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**Comparing *Trebouxia* diversity in lichen genera sympatric
with the *Niebla* species complex.**

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Abstract

Lichens are one of the most successful mutualisms on Earth. This symbiosis comprises a fungus (mycobiont) and a cyanobacterium or eukaryotic alga (photobiont), which forms a lichen (holobiont). Some lichenized fungi are generalists, potentially forming a lichen with multiple photobiont species. Others are specialists, only associating with a single or a few photobionts. The ecology and distribution of lichen symbionts likely influences their association and genetic structure. Two sister genera of lichenized fungi *Niebla* and *Vermilicinia* are endemic to the fog deserts on the west coast of North America. Species of *Trebouxia*, a genus of green algae found worldwide, are the associated photobiont partners to the *Niebla/Vermilicinia* species complex. Recent work demonstrated that *Niebla* and *Vermilicinia* are highly specific for only a few closely related *Trebouxia* molecular species (OTUs). This project examines the diversity and broad specificity of algae with lichen forming fungal genera that are sympatric to *Niebla* and *Vermilicinia* to understand if the narrow associations seen previously in this species complex is due to limited photobiont availability. We hypothesize that sympatric lichen genera will have greater diversity of *Trebouxia* than is present in *Niebla/Vermilicinia*. ITS rDNA sequence data were collected from over 100 specimens of *Niebla/Vermilicinia* and sympatric lichens collected from a region of California. Previously obtained *Niebla/Vermilicinia* sequences from Morais and Muscavitch (unpublished) and published algal sequences from *Ramalina* were also analyzed. A total of 15 *Trebouxia* OTUs were found in the lichens examined, four of which are phylogenetically distinct and potentially novel species. In contrast, only four very closely related *Trebouxia* OTUs were found with *Niebla/Vermilicinia*. These results support our hypothesis that diverse photobionts are readily available in the environment and that the *Niebla* species complex, and sympatric fungal genera, may be selective in their photobiont partners.

Introduction

Symbiosis has indisputably been crucial for the success of many organisms throughout evolutionary history. Take, for example, the endosymbiotic origin of mitochondria and chloroplasts that promoted diversification of eukaryotes (Martin et. al., 2015), or the nearly universal symbiosis of plants and mycorrhizal fungi. The mutualism between lichenized fungi and photosynthesizing green or brown algae and/or cyanobacteria forms lichens. Lichens are found on every continent and represent one of the most successful examples of mutualistic symbiosis on Earth (Honegger, 2019), making lichens especially useful as a model system to study mutualism (Peska and Skaloud, 2011). Lichens are given the name of their primary fungal partner which comprise more than 95% of their biomass and house the photobionts among its hyphae and depends on their photosynthates to survive (Ahmadjian, 1993).

Our current knowledge of photobiont distribution and diversity has been impeded by the historical focus on the mycobiont. Lichen mycobionts are more frequently documented than photobionts because 1) they are larger and immediately observable by the collector, and 2) photobionts are difficult to identify morphologically because many species diagnoses require culturing. About 19,000 lichenized fungal species have been described, however they are still understudied. The less obvious, photobionts are even more unknown with only about 200 described species (Muggia et. al., 2018), highlighting the need to further investigate photobiont diversity. *Trebouxia* (Trebouxiophyceae, Chlorophyta) is a common lichen photobiont found in association with over 20% of lichen forming fungi around the world and 80% of all temperate lichens (Rambold et. al., 1998). This genus is well suited to a symbiotic lifestyle and is rarely found outside of lichens (Bubrick et al., 1984). Muggia et. al. (2020) examined the phylogenetic diversity of *Trebouxia* across diverse lichens. They ultimately recommended recognition of 113 -

119 species forming 4 major clades, a result that is stark in contrast to the current number (as of June 2022) of 29 described species (Guiry and Guiry, 2017). Their work suggested a much higher *Trebouxia* diversity than previously known and emphasized the need for further work describing this genus and its lichen associations. Also, a greater knowledge of the photobiont diversity across lichen genera will allow for the symbiotic interaction to be better understood overall.

Although the mycobiont depends on the photosynthates produced by the photobionts to survive (Ahmadjian, 1993), photobionts can live independently from the fungus. Some mycobionts form strong associations with their algal partners while others are more plastic in their associations (Beck et. al, 1998). Lichenized fungal species that can symbiose with multiple phylogenetically distant photobiont species are typically regarded as generalists. Others are considered specialists, forming lichen with only one or a few closely related photobionts. Besides the degree of specificity, symbiont associations among lichen may be influenced by the geographic distribution of the partners and their ecological preferences.

