Establishing Phenotypic Variation from High Resolution Microscopy During Developmental Stages of Lichen Resynthesis in the Fungal Family *Physciaceae*

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I. Abstract

The process of lichenization, or the formation of a symbiotic partnership between an algal photobiont and its fungal mycobiont host, has been very challenging to replicate *in vitro*. Due to this, lichen trait evolution and composition is very difficult to track. Some of the research previously done in this area has led to an understanding of the sequential process of lichenization but has thus far been generally focused on individual species. This project intended to take a broader scale look at the morphology of a common family of lichen-forming fungi in the Chicago region, *Physciaceae*. This project consisted of three experiments that tested phenotypic variation between symbiotic associations of natural and non-natural mycobionts and photobionts (labeled here as "correct" and "incorrect"), morphological development in a nutrient-deficient environment, and variability amongst closely related fungal and algal species. In order to understand patterns of development and garner a fuller picture of the lichenization process, this project used phylogenetic and statistical analysis, as well as phenotypic data captured through high resolution microscopy, to determine levels of interaction between symbionts and the morphological implications of those interactions. The goals of this experiment extended beyond an understanding of the precise species involved into a broader study of many naturally occurring symbioses and the specificity required for those critical partnerships.

II. Introduction

In terms of evolution, one of the most critical interactions between species that can develop over millions of years is a symbiosis. A symbiosis can take many forms, often closely intertwined if not overlapping with one another. The methods of symbiosis can be mutualistic or parasitic and can range from interactions between two specific species to entire lineages or

genera (Ahmadjian 1993). Some of these interactions that are mutualistic in theory are parasitic in reality, or uneven in distribution of benefit between partners. These interactions often maintain the individual identities of species, though sometimes the lines between the two can be blurred through mutually assured survival. Lichens are a prime example of a symbiosis that has spread widely and become critical to its ecosystems. Lichens are keystone associations critical to global ecosystems, offering countless effects on their external environment far beyond their comparative biomass. They have evolved into a range of types of symbiosis between species, while simultaneously blurring the lines of what is considered a species at all, as numerous studies have shown differing results of their success outside of their symbiotic partnerships. They are crucial, in terms of biodiversity, conservation biology and evolution, for determining how to define meaningful and functional evolutionary units such as "species". It is even questionable whether the interaction itself consists of only those two expected partners, or whether others, such as bacteria and endolichenic fungi, play a role in the lichen symbiosis as well. Due to all of the above factors, the origin and evolution of this polyphyletic clustering of species offers a unique perspective on symbiotic partnerships as a whole.

Preceding all other questions that can be asked, the classification and definition of the lichen synthesis is inherently questionable. Though the International Association of Lichenology defines lichens as "an association of a fungus and a photosynthetic symbiont resulting in a stable thallus of specific structure" (Ahmadjian 1993), more recently this has been called into question regarding the inclusion of "other" organisms (Hawksworth & Grube 2020). This photosynthetic symbiont, more commonly referred to as the photobiont counterpart to a fungal mycobiont, is an alga or cyanobacterium. The photobiont contributes fixed carbon as an energy source to its mycobiont partner, where in return this mutualism offers its structure in which to live and

provides enhanced resistance to desiccation (Kranner et al. 2005). Fungi are heterotrophic, so they require an external energy source, which in the case of lichens is offered from a mutualist partner. In other fungi, this energy source can range from parasitizing another fungus or insect to decomposing wood. In the case of lichens, some researchers consider this a quasi-parasitic relationship, as the mycobiont offers little in return for the photobiont's energy other than location and does not offer freedom of movement for its partner; the mycobiont can almost be seen in this sense as a "jailer" (Ahmadjian 1993). There are also some lichens that have been found to have a third or fourth partner present, which could be yeast or cyanobacteria. Here, the exchange of nutrients includes either a green alga providing sugars from photosynthesis to its fungal symbiont or a cyanobacteria providing fixed nitrogen. The prevailing theory is that lichens are one of the first examples of a widespread distribution resulting from a mutualistic symbiosis, as both allow the other to live in places they could not have otherwise (Ahmadjian 1993). However, the distinction between parasitism and mutualism in this regard remains challenging and is yet another feature that complicates the understanding of the biology of lichens.

A major difficulty in classification is how the lichen can be delineated and formally named, as they consist of bipartite or potentially tripartite symbioses of individual species. The traditional method for naming lichens is purely according to the name of the fungal species involved, but this aspect of the field, among others, is rapidly evolving. Some other questions include: how should the naming system incorporate the photobiont partner? If each partner can form a symbiosis with multiple types of species rather than a single species, which one should be incorporated into the name? If there are other organisms present in the symbiosis, such as cyanobacterial partners or other fungi, should they be considered in the naming process? These

questions, as well as others more specific to unique lichen relationships continuously discovered around the globe, are part of why lichens are so critical to the scientific discussion surrounding symbiosis.

