geographic distribution	Associating *Trebouxia* OTUs
R. chrysoleuca (131)	–	Species complex occurs worldwide (excluding Africa and Australasia)	A02, A08, I01, I03, S02*
	'chry A' (6)	Central Asia (Russia, Altay); North America (USA, WI)	I01 (83%), I03 (17%)
	'chry B' (5)	Central Asia (Russia, Altay)	I01 (100%)
	'chry C' (1)	Central Asia (Russia, Altay)	NS (NA)
	'chry D' (9)	Western North America; GENBANK sequences	A02 (60%), I01 (40%)
	'chry E' (30)	Central Asia (Russia, Altay; Iran; Kyrgyzstan); western North America (Rocky Mountain region); GENBANK sequences	A02 (6%), A08 (18%), I01 (76%)
	'chry F' (77)	Central Asia (Kazakhstan; Kyrgyzstan; Russia, Altay, Chelyabinsk, Orenburg); western North America (Rocky Mountain region); Europe (Spain); and Turkey	A02* (3%), A08 (5%), I01 (84%), I03 (5%), S02 (3%)*
R. macleanii (63)	–	Antarctica	A02 (91%), I01 (3%)*, I03 (3%)*, S02 (3%)*
R. maheui (5)	–	Europe (Spain)	A03 (100%)
R. marginalis (2)	–	Western North America (USA, CA)	A08 (100%)
R. melanophthalma (450)		Species complex occurs worldwide (excluding Africa and Australasia)	A01*, A02, A08, A14*, I01, I03*
	L. novomexicana (9)	Asia, Europe, North America	A01 (11%), A02 (44%), A08 (11%), I01 (33%)
	R. haydenii/idahoensis (29)	Western North America; reported from China	A02 (86%), A08 (5%), I01 (9%)
	R. melanophthalma (143)	Worldwide (excluding Africa and Australasia)	A01 (1%)*, A02 (20%), A08 (18%), A13 (1%), I01 (59%), I03 (1%)*
	R. occulta (6)	USA, Great Basin region	A02 (33%), A08 (66%)
	R. parilis (66)	worldwide (excluding Africa and Australasia)	A01 (4%)*, A02 (56%), A08 (4%)*, I01 (36%)
	R. polymorpha (33)	USA, Great Basin region	A01 (3%)*, A02 (73%), A08 (10%), I01 (13%)
	R. porteri (119)	USA, Great Basin region	A01 (7%), A02 (70%), A08 (10%), I01 (13%)
	R. shushanii (39)	USA, Utah	A02 (39%), A08 (11%), I01 (47%), I03 (3%)*
P. peltata (35)	–	Species complex occurs worldwide (excluding Africa and Australasia)	A02, A08, I01, S02
	'pelt A' (7)	Central Asia (Iran, Kyrgyzstan, Turkey, Russia)	A02 (14%), A08 (29%), I01 (43%), S02 (14%)
	'pelt B' (6)	Central Asia (Russia)	I01 (100%)
	'pelt C' (22)	Central Asia (Iran, Kazakhstan, Kyrgyzstan, Russia, Turkey); and Western North America (USA, UT)	A02 (38%), A08 (29%), I01 (33%)
R. subdiscrepans (40)	–	Species complex occurs worldwide (excluding Africa and Australasia)	A08, I01, I02*, I03, S02*
	'subd B' (3)	Central Asia (Kazakhstan, Russia)	A08 (33%), I01 (67%)
	'subd A' (10)	Central Asia (Russia); and Western North America (USA, UT)	I01 (100%)
	'subd C' (1)	Central Asia (Russia, Altay)	NS
	'subd D' (6)	Central Asia (Russia, Altay), North America (USA, WI)	NS (NA)
	'subd E' (20)	Central Asia (Russia, Orenburg & Chelyabinsk); Ukraine (Prague)	A08 (9%), I01 (68%), I02 (5%), I03 (14%), S02 (5%)
Protoparmeliopsis 'sp. 6442' (1)	–	Central Asia (Kazakhstan)	NA
Rhizoplaca 'sp. 5164' (1)	–	Western North America (USA, NV)	NA
Rhizoplaca 'sp. 6842' (1)	–	Central Asia (Russia, Altay)	NA
Rhizoplaca 'sp. 6867' (4)	–	Central Asia (Russia, Altay)	NA

2013b). To improve alignment accuracy, subsequent clade-specific gene alignments were performed for *P. peltata* s. lat., *R. chrysoleuca* s. lat., *R. melanophthalma* s. lat., and *R. subdiscrepans* s. lat. clades. We used the *Trebouxia* ITS alignments reported in Leavitt et al. (2015; Dryad doi:http://dx.doi.org/10.5061/dryad.5rm6d), which included 562 sequences from photobionts associated with *Protoparmeliopsis* and *Rhizoplaca* specimens.

2.4. Mycobiont species delimitation analyses

Species within the *R. melanophthalma* complex have been circumscribed previously (Leavitt et al., 2011, 2013b), and specimens can be effectively identified using molecular-based identification tools (Leavitt et al., 2013a). We used the blast-based identification tool against the BOLD database (Ratnasingham and Hebert, 2007) to identify newly sampled specimens in the *R. melanophthalma* group using the ITS barcoding marker (Schoch et al., 2012).

The potential for previously unrecognized species-level lineages in the nominal taxa *P. peltata*, *R. chrysoleuca*, *and R. subdiscrepans* was assessed using phylogenetic reconstructions to identify genealogical concordance across independent loci (Avise and Ball, 1990), as well as Bayesian genetic clustering. Individual gene trees and concatenated topologies were reconstructed for each nominal species complex using the program RAxML v8.1.1 (Stamatakis, 2006; Stamatakis et al., 2008) to identify well-supported phylogenetic substructure in single gene topologies and also in multi-gene concatenated phylogenies. All ML analyses were performed using the CIPRES Science Gateway server (http://www.phylo.org/portal2/), using the 'GTRGAMMA' model and evaluating nodal support using 1000 bootstrap pseudo-replicates. For the multi-locus phylogenies, we used locus-specific model partitions, treating all loci as separate partitions; otherwise analyses were performed as described above. Exploratory analyses of alternative partition strategies provided highly similar topologies and nodal support values.

Genetic clusters were inferred within the *P. peltata*, *R. chrysoleuca*, and *R. subdiscrepans* groups independently using the program STRUCTURE (Falush et al., 2003; Pritchard et al., 2000). Clustering analyses were performed as described in Leavitt et al. (2011). In short, we used polymorphic sites from the aligned sequence data to infer genetic population structure in these *Rhizoplaca* taxa. In an empirical study of the lichen-forming fungal genus *Letharia*, Altermann et al. (2014) demonstrated that Bayesian clustering using polymorphic sites from multi-locus sequence alignments could be used to corroborate previously recognized cryptic genetic groups and performed consistently across a range of scenarios (see also Falush et al., 2003; O'Neill et al., 2013; Weisrock et al., 2010). Therefore, we estimated population clusters within each of these three nominal *Rhizoplaca* taxa for *K* values ranging from 1 to 10, with 10 replicates for each *K* value. The most likely number of genetic clusters was inferred by assessing likelihood values to identify where Pr(*K*) more or less plateaus (STRUCTURE documentation) and comparisons with multi-locus phylogenies.