The distribution of symbionts is a key factor affecting two symbionts ability to interact. Two taxa cannot form a lichen if they do not occur in the same geographic area. Despite the sympatry of two symbionts, their association may be shaped by many other factors (Beck et. al., 2002).

Substrate preference can be an important determinant of lichenization between two symbionts. Different taxa are optimized to different substrates, i.e., rock, bark, soil. If the substrate preference between photobiont and mycobiont is different, they are unlikely to come into contact and lichenize, whereas two taxa sharing a substrate preference are more likely occur in the same location and associate.

The *Niebla/Vermilicinia* species complex is a group of foliose lichens endemic to the west coast of North America from Canada to Baja California. They occur only in fog deserts where most of the water necessary to survive is supplied by fog drip. These lichens' primary photobiont partners are species of *Trebouxia*. In *Niebla* and *Vermilicinia* (Ramalinaceae), symbiont associations in some species are strongly influenced by substrate preference (Morais and Muscavitch., unpublished). Rock adapted and bark adapted species select distinct photobionts. The factors influencing symbiont associations can even differ within a genus. Previous work has shown that within *Ramalina*, photobiont selection patterns differ from species to species based on separate factors (Werth and Sork, 2010; Molins et. al., 2021).

The lichen genera *Ramalina*, *Hypogymnia*, and *Usnea* are sympatric to the *Niebla/Vermilicinia* species complex. These sympatric genera also associate primarily with photobionts from *Trebouxia*. A test of the *Trebouxia* diversity in lichen genera that are sympatric with *Niebla/Vermilicinia* will help us understand the factors influencing their symbiosis. Specifically, we aim to answer the question of whether *Niebla/Vermilicinia* is limited in their availability of photobionts. *Niebla/Vermilicina* has a narrow geographic range and is restricted in habitat while the sympatric lichen genera all have wider geographies and occur in more diverse habitat types therefore, I hypothesize that *Niebla/Vermilicinia* will be narrower in their photobiont associations than the sympatric species because of their limited range and unique habitat preference. The molecular barcode ITS, a highly conserved region of DNA, will be utilized to identify previously delimited and potentially novel *Trebouxia* operational taxonomic units (OTUs) among the sampled lichens.

Methods

Sample Collection and Mycobiont Identification

Collection of the lichens was done by Zachary Muscavitch from multiple sites along the California coast in the United States. The study genera *Hypogymnia*, *Usnea*, and *Ramalina*, were collected from areas sympatric with the *Niebla/Vermilicinia* species complex. Entire lichens or pieces of lichens were carefully removed from their substrate and placed into paper packets.

In the lab, A series of dichotomous keys were used to morphologically identify the mycobionts to the level of genus (McCune and Geiser, 1997; Brodo et al., 2001; Sharnoff, 2014). Full locality data and specimen identifications are shown in Appendix 1.

DNA Extraction

Pieces of lichen thalli (~2.5cm x 1.25cm) were excised from previously identified, larger samples. The thallus fragments were then submerged in acetone for 5 minutes as part of a TLC analysis for another study.

Mechanical disruption of the fungal and algal cell walls was necessary to release the DNA. This was achieved by using an OPS Diagnostics MiniG High Output Homogenizer at 2400 hz for 2 min.

A 750 μ l volume of CTAB-based DNA extraction buffer (Doyle and Doyle, 1987) was added to each tube containing powdered thallus. Next 500 μ l of a mixture of 24:1 chloroform/isoamyl alcohol was added to the tubes and homogenized. Then, the tubes were centrifuged at max rpm for 15 min. When removing the tubes from the centrifuge we were careful not to disrupt the aqueous and organic layers. We then carefully pipetted the aqueous layer or supernatant into a

new conical bottomed microcentrifuge tube. The previous two steps were repeated once. A DNA precipitate was formed by adding 0.7 volumes of cold isopropanol to the supernatant containing tubes. After chilling the samples for 20 min, we centrifuged them again to form a pellet. The pellet was then washed two times with ethanol and left upside down on a paper towel to dry for 10 min and finally resuspended in 200 μ l of 1x TE buffer.