The evolutionary history of lichens is extraordinarily difficult to track. The origins of fungi can be found in fossil records, but there are few that can be considered in the evolution of lichen symbiosis. Further elaboration concludes that both taxonomically and temporally, it is difficult to establish a standard fossil record of lichens (Lücking & Nelsen 2018). Lichens are specifically difficult to determine in the geological record because in order to have definitively found a lichen fossil, one needs to find presence of each of the fungal and algal partners together, as well as evidence of a physiological interaction. Preservation is often not adequate to display each feature enough to not be disregarded as evidence of lichenological interaction. According to the current understanding, lichens postdate the evolution of land plants, as lichen-like fossils have been found as far back as 425 million years ago (Lumbsch & Rikkinen 2017). In terms of their impact on early evolution of life on land, "lichens could have substantially modified soil biogeochemistry, stability, and hydrology, as well as the complexity of early terrestrial ecosystems-if they were present [during the evolution of land plants]" (Nelsen et al. 2020).

After their evolution, the presence and diversity of lichens increased dramatically. Diversification of lichens continued to occur as life on land flourished, in part due to the strong benefits of the lichen mutualism, including survival in harsh climates and nutrient-deficient zones. The three most common growth forms of lichens are crustose, foliose, and fruticose, 75% of which are crustose. The lichen symbiosis with algae is not monophyletic (Hawksworth 1970) and was a successful enough method for nutrient absorption that currently 20% of all known fungal species are lichenized (Lücking et al. 2016). Their success is also evidenced by the fact

that lichens are found in almost all terrestrial habitats and geographic areas (Feurer & Hawksworth 2007). The algal genus *Trebouxia* is the dominant partner of most lichenized mycobionts and was also believed by some to not survive outside of lichen thalli (Ahmadjian 1988) despite the repeated documentation of free-living algal colonies (Honegger 1998). The presence of these partners free living in a natural environment further complicates classification considerations, attributing environmental effects to lichens, and lichen evolutionary history.

The symbiotic partnership of lichens is a critical symbiosis of nearly every ecosystem on the planet, yet it is currently both underfunded and under-researched. Lichens are ecologically critical associations in many of their environments and have evolved into a range of symbiotic interactions and environmental consequences, while simultaneously offering countless effects on their external environment far beyond their comparative biomass (Ahmadjian 1993). While lichens are an exemplary model of the term "symbiosis", we still lack understanding of their basic biology, especially when it comes to lichenization. The process of how released fungal spores become a lichen, and the degree of specificity required for this process to occur, remain mostly unknown. There is a gap in our understanding of lichens, both in terms of their symbiotic process and in terms of their development, and a comparative study as performed here is a unique angle to elaborate on the gaps in knowledge of lichenization. The evolutionary origin of the polyphyletic clustering of species that perform lichenization offers a unique perspective on symbiotic partnerships as a whole and is critical to global ecosystems. An understanding not only of the symbiotic process of lichenization but the broader importance of lichens to the global ecosystem and anthropogenic uses can guide further research toward lichenology and place a symbiosis study into its broader context of understanding the nature of symbiotic associations.

III. Literature Review

A. Weathering

Aside from prevalent research questions regarding their evolution and classification, the impact of lichens on their ecosystems is also heavily studied, as their presence is both global and critical. As mentioned above, lichens are often keystone species as they are capable of being some of the first organisms present in a barren landscape. According to the USDA Forest Service, “just about anything that holds still long enough for a lichen to attach to and grow is a suitable substrate” (USDA 2018). Though the most common natural substrates are trees, rocks, and soil, lichens are also capable of growing on glass, concrete, metal, plastic, and even cloth (Machesney 2022). Lichens are also critical to environments as they provide food sources, nesting material, and habitats for birds and invertebrates in the same ecosystems. An example is seen in the ecosystem of the Negev Desert in Israel. There, lichens that fix nitrogen support small invertebrates such as snails, which are responsible for breaking down rock into soil for vegetative growth. Though small in size, this makes lichen a keystone species in the Negev ecosystem that has a large impact considering their comparatively small biomass (Shachak et a1. 1987).

Another prevalent feature of lichens important to their surrounding ecosystem is the protection they provide for the rocks and trees on which they grow. It has been hypothesized that lichen protect their substrates from harm due to weathering or other outside forces. In a study performed on historical buildings, it was found that the sandstone colonized by lichen bore less deterioration than those not colonized (Ariño 2004). This could have been caused by lichen cover creating a barrier from physical and chemical weathering effects over the course of time. Lichens also “create a favorable microenvironment by increasing bioavailability of mineral elements and nutrients to successive life-forms that may replace lichens during weathering of

rocks and soil development" (Chen 2000). Once this stone is weathered into sand and soil, the lichen that remain present can continue to sustain it. Some of their features in this regard allow for them to increase water infiltration and retention, while also preventing erosion and stabilizing soil. These features are also being continuously studied. But whatever the determination of the results of lichen on substrates or animalia, it has strong and positive consequences for the retention of an ecosystem.