2.5. Mycobiont phylogeny and divergence time estimates

Phylogenetic relationships among the sampled fungal lineages were inferred using both maximum likelihood (ML) and Bayesian inference (BI) methods. Exploratory phylogenetic analyses of individual gene topologies showed no evidence of well-supported topological conflict (conflicting nodes with $\geqslant$70% bootstrap values; data not shown). In this study, a number of putative species-level lineages were represented by small samples sizes; and furthermore, we were not able to obtain sequences representing all genes for a limited number of candidate species, despite repeated attempts and using different primer combinations. Due to the fact that concatenation exhibits statistically comparable accuracy under a range of scenarios (Mirarab et al., 2014; Tonini et al., 2015) and that missing data in our matrix limits the practical application of some commonly used species-tree methods, we chose to infer the phylogeny using a concatenated gene-tree approach. Relationships were reconstructed from the complete seven-locus concatenated data matrix (n = 732) and a reduced data matrix minimizing missing data (n = 199) using a total-evidence approach (Kluge, 1989; Wiens, 1998). ML analyses were performed on the concatenated matrix as described above.

We attempted to place the diversification history of cryptic lineages within nominal *Protoparmeliopsis* and *Rhizoplaca* taxa in a temporal context using a relaxed molecular clock using the program BEAST v1.8.2 (Drummond and Rambaut, 2007; Heled and Drummond, 2010). For the BEAST analysis, we used a reduced dataset minimizing missing data (n = 199). Relevant fossil evidence is not available for Lecanoraceae, and therefore we used an estimated substitution rate for the nuclear ribosomal ITS region (2.43×10^{-9} substitution/site/year, as per Leavitt et al., 2012b) to estimate divergence times. Similar ITS substitution rates have been estimated for other fungi (Leavitt et al., 2012c; Takamatsu and Matsuda, 2004). Substitution rates for the other loci were co-estimated under a uniform prior between 0 and 100. We estimated divergence times under an uncorrelated relaxed lognormal molecular clock (Drummond et al., 2006), implementing a constant coalescent speciation process prior, and with data matrix partitioned by individual gene regions. Two independent MCMC runs of 100 million generations were performed, sampling every 2000 steps. Chain mixing and convergence were evaluated in Tracer v1.5 (Rambaut and Drummond, 2003), considering ESS values >200 as good indicators. After excluding the first 25% of sampled trees as burn-in, trees from the two independent runs were combined using the program LogCombiner v1.8.2 (Rambaut and Drummond, 2013), and the final MCC tree was estimated from the combined posterior distribution of trees using TreeAnnotator v1.8.2 (Rambaut and Drummond, 2009).

2.6. Photobiont OTU delimitation analyses

Operational taxonomic unit (OTU) delimitation and sequence assignment for *Trebouxia* ITS sequences were reported in a companion paper (Leavitt et al., 2015), and their methods are summarized here. In short, the Automatic Barcode Gap Discovery method (ABGD; Puillandre et al., 2012) was used to circumscribe OTUs representing candidate species. ABGD infers a model-based confidence limit for intraspecific divergence, and then detects the barcode gap as a first significant gap beyond this limit to infer primary partitions. Leavitt et al. (2015) delimited *Trebouxia* OTUs from a total of 2356 ITS sequences, including 26 sequences representing 20 *Trebouxia* species generated from UTEX and SAG cultures (Dryad doi:http://dx.doi.org/10.5061/dryad.5rm6d). From this larger dataset, we explored patterns of symbiont interactions using OTU assignments of 562 *Trebouxia* sequences associating with mycobionts from Lecanoraceae – predominantly associating with *Protoparmeliopsis* and *Rhizoplaca* species. We followed the provisional names provided by Leavitt et al. (2015) for the *Trebouxia* OTUs.

2.7. Fungal–algal interactions

The abundance and diversity of *Trebouxia* OTUs in relation to their mycobiont hosts were assessed in order to identify patterns in specificity and selectivity at two levels. First, we investigated patterns in mycobiont species' specificity and selectivity towards

their algal partners in the well-sampled *R. melanophthalma* complex. A fungal–algal interaction network was constructed, where fungal partners were grouped by mycobiont species – *R. novomexicana*, *R. haydenii*, *R. melanophthalma* s. str., *R. parilis*, *R. polymorpha*, *R. porteri*, and *R. shushanii* (we were unable to generate *Trebouxia* ITS sequence from *R. occulta*) that were then compared to the associated *Trebouxia* OTUs. For the second level of investigation, we assessed patterns in specificity and selectivity at a deeper evolutionary scale, comparing patterns among each sampled nominal species or complex: *Protoparmeliopsis garovaglii*, *P. muralis*, *P. peltata*, *Rhizoplaca chrysoleuca*, *R. macleanii*, the *R. melanophthalma* complex, and *R. subdiscrepans*. For these analyses, we constructed fungal-algal interaction bipartite networks, where fungal partners were grouped by mycobiont species or species-complex and photobiont diversity was represented as OTUs. Bipartite networks were constructed using the bipartite package in R (Dormann et al., 2008). We estimated specificity coefficient (d') for each sampled lichen-forming fungal genus, derived from the coefficient of variation of interactions (Julliard et al., 2006; Poisot et al., 2012). The coefficient d' compares the observed number of interactions of a fungal species with an algal species with the average number of interactions of that particular fungal species across all algal species. A maximum d' value of 1 is achieved for a fungal species that interacts with only one algal species and therefore has the highest difference between the observed interactions and the mean across all algal species. The implementation of the index is available through function "specieslevel()" in the bipartite package in R (Dormann, 2011; Dormann et al., 2008).

We used multinomial logistic regression to evaluate the role of ecogeography and evolutionary history in structuring photobiont associations in *Rhizoplaca*. To assess the influence of ecogeography in structuring photobiont associations, we compared Akaike information criterion (AIC) values among three hierarchically nested levels – ecozone, biomes, and ecoregions – against a null model where algal OTUs were randomly distributed. To assess the influence of the fungal evolutionary history in structuring photobiont associations, we calculated AIC values from models incorporating (i) membership in a species complex (*Protoparmeliopsis garovaglii*, *P. muralis*, *P. peltta*, *Rhizoplaca chrysoleuca*, *R. macleanii*, the *R. melanophthalma* group, and *R. subdiscrepans*); and individual species/putative species-level lineages. We also examined the effect of mycobiont growth form – placodioid vs. umbilicate – on structuring symbiont interactions in rock-posy lichens. Multinomial logistic regression analyses were performed in R with the package nnet (Venables and Ripley, 2002).

3. Results

3.1. Molecular sequence data

New sequences generated in association with this study have been deposited in GenBank under accession numbers KU934303–KU935437, and all alignments have been deposited in TreeBase (study ID: 19048). The complete, 7-gene mycobiont data matrix ($n = 732$) included 4647 aligned nucleotide positions.