PCR and Confirmation of Amplification

We used a modified version of the Cold Spring Harbor Basic PCR protocol (Green and Saybrook, 2018). The 25 μ l PCR reaction used the following components: GoTaq2x – 12.5 μ l, forward and reverse primers – 0.7 μ l each, template DNA – 1 μ l, water – 10.1 μ l. The primers used to target the algal ITS rDNA genes were (forward) ITS1T (White et al., 1990), and (reverse) ITS4 (Kroken and Taylor, 2000). The PCR conditions were as follows: a hot start at 95° C for 2 min, denaturation at 95° C for 30 sec, annealing at 52° C for 30 sec, extension at 72° C for 30 sec. Denaturation, annealing, and extension were cycled 35 times, with a final extension at 72° C for 10 min.

Confirmation of amplification was done by running 3 μ l of each PCR product on a 1% Agarose TBE gel stained with 2 μ l of SYBR Safe. After gel electrophoresis was complete, we checked for a presence of a band with a transilluminator.

ExoSAP Cleanup Protocol and Sequencing

The ExoSAP master mix recipe for a single 25 μ l reaction was: water – 8.27 μ l, exonuclease 1 (Exo1) – 0.09 μ l, shrimp alkaline phosphatase (SAP) – 0.44 μ l, PCR product – 22 μ l. The master mix was homogenized with a vortexer, and briefly centrifuged. The EXOSAP reaction conditions include; Enzyme activation at 37° C for 50 min, Enzyme inactivation at 95° C for 10

min, and hold at 12° C. The cleaned algal ITS were sequenced commercially (Eurofins Genomics, LLC, Louisville, KY USA).

Alignment, Tree building and Bipartite Analysis

Raw sequences from Eurofins were examined in Geneious Prime (Biomatters Limited). As needed, bases at the 5' and 3' ends were trimmed to remove poorly sequenced regions. Ambiguously scored internal bases were also examined and verified. Fully edited individual sequences were submitted to NCBI BLAST (Altschul et al., 1990) to determine closest similarity to published algal sequences. An alignment of these sequences combined with data from (Muggia et. al., 2020) and (Werth and Sork, 2010) was prepared using the MAFFT online server (<https://mafft.cbrc.jp/alignment/server/>). The IQ-tree web server (<http://iqtree.cibiv.univie.ac.at/>) was used to build trees from the alignment. The tree was then visualized and edited in FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>). The photobiont clades identified in the phylogenetic tree were paired with their respective mycobiont partners and adapted into a bipartite analysis using R studio. A map generated from the locality data showing the distribution the collected samples was created using Simple Mappr (<https://www.simplemappr.net/>).

Results

***Trebouxia* diversity spans 4 major clades**

Using the naming convention of *Trebouxia* clades proposed by Leavitt et al. (2015) and currently defined in Muggia et al. (2020) as a framework, it was determined that the nuclear ITS sequences from the present study, along with those from Morais and Muscavitch (unpublished) and Werth and Sork (2010), could be grouped into 15 distinct subclades among 4 major clades (Fig. 1, Fig. S1), A03, A05, A13, A19, A39, A40, A46, A48, New A1, New A2, New A3, New C1, C09, I08, and S02. We found four phylogenetically distinct, potentially novel, clades - New A1, New A2, New A3, and New C1- that were previously not delimited in Muggia et al. (2020).

***Niebla* and *Vermilicinia* associate with a small set of *Trebouxia* photobionts**

The bipartite analysis in Fig. 2 illustrates, as shown previously (Morais and Muscavitch, unpublished), that *Niebla/Vermilicinia* is associated with a small subset of photobionts from only one major clade of *Trebouxia*. Specifically, *Niebla/Vermilicinia* associated with five *Trebouxia* subclades New A1, New A2, A13, A46, and A48 of major clade A. In contrast, *Ramalina* associated with seven *Trebouxia* subclades, New A1, New A2, New A3, A05, A39, and A40 of major clade A, and New C1 of major clade C. *Usnea* associated with 5 subclades, A05 and A19 from major clade A, New C1, and C09 from major clade C, and I08 from major clade I. The single sample from *Hypogymnia* associated with subclade S02 of major clade S. The genus *Usnea* was found to have the highest photobiont diversity with 5 subclades among 3 major clades. *Ramalina* had the next highest photobiont diversity with a total of 7 *Trebouxia* subclades among 2 major clades. *Niebla/Vermilicinia* had the lowest photobiont diversity, with 5 subclades

among only 1 major clade, A. An accurate analysis of the photobiont diversity in *Hypogymnia* was incomplete due to the lack of usable ITS rDNA sequences obtained from the samples.