B. Nitrogen Fixation

Lichens have a significant number of effects on their external environment outside of weathering. Most lichens rely on fixed nitrogen from other sources in the environment, but some lichens that partner with cyanobacteria as a symbiont are capable of fixing this nitrogen themselves. The cyanobionts, as the cyanobacterial partners are called, absorb nitrogen from the atmosphere and release it fixed into the nitrate NH_4^+, which is then used by their mycobiont partner (Ahmadjian 1993). The frequency of heterocysts, in which the nitrogen fixation in cyanobacteria takes place, is increased in the lichen symbiosis in comparison with free-living cyanobacteria (Hitch & Millbank 1975). This indicates the importance of nitrogen fixation for the lichen symbiosis. Cyanolichens are critical to moist locations with scarce nodulating plants, where they are significant contributors to the levels of fixed nitrogen present in the ecosystem. This process contributes to the nitrogen cycle of the ecosystem from their conversion to nitrates, which assists in the growth and development of plant and fungal life. When it rains in these moist environments, the water can leach nitrogen from both the living and dead lichens present on tree bark to make it available in the soil for plants (Machesney 2022). After death, the rest of the decomposing lichen is also able to fall to the ground via this rain, which allows for it to be a source of nutrient transference and new soil upon consumption or decomposition by other fungi

or animals. It is interesting to note that carbon uptake was the first distinguishable feature of lichens, and nitrogen fixing associations from partnerships with cyanobacteria "likely did not evolve or extensively diversify prior to the Mesozoic" (Werner 2018) though terrestrial fungi and plants evolved much earlier (Boyce 2023).

C. Humans

Lichen extracts or substances have been used historically in Chinese and Egyptian folklore art and continue to be used globally today for cosmetic and pharmaceutical purposes. Lichens are used for properties that include antiviral, antifungal, antioxidant, and maybe even anticancer effects; there is also a wide range of conditions that use lichen in their treatments, from bronchitis to skin disorders (Elkhateeb 2019). In terms of their uses as bio-indicators, lichens are easily transportable to testing sites, absorb toxic materials, and have differential sensitivities to toxic compounds, all of which make them extremely amenable to growth on agar media, and use at urban and industrial sites, as well as rarer instances like that of Chernobyl (Ahmadjian 1993). Many cities use lichens to map pollution zones. The sensitivity to air pollution can be detected by such changes in the lichen thallus including reduced thallus size, suppressed fruiting, color changes, and ultrastructural injuries (Hawksworth 1970). The presence of lichens has become symbolic of clean air as they are often "first responders" to many events that dramatically disrupt their environment. Lichens are generally very quick to respond after catastrophic events or other instances where the pollutant emitting source is removed and have been slowly reclaiming urban sites that had previously been too polluted for their survival.

The expedience with which new data is being discovered in the field of lichenology goes to show just how prevalent they are, with implications in the biodiversity of ecosystems, weathering, carbon and nitrogen cycles, medicinal purposes, and bio-indication. Its evolutionary

history is a major field within lichen research, as its determination will elaborate much of the process of vascular plant evolution after transitioning to land. This symbiotic partnership offers new insights on classifications of life as a whole and what constitutes an individual, whether it be the collective or the individual partners within that (mostly) cannot survive without each other. It also brings into question the ideas of mutualism and parasitism, redefining interactions between organisms. To quote Shakespeare, who could only have been discussing lichens, “though she be but little, she is fierce” (Shakespeare 1600).

D. Previous Work

While lichens have significant impacts on their ecological communities as well as important anthropogenic uses, there remain dramatic gaps in the understanding of lichenization. A complete understanding of this developmental mechanism would demand lichen studies to contain germination up to death, which is complicated in a symbiosis of this nature. In order to understand naturally occurring lichenization, the clearest solution for researchers would be to perform molecular analysis of a relichenization process *in vitro*. Relichenization has been the most common method of performing this process in a laboratory setting. During relichenization, mycobiont and photobiont cultures are grown from spores of an original lichen and are then rejoined *in vitro*. This experiment has been attempted many times over the course of decades. This combination of known naturally occurring symbionts is the closest possible mechanism for understanding the natural process at a microscopic level.

The early steps of the lichenization process were mapped out by Kono (2020) during a relichenization experiment, and were categorized as: (1) algal adhesion, (2) recognition of the compatible partner and ensheathment of photobiont cells by specialized fungal hyphae (pre-thallus stage), (3) photobiont cell division and coordinate fungal growth at the contact sites, (4)

development of a peripheral cortical layer at the periphery of thallus primordium, (5) polarization of the thallus primordium, (6) enlargement of the medullary layer by means of intercalary growth processes, and (7) formation of sexual reproductive stages and/or of vegetative symbiotic propagules. These stages are challenging to observe during *in vitro* relichenization rather than naturally occurring lichenization, and thus far researchers have been unable to be fully successful in replicating the natural process. Previous experiments in relichenization were performed by Stahl (1877), as reported by Stocker-Wörgötter (2001), Ahmadjian (1978), Trembely (2002), Athukorala et al. (2015), and Kono (2020), on lichen species *Endocarpon pusillum*, *Lecidea albocaerulescens*, *Baeomyces rufus*, *Cladonia rangiferina*, and *Usnea hakoensis* respectively, each with varying methods of inoculation and imaging. The methods used vary dramatically, including SEM (Joneson et al. 2011), qPCR (Armaleo 2019), and even spore-to-spore resynthesis (Bubrick & Galun 1986). Some of these studies were performed on varied substrates, such as that of Ahmadjian (1980) where cultures were transferred into soil after inoculation. Much of the previous work performed includes gene expression levels throughout the relichenization process, as well as use of various forms of microscopy to thoroughly analyze growth and morphology.