3.2. Mycobiont species delimitation

Well-supported phylogenetic substructure was inferred in *Protoparmeliopsis peltata* s. lat., *R. chrysoleuca* s. lat., *R. melanophthalma* s. lat., and *R. subdiscrepans* s. lat. (Supplementary Figs. S1–S3). Strongly supported clades inferred from the concatenated multi-locus phylogenies generally corresponded to genetic groups inferred from the STRUCTURE analyses, inferring the most appropriate *K* value by identifying a general pattern of plateau in likelihood values (Fig. 2; Supplementary Fig. S4). Patterns of genealogical concordance are summarized for *P. peltata* s. lat., *R. chrysoleuca* s. lat., and *R. subdiscrepans* s. lat. in Fig. 2. Candidate species in the *R. chrysoleuca* s. lat. and *R. subdiscrepans* s. lat. groups were consistently recovered as well-supported monophyletic clades in the ITS and RPB2 topologies, although less consistently recovered as monophyletic clades in the other single gene topologies (Fig. 2).

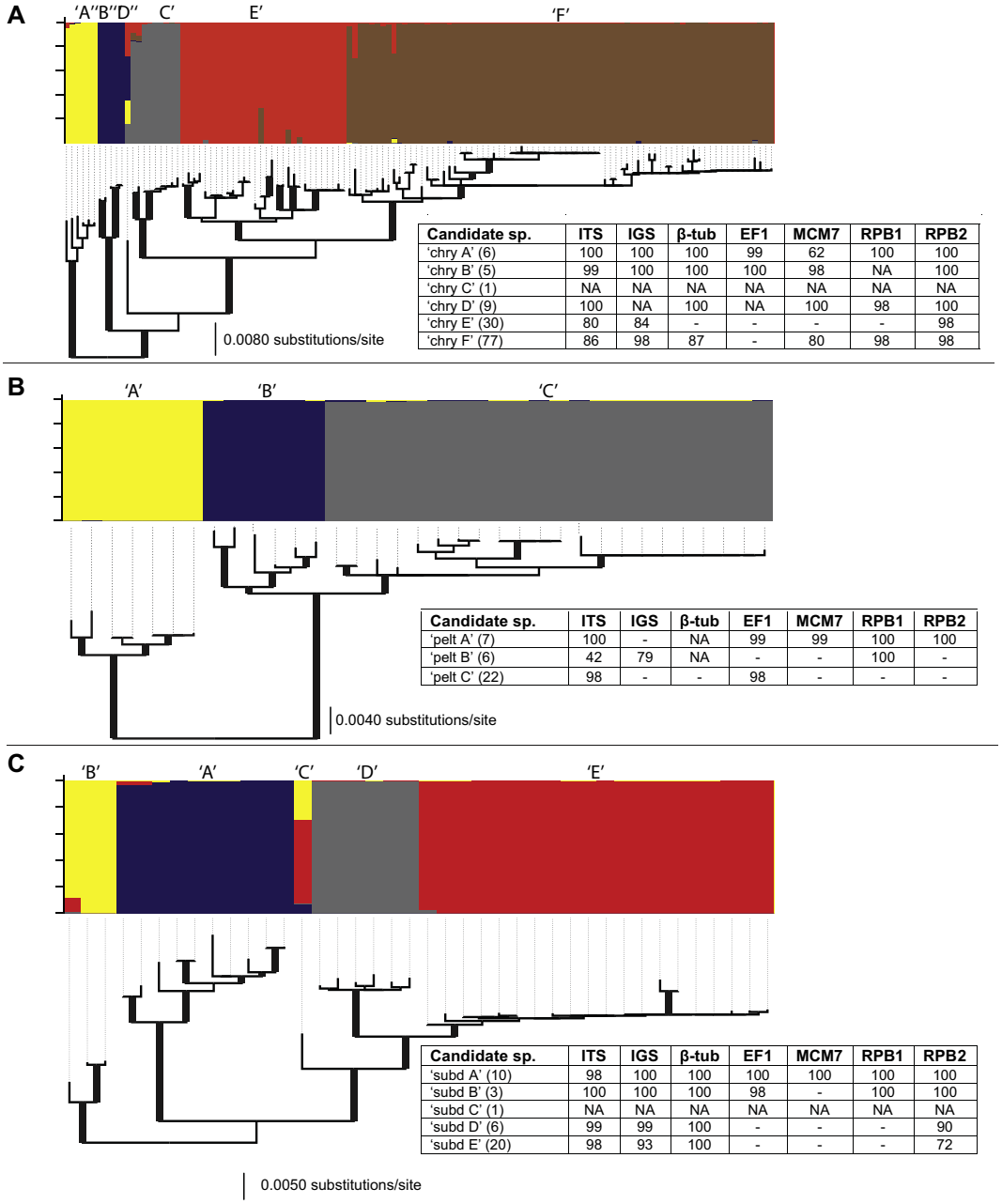
Six candidate species, including a singleton, were circumscribed in *R. chrysoleuca* s. lat. (Fig. 2A). In *P. peltata* s. lat., we circumscribed three candidate species-level lineages (Fig. 2B). A total of five candidate species were circumscribed in *R. subdiscrepans* s. lat., including one represented by a single specimen (Fig. 2C). Geographic distributions of all candidate species are summarized in Table 1. Phylogenetic relationships and genetic clustering for the *R. melanophthalma* group are shown in Supplementary Figs. S5 and S6. *Rhizoplaca haydenii* was not recovered as monophyletic and *R. occulta* was recovered as a well-supported clade nested within *R. porteri* in the phylogeny inferred from concatenated multi-locus sequence data representing the *R. melanophthalma* group (Fig. S5). In the STRUCTURE analysis, *R. polymorpha* and the vagrant *Rhizoplaca* species (*R. haydenii* and *R. idahoensis*) were recovered as a single genetic cluster, although a number of individuals were recovered with admixed ancestry (Fig. S6). Also, *R. occulta* and *R. porteri* were assigned membership in a single genetic cluster, and specimens identified as *R. occulta* were inferred to have a more admixed ancestry, when compared to *R. porteri* (Fig. S6). Based on our current sampling, no strong evidence of previously unrecognized lineages was found in *R. macleanii*, *R. maheui*, and *R. marginalis*; however, in this study the latter two taxa were represented by small samples sizes. Four lineages appear to represent undescribed or unidentified species: *Protoparmeliopsis* 'sp. 6442'; *Rhizoplaca* 'sp. 5164'; *Rhizoplaca* 'sp. 6842'; and *Rhizoplaca* 'sp. 6867' (Tables 1 and Fig. 3).

3.3. Mycobiont phylogeny and divergence time estimates

The ML phylogeny estimated from the complete concatenated 7-locus data matrix is shown in Supplementary Fig. S7. The majority of nodes were recovered with bootstrap (BS) values $\geqslant$70%, and each nominal *Rhizoplaca* species complex was recovered as monophyletic with strong support (BS $\geqslant$ 99%). Two major clades were recovered: the *Protoparmeliopsis* clade (BS = 100%), which was comprised of *P. garovaglii*, *P. muralis, P. peltata*, and other unidentified *Protoparmeliopsis* specimens; and the *Rhizoplaca* clade (BS = 95%), which included *Lecanora saligna*, the *R. chrysoleuca* complex, *R. marginalis*, *R. maheui*, the *R. melanophthalma* complex, and the *R. subdiscrepans* complex.