Photobiont geography and diversity

As shown in the map illustrating photobiont distributions (Figure 3) all of the *Trebouxia* subclades with the exception of I08 and New A2 had a coastal distribution. *Trebouxia* clade New A2 had a primarily inland range, with only 3 of 138 samples being found along the coast. The coastally distributed samples from New A2 were found among *Niebla/Vermilicinia*, whereas samples distributed inland were associated with *Ramalina*. The inland sample of I08 was from *Usnea*.

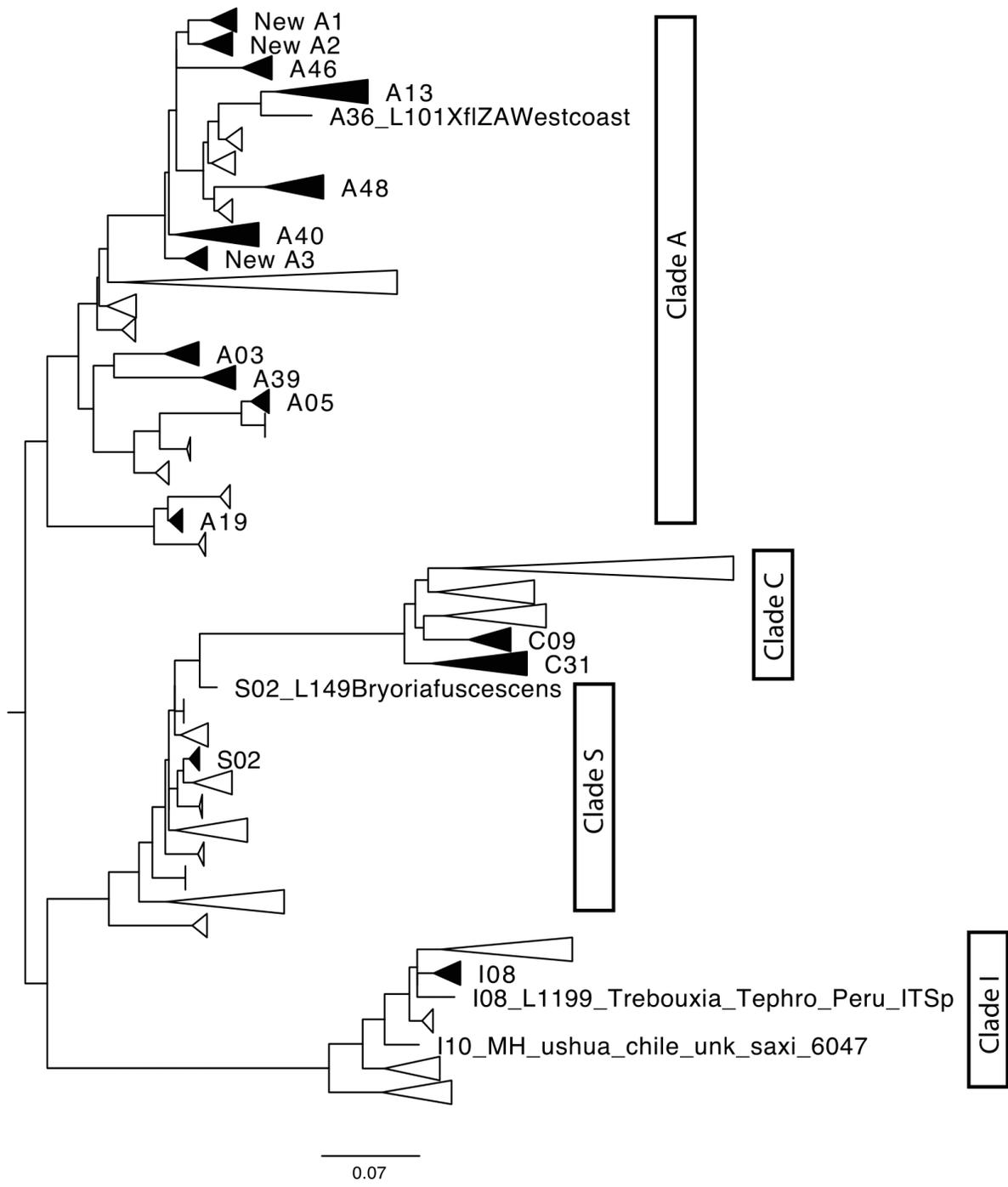


Figure 1. Phylogenetic tree of *Ramalina*, *Usena*, *Hypogymnia*, and *Niebla/Vermilicinia* photobiont nuclear ITS sequences. The sequences collected in this study were aligned with the sequences from Muscavitch, (unpublished), Werth and Sork (2010), and Muggia et al. (2020). The sequences from (Muggia et al., 2020) act as a phylogenetic framework on which the clades were named. The major clades: A, C, I, and S are indicated, and clades represented in the data of this study are shown in filled triangles.