As determined through this extensive experimentation, it is challenging to grow the mycobiont and photobiont individually in a laboratory setting, especially while attempting to avoid contamination and confirm identities of the species cultured. In the case of the mycobiont, it loses the identifiable structure formed from this symbiosis while cultivate *in vitro*. Photobionts are also difficult to confirm as the actual symbiont involved or as another that may have been resting on or within the lichen structure. This differentiation between morphology of a lichen thallus grown *in vitro* and *in situ* is outlined in Figure 1. This project, though similar in nature to

previous experimentation, intended to use a more comparative analysis, rather than documenting a single species during its development with few benchmarks for the process. By broadening the experiment to multiple species of lichen-forming fungi within the fungal family *Physciaceae* and related family *Caliciaceae*, alongside algal symbionts from the family *Trebouxiaceae*, this project aimed to use a varied approach from the trajectory of previous attempts. This approach both broadens the scope of the experiment to include differences between species and uses microscopy techniques that were not available during much of the earlier imaging experiments. As the most recent step in the relichenization attempts beginning back in 1877, this project attempted to put the lichenization process into context by presenting *Physciaceae* as a model family for exploration of the mechanisms that make lichen formation so intricately complex and ecologically critical.

IV: Methods

A. Sample Collection

At the start of this project, the *Physciaceae* samples were collected in 2021 from varied woodland locations throughout the Chicagoland area. Some of these locations include Bunker Hill Park, Swallow Cliffs, LaBagh Woods, and the Nachusa Grasslands. These lichen samples were identified by species by Dr. Matt Nelsen, with input from Dr. Thorsten Lumbsch and Dr. Todd Wilhelm. The cultures were established on malt yeast (MY) agar from ascospores by Dr. Sabine Huhndorf. They were then stored in liquid MY media for approximately one year to allow substantial time for cultures to collect enough material for experimentation. The spore germination protocol for origination of these cultures from naturally occurring thalli is described in previously performed protocols (Trembley 2001). Algal cultures were purchased from culture

collections and plated onto Bold's Basal Media (BBM). This includes the UTEX Culture Collection of Algae in Texas, USA (*Trebouxia gelatinosa*– UTEX 906, *Trebouxia decolorans* – UTEX 901, *Friedmannia israelensis* – UTEX 1181) and the NIES Microbial Culture Collection in Japan (*Trebouxia higginsiae* – NIES 1289). Algal cultures were transferred to new media plates approximately every three months. After the growth period some material was removed for sequencing and other material was measured by weight and used for experimentation as described below.

The well-documented local family of lichen-forming *Physciaceae* fungi were selected as the family of choice for this project as this family is highly prevalent in the Chicago region, is fast growing, and some species within the family already have a complete genome sequence published. These factors make *Physciaceae* not only accessible but an excellent model family for future experiments that come from this research. The neighboring family of *Caliciaceae*, also common to North America, was used as a comparative group. Similarly, *Friedmannia* was used as a comparative outgroup for the *Trebouxiaceae* photobionts.

B. Culturing

The culturing process consisted of ejection onto BBM agar from apothecia attached to the lid of the Petri plate with petroleum jelly. The spore collection was performed according to Marin-Felix (2020). Following ejection and germination of spores, cultures were transferred to liquid MY medium and maintained at ambient temperature. Subcultures of isolated fungal spores from the original lichens were allowed to grow in this liquid media for approximately one year preceding the preliminary experiment, with transfers into fresh media approximately once every other month or when plates were overcrowded with culture material.

The DNA for most samples was extracted from the collected thallus dried material according to the protocols for rapid extractions using Sigma Aldrich Red Extract N Amp Kit and tested via Qubit for concentrations. Other samples that produced unsuccessful extractions according to the Qubit results were extracted using a chrome bead extraction method, which included lysing tissue with beads in the TissueLyser as a first step, and then continuing with the rapid pestle extraction protocol using the lysed material.

The PCR protocol for the extracted samples with Taq Red mix, was as such: 95°- 52°C- 72°C cycle repeated 35X and finalized with 72°C before storage at 4°. Reactions of 12.5 µL included 1 µL of template DNA, 0.25 µL (each) forward and reverse primers, 6.25 µL of Taq Red Mix, and 4.75 µL DNAse free water. The nuclear ribosomal internal transcribed spacer region (ITS) genetic marker was used for primer development. The primers used to amplify fungal regions included ITS4 (White 1990), ITS4A (Kroken and Taylor, 2001), and ITS1F (Gardes & Bruns 1993), along with amplifying algal regions using ITS4T and a-nu-ssu-1752- 5' (Nelsen & Gargas 2006). The sequences of the primers are available from Nelsen (2022). All samples were cleaned using the ExoSAP-IT protocol according to manufacturer instructions.