In BEAST analyses, run lengths were sufficient to generate ESS > 200, and topologies and divergence estimates were congruent across independent runs. A maximum clade credibility (MCC) tree from the combined BEAST runs is shown in Fig. 3 (the full tree is shown in Supplementary Fig. S8).

Estimated divergence dates among the sampled lineages are shown in Fig. 3. Based on our rate-calibrated topology, the divergence between the *Protoparmeliopsis* clade and the *Rhizoplaca* clade occurred during the Late Cretaceous (c. 76 Ma, 95% highest posterior density interval [HPD]: 57.4–97.9 Ma). Our divergence time estimates indicate that the initial diversification in the different *Rhizoplaca* s. lat. species complexes (e.g., *P. peltata*, *R. chrysoleuca*, *R. melanophthalma*, and *R. subdiscrepans* groups) occurred during the Miocene, although the initial splits in the *R. chrysoleuca* (c. 21.9 Ma; HPD: 17.0–27.7 Ma) and *R. subdiscrepans* (c. 18.5 Ma; HPD: 6.1–23.9 Ma) groups were estimated to have occurred c. 8–10 million years before the initial split in the *R. melanophthalma* (c. 10.9 Ma; HPD: 7.9–14.5 Ma) and *P. peltata* (c. 10.4 Ma;



Candidate sp.	ITS	IGS	β-tub	EF1	MCM7	RPB1	RPB2
'chry A' (6)	100	100	100	99	62	100	100
'chry B' (5)	99	100	100	100	98	NA	100
'chry C' (1)	NA	NA	NA	NA	NA	NA	NA
'chry D' (9)	100	NA	100	NA	100	98	100
'chry E' (30)	80	84	-	-	-	-	98
'chry F' (77)	86	98	87	-	80	98	98

Candidate sp.	ITS	IGS	β-tub	EF1	MCM7	RPB1	RPB2
'pelt A' (7)	100	-	NA	99	99	100	100
'pelt B' (6)	42	79	NA	-	-	100	-
'pelt C' (22)	98	-	-	98	-	-	-

Candidate sp.	ITS	IGS	β-tub	EF1	MCM7	RPB1	RPB2
'subd A' (10)	98	100	100	100	100	100	100
'subd B' (3)	100	100	100	98	-	100	100
'subd C' (1)	NA	NA	NA	NA	NA	NA	NA
'subd D' (6)	99	99	100	-	-	-	90
'subd E' (20)	98	93	100	-	-	-	72

Fig. 2. Multilocus phylogenies, results from STRUCTURE analyses, and summaries of genealogical concordance for three *Rhizoplaca* species: (A) *R. chrysoleuca*; (B) *P. peltata*; and (C) *R. subdiscrepans*. STRUCTURE plots include individual assignments for the most appropriate *K* value inferred from identifying a general pattern of plateau in likelihood values; each accession is shown by a thin vertical line that is partitioned into colored segments representing the proportion of each individual's genome assignment to a genetic cluster. Bootstrap support values for individual gene topologies (ribosomal ITS and IGS, and protein-coding loci β-tubulin, EF1-alpha, MCM7, RPB1, and RPB2) are reported for each candidate species represented by >2 individuals. 'NA' indicates sample size <2; and '–' indicates the candidate species was not recovered as monophyletic in that gene tree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HPD: 6.8–15.2 Ma) groups (Fig. 3). Species-level diversification was estimated to have occurred in each complex through the Miocene and into the Pliocene for each species complex, with evidence supporting limited diversification during the Pleistocene in the *R. melanophthalma* complex (Fig. 3).

Substitution rates for other loci, relative to the ITS region (2.43×10^{-9} substitution/site/year), co-estimated under a relaxed lognormal clock were as follows: IGS = 3.00×10^{-9} (HPD = 2.24–3.82); β-tubulin = 2.11×10^{-9} (HPD = 1.51–2.78); EF1-alpha = 0.98×10^{-9} (HPD = 0.69–1.30); MCM7 = 1.78×10^{-9} (HPD = 1.38–2.22); RPB1 = 1.57×10^{-9} (HPD = 1.20–1.94); and RPB2 = 1.47×10^{-9} (HPD = 1.11–1.85).

3.4. Ecogeographical and ecological distribution of *Trebouxia* OTUs$_{ABGD}$

Rock posy lichens were found to associate with 15 of the 69 previously circumscribed OTUs (Supplementary Table S1). The four most commonly sampled *Trebouxia* OTUs – 'A02' (in 212 specimens), 'I01' (197), 'A08' (57), and 'A01' (40) – were found in 90% of sampled lichens, with the two most common OTUs, 'A02' and 'I01', found in 72.5% of all sampled rock posy lichens. Nine of the 15 OTUs were observed less than ten times ('A03' [in 5 specimens], 'A10' [1], 'A11' [2], 'A13' [6], 'A14' [2], 'A15' [4], 'A18' [5], 'I02' [1], and 'S02' [4]). The majority of the sampled *Trebouxia* OTUs were found to occur across broad, intercontinental geographic