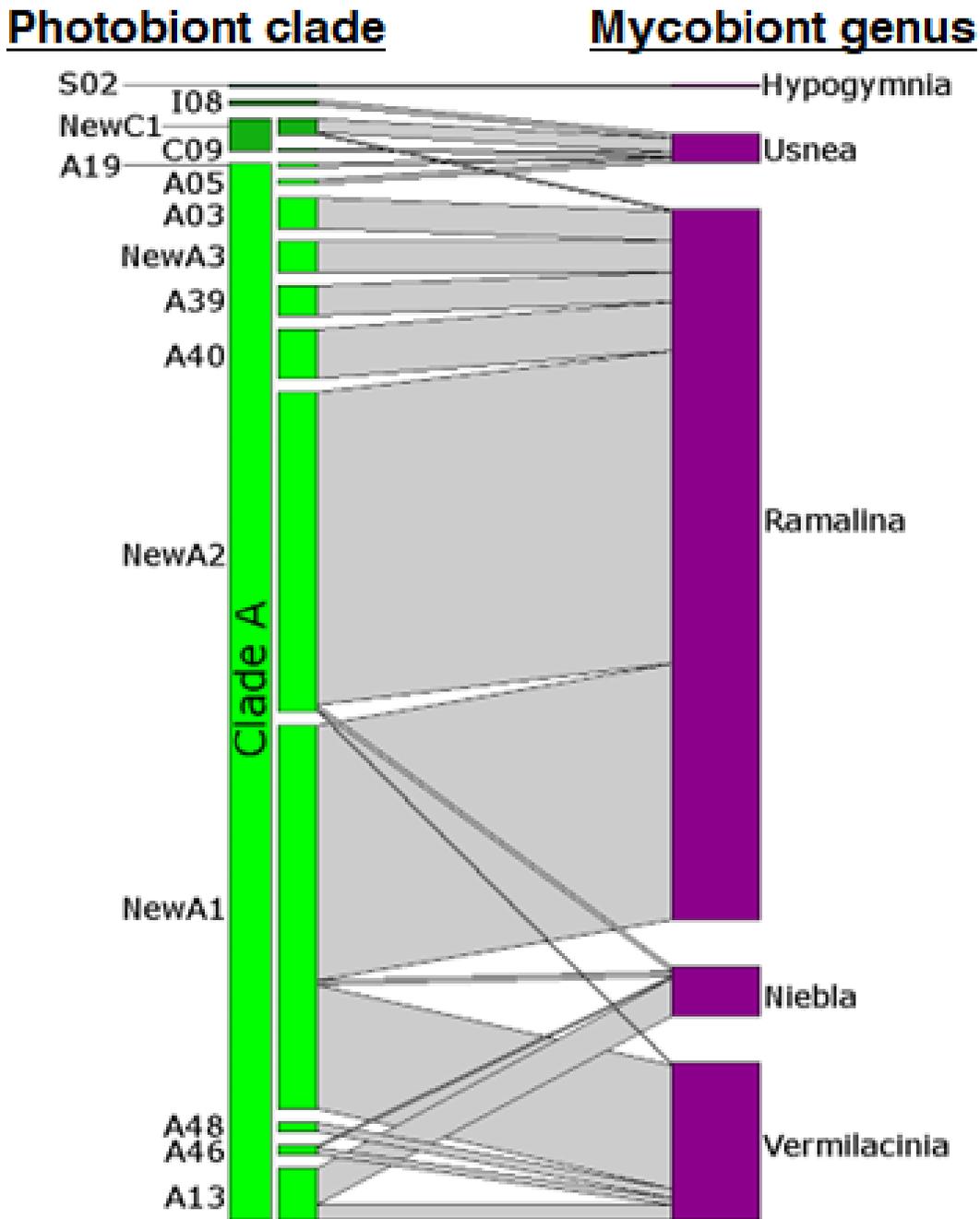


Fig. 2. Bipartite association network showing mycobiont genera (named, purple, boxes at the right side of the graph) and their associated photobiont clade (named, green, boxes at left). The major clades correspond to those defined by Muggia et al. (2020) and in Supplementary Figure 1. NewA1, A2, A3, and C1 were not observed in Muggia et al. (2020). *Niebla/Vermilacinia* associates with 5 different *Trebouxia* subclades of clade A. *Ramalina* associates with 7 *Trebouxia* subclades, 6 of which are from clade A and one from clade C. *Usnea* associates with 5 subclades, 2 from clade A, 2 from clade C and 1 from clade I. The single sample from *Hypogymnia* associates with 1 subclade of clade S.