Fungal DNA was extracted from culture material using the High Quality DNA Extraction from Fungi (Hu, 2016) protocol with slight adjustment. The adjustment of the grinding step was done with a mortar and pestle that was allowed to warm to room temperature after the addition of liquid nitrogen to the sample. Lysis buffer was added directly to the mortar and transferred to a 1.5mL Eppendorf tube for incubation after with a wide-bore pipette tip. PCR of these HMW samples was performed according to the same protocols as the original sample DNA extractions but with the addition of 1 µL of 0.5 µmol/L MgCl.

Sanger sequencing was performed on both types of replicated DNA according to the manufacturing protocol for BigDye v3.1 (Applied Biosystems, Foster City, CA, USA), and products were then run on an ABI 3730 automated sequencer (Applied Biosystems) at the Pritzker Laboratory for Molecular Systematics and Evolution at the Field Museum, Chicago, IL, US. The sequences were verified using the Geneious Prime BLAST function.

C. Phylogenetic Tree

The phylogenetic aspect of this project included sequences gathered from the fungal cultures in the experiment as well as other data extractions. Though the DNA extraction was primarily intended for species identification purposes, the tree was intended to demonstrate degrees of relatedness between fungal species in the family *Physciaceae*. The sequences used came from dried fungal culture material of the collections used in this experiment along with other non-cultured lichen samples collected at the same locations. The High Quality DNA Extraction protocol listed above provided DNA material from these samples. Sequences were analyzed and cleaned using the Genious Prime application (Kearse 2012), and aligned using the MAFFT sequence alignment program. Sequences were input into Gblocks (Talavera 2007) for cleanup and additional NCBI published sequences were added, labeled in the figure by accession number. Construction of the phylogenetic tree was performed via the CIPRES Portal (Miller, Pfeiffer, & Schwartz 2010) according to a RAxML-HPC2 on XSEDE (Stamatakis 2014) maximum likelihood analysis using a GTR+GAMMA substitution model and visualized in FigTree v1.4.4 (Rambaut 2012). The sequenced samples used in this experiment include *Physcia millegrana*, *Physciella chloantha, Phaeophyscia pusilloides, Phaeophyscia ciliata*, and *Amandinea dakotensis*.

D. Experimental Procedures

The compatible and incompatible algal symbionts, according to Muggia et. al (2020), were ordered from the UTEX Culture Collection and the NIES Microbial Culture Collection as listed above. All cultures were inoculated onto agar plates with BBM as subcultures on two occasions to maintain purity of original cultures throughout experimental procedures. Preliminary test experiments were performed via co-inoculation of the isolated fungal spore culture with the compatible lichen alga (*T. gelatinosa*) and the incompatible lichen alga (*T. decolorans*). The co-inoculation procedure was loosely based on combined protocols from previous work by Athukorala et al. (2014), Kono (2020), and Joneson et al. (2010). These preliminary experiments and their findings are further outlined in the Results section, and their success on both BBM and water agar media served as a benchmark for later experiments. These methods are outlined below in Figure 2.

The preliminary experiment was used to test methodologies for co-culturing. For measurement purposes, the increase in weight was calculated after fungal culture (*Physcia millegrana*) inoculation of the culture tube containing 1 mL of sterile, autoclaved water (Athukorala et al. 2015). An approximated 7:3 ratio in size (Joneson et al. 2010) was used to inoculate the sterile water first with fungal culture from the liquid MY media and then with scraped algae from BBM plated subcultures (*T. gelatinosa, T. decolorans*). The increase in weight was then measured after inoculating with only culture, and algal culture was measured as approximately 3 parts in comparison to the 7 parts fungal culture. The weight distinction after only the algal addition was not substantial enough of a change to be detected by the scale, as it only measured down to the tenth of a gram, so only fungal weight measurement was used for the preliminary tests.

Previous methods for mixture of cultures via mortar and pestle, as described in Kono (2020), were not used in order to minimize contamination from outside tools. Instead, both cultures were inoculated in sterile water and the culture tube was shaken vigorously by hand. For the purposes of plating, nitrocellulose sheets, titled “Porous Cellophane Circles, 8 cm diameter”, were ordered from Gel Company, Inc. The sheets were autoclaved in water according to website instructions and hand cut to fit size of petri dishes. After mixing, the water containing both cultures was poured onto the nitrocellulose sheet covered plate. The same fungal and algal naturally occurring symbionts were plated on two plates, one containing BBM media for algal growth and the other containing nutrient-poor water agar. These plates were left in incubation for five months to develop, and imaged weekly. The first four months imaging was performed via dissecting scope, and for the last month progress was imaged on the Leica imaging system used for Experiment II. Both the water agar and BBM plates had a section of the nitrocellulose sheet removed and were imaged each week, along with each of the previously removed sections. These experiments are elaborated on in Results and are referred to as “preliminary”.