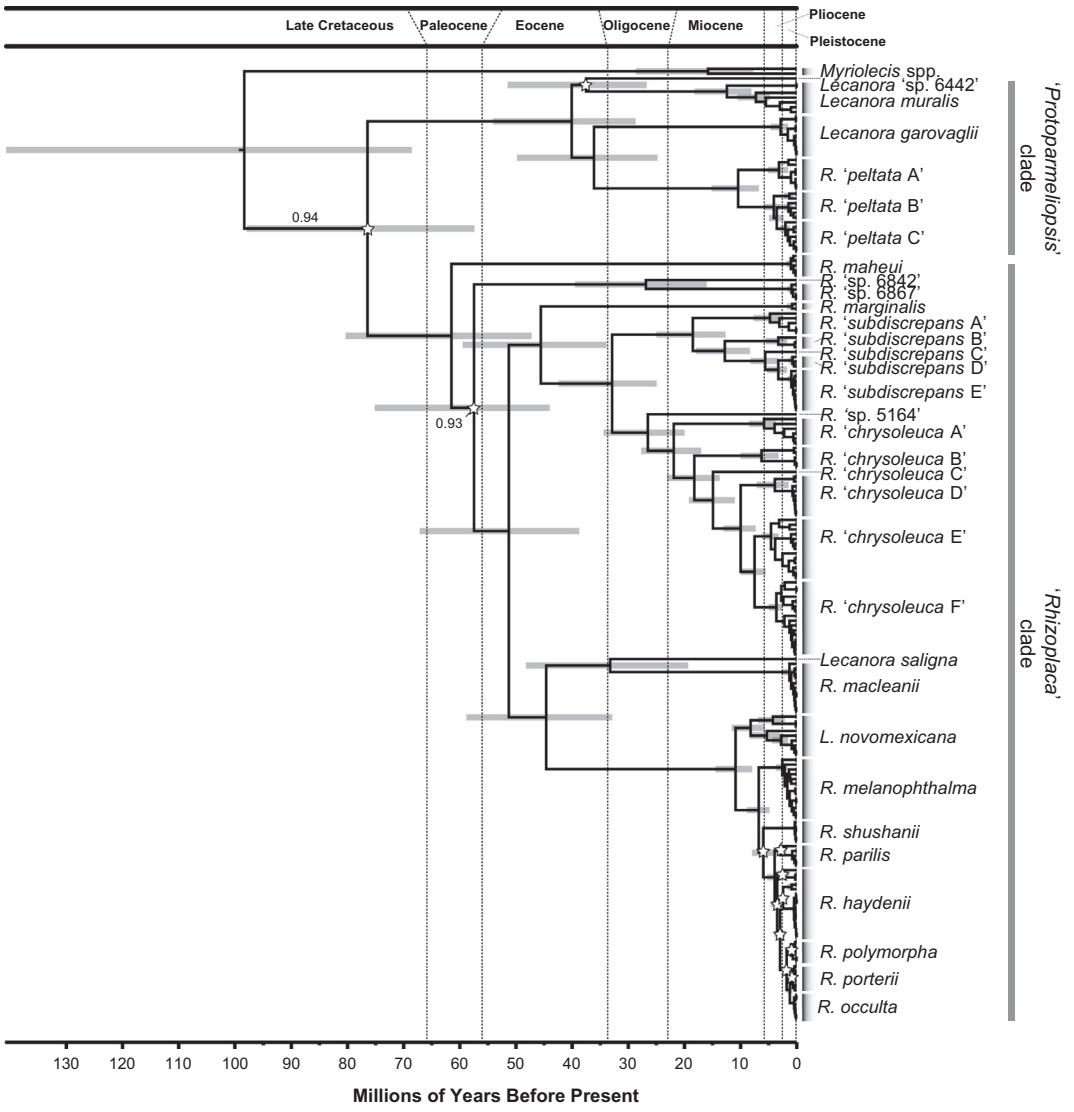


Fig. 3. Rate-calibrated chronogram for *Rhizoplaca* s. lat. inferred from seven loci (ITS, IGS, β-tubulin, EF1-alpha, MCM7, RPB1, and RPB2) using the program BEAST. Posterior probabilities (PP) were generally $\geqslant 0.95$; and PP < 0.95 are indicated with stars.

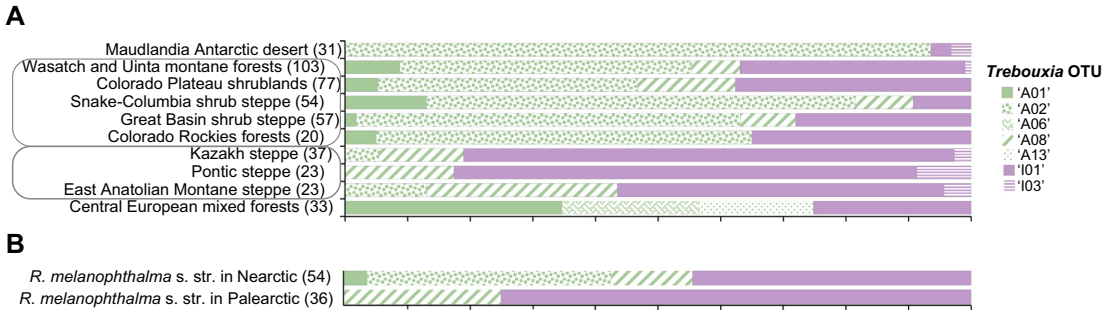


Fig. 4. A. Comparison of *Trebouxia* OTU composition associated with *Protoparmeliopsis* and *Rhizoplaca* species across ten different ecoregions. B. Comparison of *Trebouxia* OTU composition associated with *Rhizoplaca melanophthalma* s. str. between the Nearctic and Palearctic. Photobiont OTUs 'A01', 'A02', 'A06', 'A08', and 'A13' belong to the *Trebouxia* 'arboricola/gigantea' clade; and OTUs 'I01' and 'I03' belong to the *T.* 'impressa/gelatinosa' group (Leavitt et al., 2015). Only photobiont OTUs represented by >5 sequences are shown.

distributions, although distinct patterns of OTU composition across ecoregions were apparent (Fig. 4A). Algal OTU 'I01' was recovered from Central Asia, Europe, North America, Antarctica, and southern South America, and every ecoregion substantially sampled for *Rhizoplaca* photobionts. Similarly, algal OTU 'A02' was recovered from Central Asia, Europe, North America, Antarctica and southern South America and nearly every ecoregion with substantial sampling for *Rhizoplaca* photobionts with the exception of the Pontic

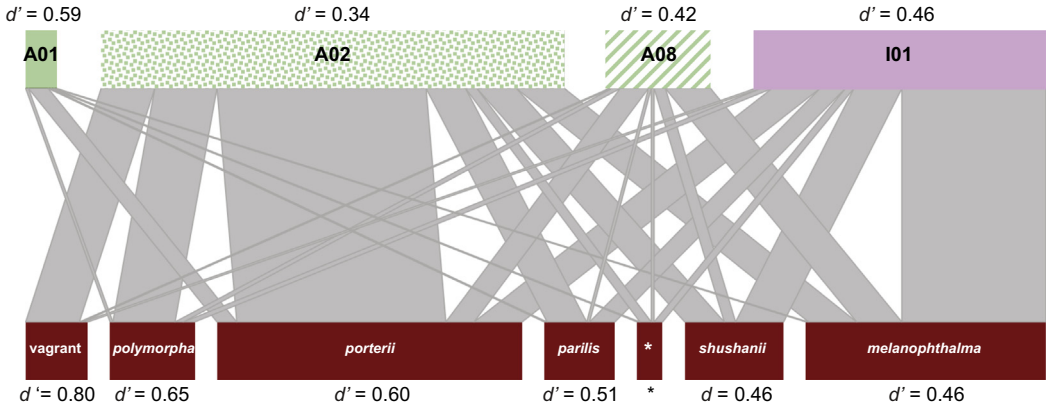


Fig. 5. Interaction network structure between lichen mycobiont species in the *Rhizoplaca melanophthalma* s. lat. species group and photobiont OTUs. The width of the links is proportional to the number of specimens forming the association. Specificity values (*d'*) for both mycobiont species and *Trebouxia* OTUs are report for each species-level lineage. The mycobiont taxon *L. novomexicana* is indicated with a '∗'; *R. occulta* was not included due to small sample sizes. Photobiont OTUs 'A01', 'A02', and 'A08', belong to the *Trebouxia* 'arboricola/gigantea' clade; and OTUs 'I01' belongs to the *T.* 'impressa/gelatinosa' group (Leavitt et al., 2015).

steppe and Central European mixed forests (Fig. 4A). However, OTU 'A02' was completely absent from the associations with *R. melanophthalma* s. str. sampled in the larger Palearctic ecozone (Fig. 4B).