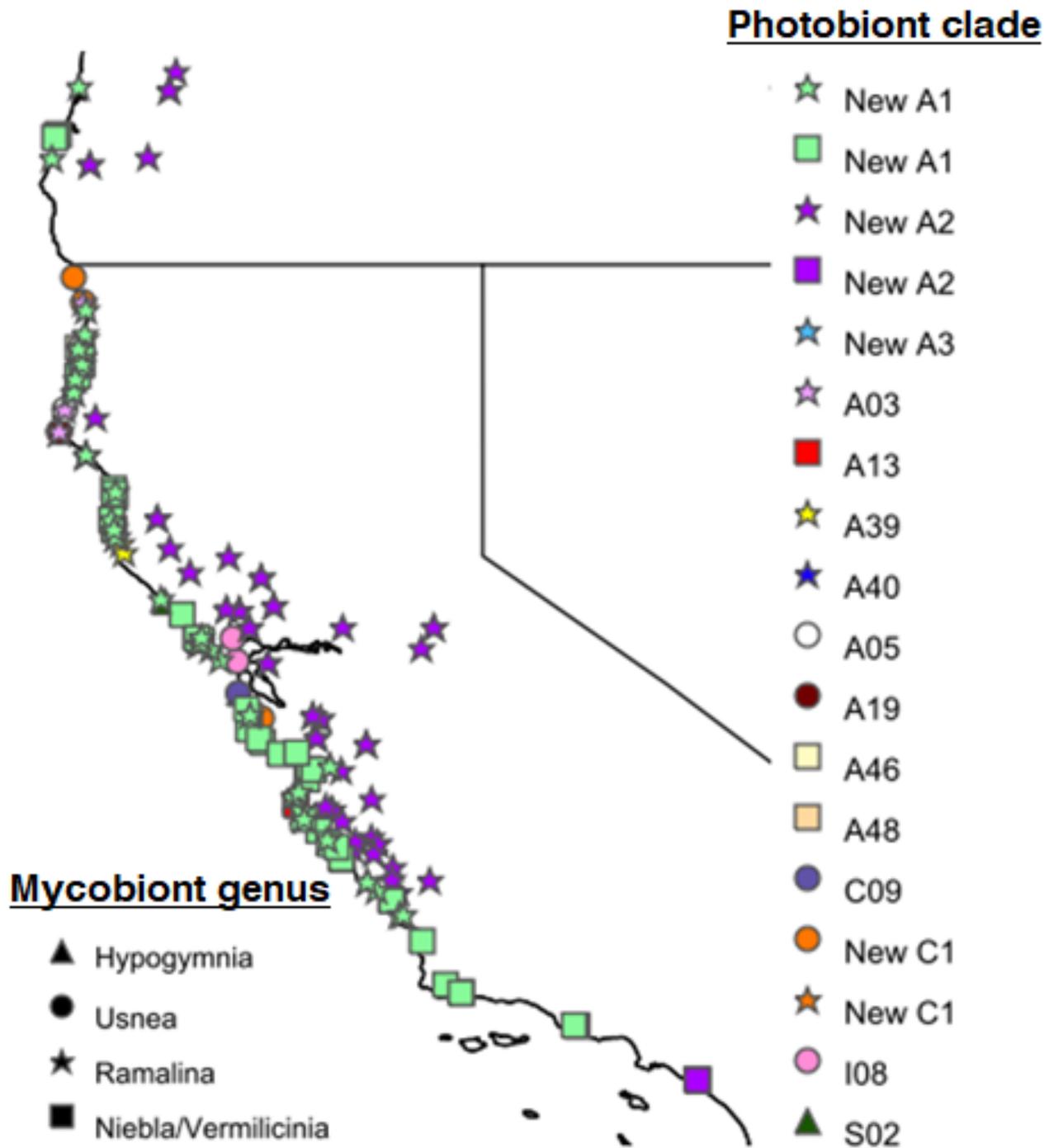


Fig. 3. Geographic distribution of sampled lichens and their photobionts in California and Oregon. Symbols are colored to represent the photobiont subclade, and the symbol shapes represent the mycobiont genera. Two photobiont clades, New A2 and I08, are distributed inland while all other clades have a mainly coastal distribution.

Discussion

The results of our study indicate that the phylogenetic diversity of photobionts in *Niebla/Vermilicinia* was lower in comparison to that of the sympatric lichen genera. Only one major *Trebouxia* clade, A, was found in *Niebla/Vermilicinia*. The sympatric genus *Usnea*, on the other hand, associated with three divergent clades: A, C, and I. Additionally *Ramalina* associated with the two major *Trebouxia* clades A and C. The *Hypogmnia* samples did not produce enough usable ITS rDNA sequences for a full comparison, so interpretation is difficult, however the sample still allowed for a more complete assessments of the photobionts sympatric to *Niebla*. If we define lichen photobiont diversity in this case as the number of unique major clades, the order of most diverse to least diverse in our study lichen genera is as follows: *Usnea*, *Ramalina*, *Niebla/Vermilicinia*. This supports the hypothesis that *Niebla/Vermilicinia* has a lower photobiont diversity than the sympatric genera.

The observed patterns of photobiont diversity among lichen genera may be influenced by many factors. The geographic distribution of *Trebouxia* photobionts was found to be a determinant factor of the photobiont diversity found in the sampled lichens. *Niebla* and *Vermilicinia*, being endemic to the coastal regions of western North America are geographically limited in their otherwise potentially compatible algal partners. Clades New A2, and I08 are primarily located inland, away from the coastal regions occupied by *Niebla/Vermilicinia*. I08 was not found in any *Niebla/Vermilicinia* samples, however clade New A2 was discovered in three samples of *Niebla/Vermilicinia* found in fog deserts suggesting that *Niebla/Vermilicinia* could potentially associate with a higher proportion of photobionts from this clade, if their ranges had more overlap, as is seen in *Ramalina* (Figure. 2). Photobiont clade I08 was not found in fog deserts, where *Niebla/Vermilicinia* occur, indicating that low geographic availability may be the reason