For the subsequent experiment, the same original procedure was performed for growth of fungal and algal cultures, in this case all on BBM plates. For the mixing procedure, in this experiment the algal culture concentration was measured before addition of fungal culture to the inoculation tube. Algal cultures were transferred from subcultures made from the purchased culture tubes into 5 mL of sterile water, and then had 1 mL transferred into a cuvette for the DU Series 700 UV/Vis Scanning Spectrophotometer. Wavelength values of each algal sample, used as a proxy for algal concentration, are listed below in Supplemental Figure 1. After this measurement, the other 4 mL were transferred into individual inoculation tubes and weighed. These tubes were weighed again after addition of each fungal species individually, to estimate a

wet weight of fungal culture addition for standardization purposes. The weights of fungal cultures are also listed below in Table 1 and approximated to the nearest milligram. The 7:3 ratio was also approximated in this experiment but was not strictly quantifiable according to these methods. These inoculation tubes were then vigorously shaken and the 1 mL of cultures in sterile water were poured onto nitrocellulose sheet covered BBM agar plates. There were 16 plates total, one of every combination of fungal and algal symbiont listed above. Once every week for one month, according to guidelines for "early relichenization" set by Athukorala et al. (2015), a section of the nitrocellulose sheet was removed from the plate and imaged. This section was not returned to the remaining culture for fear of introducing contamination.

E. Imaging

Imaging for the samples was done for the preliminary experiment once a week on a dissecting microscope. This process consisted of labeling individual clusters on each plate and providing a time lapse image series weekly over the course of two months. Preliminary results focused on coloration differences between the fungal culture and its coverage in green algae. The images were input for analysis into Fiji ImageJ software. ImageJ was used to calculate ratio of fungal to algal area through selecting algal regions by hand with the wand tool and calculating pixel area of those regions. This process was then repeated for fungal area and measurements were collected into Supplemental Table 1. Data were collected and input into R to calculate algal coverage rate of increase over time. This process was only performed for the preliminary experiment consisting of *Physcia millegrana* mixed with *Trebouxia decolorans* and *T. gelatinosa*. Subsequent experiments did not allow for these calculations as the high-resolution microscopy demanded removal of the plate lid for imaging, which would introduce

contamination, and therefore had sections removed from the plate rather than imaging the plate in its entirety.

The goal for Experiments II and III was to obtain higher resolution images for calculation and presentation purposes, and in order to further understand the extent of the interactions in a qualitative way other than purely by area calculations. These images were obtained on a Leica DFC450 Digital Camera, a Leica 1400 Column, a Leica LED5000 HDI Dome Illuminator and a number of Leica lenses. The software is the Leica Application Suite, which manages imaging and photo compositing. Some photos were alternately stacked with Helicon Focus Pro. These images were all taken at the 9.2X focus lens of this system, and qualitative descriptions and analysis is collected from this time series in the Results below.

F. Data Analysis

The dataset collected from the preliminary experiment was organized by fungal species and corresponding algal pairings for each of those species. The data was tidied into an Excel spreadsheet with categorical variables including "cluster" (the region of the plate used to keep track of smaller sections of fungal culture), "symbiosis" (whether this pairing was the 'correct' symbiosis seen in natural settings), and "algal distribution" (description of the algal spread over fungal area). This table is shown below in Supplemental Table 1. The calculations done for area and growth rates were done in Fiji ImageJ. From there, the data processing for Experiment I was transferred into R for visualizations of patterning between morphological features of differing species and is shown in Figure 3. Once in a readable format for R, the packages needed for analysis of said experiment were installed and data manipulation, calculations of p-values, and image generation were done with functions in the tidyverse, dplyr, and ggplot2 packages.

V: Results

This project included *in vitro* co-cultured re-lichenizations of fungal and algal partners that had been separated into different cultures and grown independently. There were a total of four algal cultures and five algal cultures used for all three experiments. The experimental procedures, as well as the data and images resulting from these experiments, are outlined below. Algal identifications were confirmed by the culture collections they were purchased from, and as shown in Figure 7, phylogenetic analysis confirmed the identification of the fungal species cultured for use here before said use in these experiments and degrees of relatedness between species used. The phylogenetic tree was used in confirming identities of the fungal cultures used for these experiments.

A. Preliminary Experiment – Growth Rate Variation

The preliminary experiment consisted of the singular fungal species *Physcia millegrana* cultures with two different photobionts in two different trials. These were *Trebouxia gelatinosa*, its naturally occurring photobiont, and *T. decolorans,* which does not associate with the species studied but is found in lichen symbioses with distantly related lichenized fungi (Ahmadjian 1960). This experiment was performed on a dissecting microscope. As the same section was imaged every week, the set up allowed for the quantitative data collection outlined in the Data Analysis section of Methods. It was expected that there would likely be minimal difference between the two plates, as both algal species are within the same genus and are both found in similar geographic regions. The goal of this experiment was to look at precursory phenotypic variation for the planned Experiment III and track rates of change, as the imaging for this experiment alone could be performed on the same sections of the plate each week. As was expected over the course of five weeks and seen in Figure 3, these results showed no pattern of

increase or decline in the growth rates between the two species, and the p-value for the compared dataset of growth rates was far from significant. There was no steady rate of increase for algal coverage of fungal samples, as seen in the variability of the histogram in Figure 3, which would be quantitative evidence for varied interaction between the two algal species. Figure 4 shows the phenotypic variation through a series of images taken once each week, and clearly demonstrates that the "incorrect" symbiosis with *T. decolorans* had a greater increase in algal growth than *T. gelatinosa*. This seems to qualitatively indicate that the incorrect pairing had more algae spread around the culture rather than on top of fungal clusters. For the fungal cluster, as calculated in ImageJ, the radius growth of the correct pairing was 1.639 mm, while the incorrect was 0.28 mm, indicating a more successful fungal cluster growth in symbiosis with its correct photobiont. Though radial growth and visible qualitative analysis do seem to indicate interaction, the calculated p-value of 0.228 from the chi-square test between the growth rates of these symbiont pairings fails to show any definitive correlation.