3.5. Myco-photobiont interaction in Rhizoplaca

The *R. melanophthalma* complex associated with four of the six *Trebouxia* OTU's represented by more than 10 sequences, and a bipartite network analysis of symbiont interactions in the *R. melanophthalma* complex is reported in Fig. 5. All mycobiont species within the *R. melanophthalma* group associated with multiple *Trebouxia* OTUs, with each species in this group associating with at least three of the four *Trebouxia* OTUs ('A02', 'A08', 'I01'). In addition, all species in this complex, except *R. shushanii* and the vagrant forms (*R. idahoensis* and *R. haydenii*), also associated with algal OTU 'A01' (Fig. 5). Despite the near ubiquity of associations in this complex, the frequency at which fungal species occurred with these algal lineages varied substantially among taxa. For example, *R. parilis*, *R. polymorpha*, *R. porteri*, and the two vagrant *Rhizoplaca* species are most commonly associated with the *Trebouxia* OTU 'A02', while *R. melanophthalma* s. str. most frequently associates with *Trebouxia* OTU 'A01' (Fig. 5).

When comparing individual species complexes, the four *Trebouxia* OTU's associated with the *R. melanophthalma* complex were not exclusively restricted to this fungal clade, and occurred with several of the other fungal species complexes sampled for this study (Fig. 6). Algal OTU 'I01' was exceptionally widespread—occurring with six of the seven fungal species complexes sampled; and algal OTU 'A02' occurred with every *Rhizoplaca* species complex sampled, except *R. subdiscrepans* and *R. chrysoleuca* complexes which formed a monophyletic clade in phylogenetic analyses. The placodioid taxa *P. garovaglii* and *P. muralis* were shown to generally associate with a pool of photobiont OTUs distinct from those associating with *Rhizoplaca* s. lat. species, including the umbilicate taxon *P. peltata* (Fig. 6). In contrast to the placodioid *Protoparmeliopsis* species, the placodioid taxon *R. novomexicana* within the *R. melanophthalma* species complex shares a similar pool of photobionts with other closely related *Rhizoplaca* taxa.

Varying levels of specificity (*d'*) were inferred, both within the *R. melanophthalma* species complex and among different *Rhizoplaca* species complexes (Figs. 5 and 6). The Antarctic endemic *R. macleanii* was shown to have the highest specificity towards photobionts,

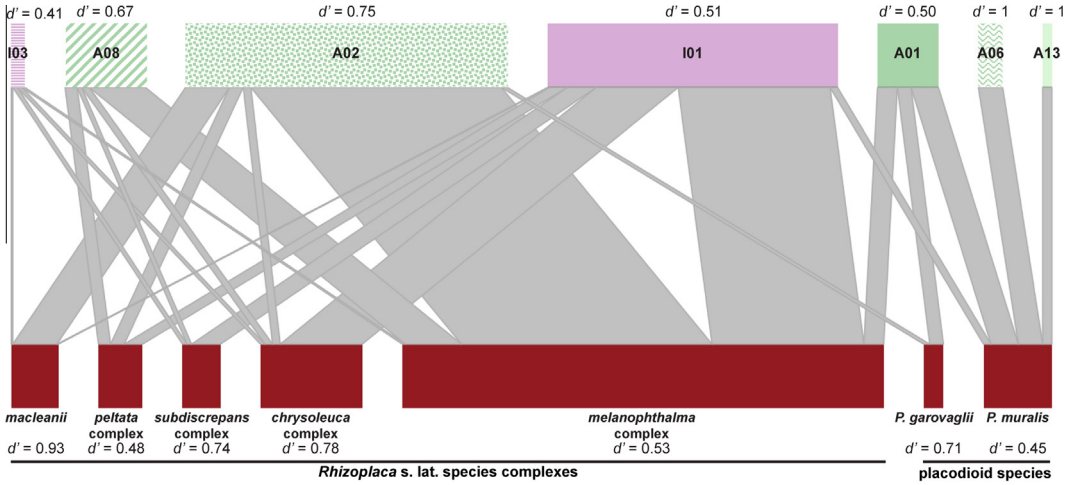


Fig. 6. Interaction network structure between lichen mycobiont species complexes (*P. garovaglii*, *P. muralis*, *P. peltata* s. lat., *R. chrysoleuca* s. lat, *R. macleanii*, *R. melanophthalma* s. lat., and *R. subdiscrepans* s. lat.) with photobiont OTUs. The width of the links is proportional to the number of specimens forming the association, and only photobiont OTUs represented by >5 sequences are shown. Note: *R. maheui* was found to associate exclusively with *Trebouxia* clade 'A03' but is not shown due to the small sample size ($n = 5$). Specificity values (*d'*) for both mycobiont species complexes and *Trebouxia* OTUs are report for each species group. Photobiont OTUs 'A01', 'A02', 'A03', 'A06', 'A08', and 'A13' belong to the *Trebouxia* 'arboricola/gigantea' clade; and OTUs 'I01' and 'I03' belong to the *T.* 'impressa/gelatinosa' group (Leavitt et al., 2015).

Table 2
AIC values from the multinomial logistic regressions of *Trebouxia* OTU occurrences against five different models and the null. Models with AIC values lower than the null model are shown in bold.

Model	AIC
Mycobiont species	2061
Mycobiont species-complex	**1541**
Ecozone	**1705**
Biome	**1639**
Ecoregion	2099
Null	1856

while *P. muralis*, *P. peltata* s. lat., and *R. melanophthalma* s. lat. had the lowest specificity. It is important to note that *R. macleanii* occurs strictly in Antarctica where the pool of available photobionts has been shown to be limited, while mycobiont species with lower specificities are distributed across broader geographic and ecological distributions.

3.6. Photobiont distributions across ecogeographical regions and mycobiont species complexes

AIC values from the multinomial logistic regression analyses showed that the mycobiont species complex, ecozone, and biome play a significant role in determining fungal-algal associations in *Protoparmeliopsis* and *Rhizoplaca* (Table 2).

A comparison of *Trebouxia* OTUs associated with *R. melanophthalma* s. str. in the Palearctic vs. Nearctic revealed a number of symbiont interactions occurring in one region but not the other (Fig. 4B). Specifically, *Trebouxia* OTUs 'I01' and 'I02' associated with *R. melanophthalma* s. str. in the Nearctic but were absent in our samples collected from the Palearctic. Similarly, 'I14' and 'A03' associated with *R. melanophthalma* s. str. in the Palearctic but were not detected in the Nearctic. We found no evidence of reciprocal symbiont specificity in co-occurring mycobiont species in the *R. melanophthalma* complex (Table 3). Three of the four most common *Trebouxia* OTUs associating with species in the *R. melanophthalma* group – 'I01', 'A02', and 'A08' – were distributed across sites along an altitudinal gradient on the Aquarius Plateau in southern Utah, USA, ranging from 2220 to 3400 m (Supplementary Fig. S9). Likewise, identical *Trebouxia* OTUs were found in rock-posy lichens occurring in distinct habitats on the Aquarius Plateau, ranging from arid, exposed pinyon-juniper woodlands to subalpine meadowlands, suggesting ecological specialization is unlikely at the sampled scale.