this clade is absent from *Niebla/Vermilicinia*. The remainder of the clades found among the samples were in fact found along the coastline in fog deserts suggesting that additional factors beyond algal distribution influence the ability of *Niebla/Vermilicinia* to associate with those photobionts.

In other studies photobiont associations were influenced by mycobiont substrate preference. Morais and Muscavitch (unpublished) found that *Vermilicinia* species adapted to rock and species adapted to bark were associated with two distinct sets of photobionts. Our analysis only considered mycobiont identities to genus. Given the variation of substrate preference among species within a genus, it is impossible to group one genus into a single substrate preference. Considering that substrate preference has been previously identified as an important influence on photobiont selection in *Vermilicinia*, it is probable that *Vermilicinia* photobiont diversity in our samples is also influenced by this factor.

Lichen thallus growth stage has been found to influence the photobiont diversity in *Ramalina faranacia*. Young, newly emerging thalli can contain a higher photobiont diversity than mature thalli (Mollins et. al. 2021). It is unclear whether all the collectors whose data were included in this study considered thallus age and excision location when collecting and preparing samples to extract, which may have affected the photobionts found. Since mycobionts in our analysis were not identified to the species level, it is unknown whether this factor is important to our samples. Even within a genus, the factors influencing association can be varied. Association of *Ramalina menzeisii* and their *Trebouxia* photobiont is not influenced by geography or thallus growth stage, but substrate based on phorophyte (epiphytic host) species on which the alga is adapted (Werth and Sork, 2010). They found that even across a large geographic range, lichens on three different oak species showed distinct patterns of photobiont selection based on the phorophyte.