B. Experiment II – Media Variation

The second experiment was mainly intended to assess phenotypic variation from one symbiotic partnership plated onto different media. The same fungal species from the preliminary experiment, *Physcia millegrana*, was plated with its "correct" photobiont, *Trebouxia gelatinosa* on one plate of water agar and one plate of BBM media. The high-resolution Leica imaging system required removal of the sample from their sterile plating, so they could not be returned to the agar after their section was imaged. This meant that the second experiment could not include quantitative measurements for algal and fungal growth, as each week consisted of imaging a different section than one previously done. Both algal coverage over the fungal cluster, as well as growth patterning of algae around the fungus compared to widely across the plate, were

approximated from this collection of images. Weekly progression images of the water agar and BBM plates are shown in Figure 5. The first week of images was taken approximately three months after the two cultures were mixed and plated. Qualitative results from this experiment show a dramatic difference in morphology of the two plates, in terms of coloration, algal growth, and hyphal growth, but little differentiation between the weekly images for each plate. The minimal differentiation between weeks is mostly likely due to the fact that the imaging occurred relatively late after the plating of the merged cultures. For the fungus, the water agar sample is much lighter in color and begins growing hyphae already in the first week after inoculation. The fungus on the BBM plates does not retain the same cluster shape as the water agar counterpart, is also much darker in coloration, and only shows evidence of hyphal growth after week five. Interestingly, algal growth is much more centered around the fungal cluster when deprived of nutrients, indicating that it is attempting to interact with the fungal symbiont in some manner as a nutrient source. The extent of this interaction and nutrient sourcing success requires further research.

A. Experiment III – Phenotypic Variation

The natural photobiont of each of the *Physcia* species used in this experiment is *Trebouxia gelatinosa*, and this pairing is used as the comparative measure for each of the fungi and corresponding symbionts. This experiment consisted of a series of 16 plates, four fungal symbionts each paired with four algal symbionts, all mixed and plated onto BBM media as laid out in Methods. The algal symbionts include one from each known clade of *Trebouxia* (including *T. higginsiae*, *T. gelatinosa*, and *T. decolorans*) and *Friedmannia israelensis*, a more distantly-related soil alga (Chantanachat and Bold 1962). These algae were tested against three Physciaceae fungi (*Phaeophyscia ciliata*, *Physciella chloantha,* and *Phaeophyscia pusilloides*)

and one related species from the sister family *Caliciaceae* (*Amandinea dakotensis*). Table 1 below lists the phenotypic fungal and algal traits recorded for each of the experimental pairings, including color variation over the course of the experiment, algal growth, algal interaction with fungi via spread, and hyphal growth. The resulting phenotypic traits, categorized after one month of culture growth, show unique trends in terms of relationships between different algal symbionts. Figure 6 shows images collected of the pairings after one month of the experiment, which are used for the assessments listed below. The results of this experiment can be divided into a series of observations on individual factors of lichen morphology and levels of interaction. The category "Algal Growth" describes the relative rate of algal cells increasing across the plate, be it qualitatively described "High" or "Low" levels of growth. Growth was considered "High" if the algae covered more than 50% of the plate, and "Low" otherwise. The same factor is accounted for in terms of closeness to the fungal clusters, which tended to grow much more intact than the algal symbionts. This is described as "Algal Spread", where "Close" would mean that the algal growth occurred centrally around the fungal cluster taken for imaging each time period, and "Broad" would mean that the algal growth is evenly spread across the plate rather than focusing around or on top of fungal clusters. The final category, "Hyphal growth", is assumed to be the least accurate of these readings. Any growth that appears hyphal in nature, via an understanding of these hyphae as long, white filaments extending from fungal cultures, is marked as either "Yes" or "No". Supplemental Figures 2, 3, 4, and 5 show the total images taken every week, depicting the full progression of each of the four algal symbionts as plated with varying fungal cultures.

The fungal cultures grown with *Trebouxia gelatinosa*, the assumed "correct" natural pairing, resulted in samples with high algal growth rates that were centralized around the fungal

clusters, and all had various levels of fungal hyphae growth. The uniqueness of both hyphal growth and close spread of algae of only these pairings could be indicative of recognition of the symbionts. Though this is not a clear and irrefutable indication, of each of the individual pairings of symbionts these phenotypic traits are most indicative of interaction between symbionts. The precise nature of this interaction, be it nutrient exchange or parasitism due to lack of other available sources, remains in question. The other phenotypic traits, discussed below, show closer patterning to solo algal culture growth by being both broad in scope rather than closely intertwined with fungal clusters, as well as having high rates of hyphal growth alongside algal growth. Other symbiotic pairings did not exhibit these trends as clearly.