Trebouxia OTU 'I01' was found to occur across all of the sampled ecoregions (Fig. 4) and was found in association with most candidate *Rhizoplaca* species (Table 1). In contrast, the widespread algal OTU 'A02' was generally not associated with the *R. chrysoleuca* and *R. subdiscrepans* species complexes; these two mycobiont complexes comprise a well-supported monophyletic clade (Fig. 3). The *R. subdiscrepans* complex was largely sampled from ecoregions where *Trebouxia* OTU 'A02' was absent (Pontic steppe and Central European mixed forests) or recovered at low frequencies (Kazakh steppe). In contrast, samples from the *R. chrysoleuca* complex originated from a mixture of ecoregions with low or no 'A02' samples (Kazakh steppe; Pontic Steppe), as well as ecoregions where 'A02' was found at high frequencies with other *Rhizoplaca* species complexes sampled (Wasatch and Uinta montane forests, Colorado Rockies forests, Great Basin shrub steppe, among others). *Protoparmeliopsis muralis* was frequently sampled from Central European mixed forests, an ecoregion in which *Trebouxia* OTU 'A02' was not recovered, although *P. muralis* specimens from western North America occurred in ecoregions where 'A02' was found to associate with *Rhizoplaca* species.

Table 3
Local patterns of photobiont sharing among co-occurring mycobiont species in the *R. melanophthalma* complex at four separate sites in western North America. Columns represent *Trebouxia* OTUs, and numbers indicate the proportion of photobiont OTUs associated with the specified mycobiont ('–' indicates the photobiont OTU was not sampled with the specified mycobiont host).

	A02	A08	I01
Birch Creek, ID, USA: collection #8935 (F)			
R. haydenii (2)	1	–	–
R. polymorpha (12)	0.83	0.08	0.08
R. porteri (9)	0.67	0.22	0.11
Mosquito Creek, NV, USA: collection #8668 (F)			
R. melanophthalma (5)	1	–	–
R. polymorpha (7)	1	–	–
R. porteri (11)	0.91	–	0.09
Barley Creek, NV, USA: collection #8665 (F)			
R. melanophthalma (4)	0.5	–	0.5
R. parilis (1)	–	–	1
R. polymorpha (3)	0.33	0.33	0.33
R. porteri (13)	0.46	0.08	0.46
Nutter's Ridge, UT, USA: collection #8663 (F)			
R. melanophthalma (2)	0.5	–	0.5
R. polymorpha (2)	0.5	–	0.5
R. porteri (16)	0.63	–	0.37

4. Discussion

Understanding key factors that influence species interactions is a major focus of research in symbiotic systems. While lichens represent iconic models of symbiosis and can play an essential role in understanding the biology of symbiotic interactions (Arnold et al., 2009; Peksa and Škaloud, 2011; Wang et al., 2014), current taxonomic approaches for recognizing diversity in lichen symbionts commonly fail to accurately reflect actual species diversity (Lumbsch and Leavitt, 2011; Sadowska-Deś et al., 2014). In this study, we demonstrate that previously unrecognized species-level lineages are prevalent in a group of well-known lichens that occur in arid habitats throughout the world. By delimiting candidate species-level lineages in both the myco- and photobionts in rock-posy lichens, we were able to elucidate detailed patterns in symbiont interactions in these lichens. Here we also provide the most complete phylogeny to-date for the mycobiont genus *Rhizoplaca* and other closely related taxa.

4.1. Cryptic diversity in lichen symbionts

Previous studies have revealed cryptic species-level diversity in the mycobiont *R. melanophthalma* s. lat. (Leavitt et al., 2011, 2013a), and in this study we show that cryptic species-level lineages are common in a number of other *Rhizoplaca* s. lat. species with cosmopolitan distributions. In fact, we circumscribed a total of 14 candidate species in the three nominal taxa – *P. peltata*, *R. chrysoleuca*, and *R. subdiscrepans* (Table 1). Six of the 14 candidate species appear to have broad, intercontinental distributions, seven are restricted to Central Asia and one occurs exclusively in western North America, based on current sampling. However, more complete sampling will be required to confirm these distribution patterns, especially in the relatively sparsely sampled *P. peltata* and *R. subdiscrepans* groups. In addition to previously unrecognized species-level lineages circumscribed in nominal *Rhizoplaca* s. lat. species, we also sampled a number of *Protoparmeliopsis* and *Rhizoplaca* specimens that did not fit within any of the currently described species and these were recovered as distinct lineages within the *Rhizoplaca* s. lat. phylogeny (Fig. 3).

All of the *Trebouxia* OTUs associated with *Protoparmeliopsis* and *Rhizoplaca* mycobiont species appear to belong to species-level

lineages lacking formal taxonomic recognition. Twenty of the 24 described *Trebouxia* species are represented by ITS sequences in GenBank generated from UTEX (http://www.utex.org) and SAG (http://www.uni-goettingen.de/en/184982.html) cultures (Leavitt et al., 2015). However, none of the *Trebouxia* OTUs associating with *Protoparmeliopsis* and *Rhizoplaca* species include sequences generated from these important reference cultures. In spite of the fact that ITS sequence data has been used to investigate *Trebouxia* diversity for over twenty years (Beck et al., 1998; Bhattacharya et al., 1996), incorporating information from phylogenetic studies into a working taxonomy for *Trebouxia* has not yet been developed. Studies involving *Trebouxia* photobionts continue to document previously unrecognized phylogenetic lineages, requiring the application of informal or provisional species-level names that may be applied to vastly different phylogenetic levels across studies (Kroken and Taylor, 2000; Leavitt et al., 2013c; Muggia et al., 2014; Nyati et al., 2014). A provisional naming system recently proposed for *Trebouxia* species-level OTUs (Leavitt et al., 2015) provides a useful starting point for more consistent recognition of diversity in this important algal genus.

4.2. Symbiont interactions in rock-posy lichens

In this study we found no evidence of reciprocal symbiont specificity in closely related mycobiont species groups – e.g., species in the *P. peltata*, *R. chrysoleuca*, *R. melanophthalma*, and *R. subdiscrepans* species complexes (Table 1). Similarly, reciprocal symbiont specificity was not observed among co-occurring *Rhizoplaca* species in rock-posy communities in western North America (Table 3). While reciprocal one-to-one specificity does not appear to play a major role in diversification in closely related *Rhizoplaca* species complexes, placodioid *Protoparmeliopsis* species were shown to associate with a suite of *Trebouxia* OTUs largely distinct from those associating with *Rhizoplaca* species (Fig. 5). It is generally accepted that lichen mycobionts have a higher degree of specificity towards photosynthetic partners than the photobionts do towards their mycobiont hosts (Beck et al., 2002; Otálora et al., 2010). Interestingly, it is generally noted that higher levels of specificity are commonly observed for many mycobiont lineages, relative to their photobionts, when compared to specificity among rock-posy lichen symbionts (Figs. 4 and 5).

Two *Rhizoplaca* species with geographically and ecologically restricted distributions, *R. macleanii* (Antarctic endemic) and *R. maheui* (central Spain), also have the highest specificity of all sampled mycobiont taxa (Fig. 6). The relatively high specificity of *R. macleanii* and apparent opportunistic associations with available photobionts in Antarctica suggest that the high specificity observed here may be due to limited pool of available photobionts (Pérez-Ortega et al., 2012). Alternatively, symbionts isolated to extreme habitats may have higher photobiont specificity due to the fact that they have co-evolved complementary adaptations to the extreme habitat conditions. Future research aimed at characterizing the overall diversity of *Trebouxia* photobionts at specific sites/regions will help elucidate whether specificity is driven by active selection of the best suited photobiont(s) from a diverse pool of potential partners or more simply by the availability of appropriate photobionts.