Another potentially important factor in photobiont selection that may have been overlooked by our focus on the generic level is reproductive mode. Both asexual (vertical) and sexual (horizontal) reproduction occurs in lichens, and some species do both while others favor one method. In asexually reproducing lichens the photobiont is vertically transmitted to the next generation via vegetative propagation. After lichens undergo sexual reproduction, the resulting spore must germinate and acquire a new photobiont that may be different from the photobiont of the previous generation. Sexually reproducing lichens have been shown to be more flexible in their photobiont associations than asexually reproducing lichen (Werth and Scheidegger, 2012).

Photobiont selection in some lichens has been found to change with altitude. Altitude can have a major influence on biome. On a single mountain, different biomes may occur at different altitudes and the transition between two can be quite extreme. In a study of the lichenized fungal genus *Lasallia*, photobiont selection in was found to change along an altitudinal gradient with different algal OTUs being detected at specific altitudes within different biomes (Dal Grande et. al., 2018). OTU 5 and OTU 4 were the only photobionts detected in the boreal zone above 2000 km. In the warm temperate zone, below 900 km, only OTU 2 and OTU 3 were found. Altitude can change many abiotic factors such as temperature and humidity, these ‘secondary’ factors likely influence the photobionts’ ability to survive in a certain environment.

Many factors can influence photobiont associations in lichens and may vary from species to species. These factors are complex, rarely is anything in nature influenced by a single factor and the same is true in lichen photobiont associations. Therefore, pinpointing an exact influence on photobiont association is difficult. Identifying samples only to the level of genus limited our ability to further investigate the complex factors that vary within a genus.

The results could have been influenced by other facets of lichen biology. More than one photobiont species may be found within a lichen thallus and can occur in different locations of the lichen thallus, there may be different photobionts in basal, distal, intrathalline and epithalline portions of a lichen. It is unclear whether all the collectors whose data were included took samples from same thallus location. One collector (Zachary Muscavitch) focused on taking samples from the younger lobes or branches of lichens. The loss of multiple potentially present photobionts likely narrowed our observed results within the true spectrum of diversity. Focusing on only a subset of closely related sympatric genera also limited the observed photobiont diversity in our study area. Including samples from all sympatric lichens would have been ideal to capture the true scope of photobionts available in the area.

Despite the inherent challenges of lichen biology and the lack of species level identification, the results support that overall, the genera *Niebla* and *Vermilicinia* may be selecting a small subset of partners from a larger pool of sympatric photobionts. Further research addressing *Niebla/Vermilicinia* on the species level will help elucidate the many aspects that shape their selection of photobionts.

An important result was the discovery of four distinct clades, New A1, New A2, New A3, and New C1, that did not fall into the previous clades outlined in Muggia et. al. (2020). Upon using NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the closest matched species, the 3 phylogenetically distinct subclades in major clade A all matched closest with *Trebouxia decolorans* suggesting that this previously defined species harbors more diversity than previously thought. Subclade New C1 matched closest with unidentified *Trebouxia sp.* indicating that it has not yet been formerly described. Culturing and detailed morphological description is required to truly confirm these OTUs as novel species. Distinct species of algae can be

morphologically similar and there are many known cryptic species of unicellular algae, so the process can be lengthy and difficult making true photobiont description quite hard, especially without the use of molecular data. These newly discovered *Trebouxia* species add to the growing body of knowledge about the alga genus. Four distinct and potentially novel clades were found among a small set of only 410 samples, supporting the study by Muggia et al. (2020), that lichen photobiont diversity may be much wider than is currently understood.

Similar studies on *Niebla/Vermilicinia* in the future should focus on using a larger dataset to identify the unique species level factors influencing photobiont selection. Because the current study was only focused at the generic level, the factors that may vary from species to species were unable to be identified with confidence. The results do support the hypothesis that *Niebla* and *Vermilicinia* are narrower in their *Trebouxia* association than the sympatric fungal genera *Ramalina* and *Usnea*. This study hopefully helps to set the stage for more in-depth research into the factors affecting the observed distribution of algal photobionts in these genera, and promotes the exploration and description of more photobionts in lichens.

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