Some *Trebouxia* OTUs ('A01', 'A06', 'I01', 'I03') associating with *Rhizoplaca*/*Protoparmeliopsis* taxa were previously found to associate with a number of co-occurring mycobiont species in genera from Parmeliaceae, including *Melanohalea* and *Xanthoparmelia* (Leavitt et al., 2015). In contrast, others ('A02', 'A08', 'A13') were entirely or largely absent from associations with these two parmelioid fungal genera (Leavitt et al., 2015). Our data do not provide insight into the reason for general abundance of *Trebouxia* OTUS 'A02' and 'A08' in *Rhizoplaca* species and absence in some other co-occurring lichens; and similarly, why another common photobiont OTU associating with *Rhizoplaca* species, 'I01', also associates with a broad range of other co-occurring mycobiont genera.

A number of lichen mycobiont genera in the family Parmeliaceae have also recently been found to associate with distinct suites of *Trebouxia* OTUs (Leavitt et al., 2015). However, relative to a number of genera from the lichen-forming fungal family Parmeliaceae, *Protoparmeliopsis* and *Rhizoplaca* mycobiont species associate with a relatively narrow range of *Trebouxia* species (Fig. 5 and Leavitt et al., 2015). The origin of Parmeliaceae roughly coincides with the most recent common ancestor (MRCA) of the *Protoparmeliopsis* and *Rhizoplaca* clades, and many clade ages in the *Rhizoplaca* phylogeny are comparable to generic groups in Parmeliaceae (Fig. 3 and Divakar et al., 2015). In spite of similar ages, lineages in Parmeliaceae generally appear to associate with a much broader ranges of photobiont OTUs. For example, ecologically most of the *Rhizoplaca* species sampled for this study commonly co-occur with the species from the foliose mycobiont genus *Xanthoparmelia* (Parmeliaceae); yet co-occurring *Xanthoparmelia* species associate with at least three times the number of *Trebouxia* OTUs than *Rhizoplaca* mycobionts (Leavitt et al., 2015).

Most *Trebouxia* OTUs from this study have broad geographic and ecological distributions (Supplementary Table S1; see also Leavitt et al., 2015); however, fine-scale availability of most lichen photobionts has not been studied in depth. In this study no clear ecological pattern of availability of *Trebouxia* OTUs was observed across a localized altitudinal transect in the western USA, with most of the photobiont OTUs occurring across sites ranging from arid sage-steppe habitat to subalpine meadowlands (Fig. 6). Identifying and sampling at appropriate ecological scales remains a common challenge to ecological research (Jackson and Fahrig, 2014). Ecological differences at a microhabitat scale are central to determining fine-scale distribution patterns of most lichens (Bergamini et al., 2007; Nadyeina et al., 2014; Peck et al., 2004; Ranius et al., 2008). Furthermore, detailed examinations of the distribution of lichen photobionts across microhabitat scales will likely provide a more nuanced perspective into patterns driving interactions in lichen symbioses (e.g., Nadyeina et al., 2014; Werth and Sork, 2014).

4.3. Phylogenetic relationships among Rhizoplaca species

Here we provide the most robust phylogeny to-date for *Protoparmeliopsis* and *Rhizoplaca*. We place the diversification of *Rhizoplaca* within a temporal context, estimating the split between the *Protoparmeliopsis* and *Rhizoplaca* clades in the Late Cretaceous (c. 76 Ma, 95% HPD: 57.4–97.9). Initial diversification of extant lineages within each *Rhizoplaca* species complex was estimated to have begun in the Miocene, with diversification in the *R. chrysoleuca* and *R. subdiscrepans* groups beginning c. 20 Ma, while the initial diversification within the *R. melanophthalma* and *P. peltata* groups began more recently, ca. 10 Ma.

The Neogene has also been shown to be an important period for diversification in the hyper-diverse lichen-forming family Parmeliaceae (Amo de Paz et al., 2011; Divakar et al., 2015; Kraichak et al., 2015; Leavitt et al., 2012a, 2012b, 2012c). The Miocene was a time of major climatic and vegetative changes worldwide, especially in the northern Hemisphere, including major tectonic activity and orogeny (Zachos et al., 2001). Increasing aridity in the middle Miocene (15–8 Ma; Axelrod, 1979; Dunai et al., 2005) resulted in woodlands giving way to more open habitats and grasslands (Jacobs et al., 1999; Pagani et al., 1999; Zachos et al., 2001). It is reasonable to expect that diversification in *Rhizoplaca* would coincide with these major climatic and ecological shifts towards increased aridity and more open habitats.

Consistent with previous studies (Arup and Grube, 2000; Zhao et al., 2016), our phylogenetic reconstructions recovered *Rhizoplaca* s. lat. as polyphyletic, with the *P. peltata* group recovered in the *Protoparmeliopsis* clade (Fig. 3). The most recent common ancestors (MRCA) for the *P. peltata* (c. 10.4 Ma), *R. chrysoleuca* (c. 21.9 Ma), *R. melanophthalma* (c. 10.9 Ma), and *R. subdiscrepans* (c. 18.4 Ma) complexes are similar to the age of the MRCA of many genus-level lineages in *Parmeliaceae* (Amo de Paz et al., 2011). The MRCA of the *Rhizoplaca* clade was estimated at c. 62.5 Ma; 95% HPD: 44.0–75.2 Ma), similar in age to the diverse parmelioid core group in Parmeliaceae (Amo de Paz et al., 2011). Generic circumscriptions in the Lecanoraceae are in need of critical revisions with the core genus *Lecanora* being polyphyletic and several genera being nested within *Lecanora* (Zhao et al., 2016). The results of our study provide a robust phylogenetic framework for genus-level taxonomic revisions in *Rhizoplaca* s. lat.

4.4. Conclusions

Our study provides important perspectives into species diversity in iconic lichen symbiotic systems and establishes a valuable framework for continuing research into rock-posy lichens. While our study indicates high levels of cryptic diversity in the umbilicate mycobiont genus *Rhizoplaca* s. lat., diversity in closely related placodioid growth forms remains underexplored. While we are able to better characterize mycobiont species diversity using multi-locus sequence data, specific factors driving diversification in rock-posy lichen mycobionts remain elusive (Leavitt et al., 2013b).

Species diversity in the common lichen photobiont *Trebouxia* remains poorly characterized from a taxonomic perspective. This taxonomic gap is a major impediment to understanding biodiversity, evolution, adaptations, physiology, and ultimately symbiotic interactions. Furthermore, understanding distribution patterns and fine-scale availability of *Trebouxia* photobiont OTUs will be central to finally understanding the nuanced patterns in selectivity and specificity in lichen symbioses. Some photobiont lineages have clear preferences for environmental factors (Peksa and Škaloud, 2011); however, this preference may occur below the species-level. Intraspecific (or intra-OTU) variation in physiological performance or other selective pressures may confound interpretations of symbiont interactions when photobiont diversity is characterized using OTUs. Additional research will be required to characterize the physiological differences among and within *Trebouxia* OTUs and how these differences may relate to patterns of symbiont interactions in lichens.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.03.030.

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