The samples interacting with *Trebouxia higginsiae* are unique in that algal growth levels were visibly slower, with algae presenting less predominantly on the plate than in every other sample. As this is an example of another algal clade within *Trebouxia*, rather than an entirely unrelated algal symbiont, it is unclear as to why this would be the case and will require further analysis. In terms of other phenotypic traits, the minimal algal growth closely corresponded to fungal cluster sites rather than across the full plate, and also did not exhibit any strong levels of hyphal growth.

The fungal cultures grown with *Trebouxia decolorans* exhibited mostly broad algal spread and high algal growth, both indicative of a lesser interaction between fungus and algae. The algal growth in these cases could be inferred as centrally driven by the media nutrients rather than extraction of any nutrients or secondary metabolites from fungal counterparts. They also did not exhibit discernable hyphal growth, which was defined as any filamentous protrusions from fungal clusters. This lack of hyphal growth, used here as a proxy for overall fungal culture success and growth, could be from a lack of interaction with algal symbionts or from poor

absorption of nutrient media. It is less likely to be a byproduct of fungal interaction with media as each of the different species of fungus exhibited the same phenotype of minimal hyphal growth rather than some fungal species growing preferentially over others.

The fourth symbiotic pair plated was that of the fungal samples individually with *Friedmannia israelensis*, a more distantly-related algal species. This alga was selected for its distinction from the others both phylogenetically and regionally, and was used as a marker for what could be assumedly minimal interaction between symbionts, as they would have no potential for natural interaction. In these sample pairings, similarly to those partnered with *T. decolorans*, the samples the algal growth rates were high with broad spread of growth not distinctly tied to fungal clusters. Though this is an expected result from what could be considered a control, there are some factors that are worth noting, one of which is the striking resemblance in patterning to *T. decolorans,* which is a commonly found algae in the North American region expected to have occasional interaction with *Physciaceae* species. The difference between these two pairings can be noted in their hyphal growth, which was present in *T. decolorans* pairings but not in those with *F. israelensis*. As mentioned, hyphal growth is used as a proxy for fungal growth and could be indicative of broader fungal success in this pairing. By lacking hyphal growth in the *F. israelensis* cultures, as would have been expected by it being the outlier, it is further indication that hyphal growth can be used for this proxy.

An interesting distinction was that there was no clear pattern of distinction between features of *Amandinea dakotensis* cultures, which might have been expected due to it being from a different family than Physciaceae. The trends of similar phenotypic variations followed the variability in photobiont more closely than they did mycobiont. There was no trend that

singularly followed the *Amandinea* pairings other than a retention of dark fungal color from beginning to end of the experiment, which occurred in a few of the other fungal species as well.

VI: Discussion

From the data gathered in the preliminary experiment, the central determination was the lack of a linear growth pattern for fungal and algal cultures and the lack of a direct trend in algal coverage of fungal clusters over time. The proportion of algal growth to fungal growth shown in Figure 3 mostly increased over time was but not consistent in this increase or in rate between each week's measurements. A central takeaway from the preliminary experiment is the characteristic development of algal cultures broadly across the plate in the "incorrect" artificial symbiont pairing of *Physcia millegrana* rather than the "correct" natural pairing, which instead had algal growth bound to the fungal clusters. This inherent connection between symbionts is a qualitative depiction of interaction.

Experiment II also indicated interaction between symbionts, only in this case it occurred in both a nutrient-deprived and nutrient-rich environment. In both nutrient-rich and nutrient-poor co-culturing, there was morphological evidence for interaction between symbionts to varying degrees. Hyphal growth was taken as the clearest indication for some level of interaction between symbionts, which occurred from Week 1 in the water agar sample, but also had some growth in the final Week 5 image of the BBM sample. This is similar to what has been seen in previous research where hyphal growth was used as an indication that the fungus may be doing so to "facilitate the capturing of as many photobiont cells as possible" (Trembley 2002) when limited in other food or nutrient sources.

Experiment III also similarly measured levels of interaction, only on a much broader scale with multiple fungal and algal pairings. These comparisons showed little trends that differentiated fungal families from one another other than coloration, but differentiation was significantly more noteworthy between photobiont groups. In this case, there were also the greatest number of indicators of symbiotic interaction when the "correct" natural symbionts were used. It is interesting to note that the number of indicators of symbiont interaction was approximately the same between each of the "incorrect" symbiont pairings and the "correct" symbiont pairings, though these indicators varied most strongly between each photobiont. This evidence correlates well with previous data from Joneson et al. (2011), that used differential gene expression levels during relichenization contact to claim that "mycobionts and photobionts communicate both before and including cellular contact". It differentiates, however, with data from Ahmadjian (1980) that described *Cladonia* fungal cultures only forming advanced structures with their "correct" photobiont *Trebouxia glomerata*. This study also described the fungi interacting similarly with various algae from *Trebouxiaceae* but not with the sister group *Pseudotrebouxia* or the outside algal group *Pleurastrum*, which also differentiates it from these experiments that showed similar levels of interaction. James and Henssen (1976) also similarly state that the photobiont does assist in directing the phenotypic expression of its mycobiont, and though they specified types of algae they used in their experimentation, it can also be confirmed for *Physciaceae* from the data gathered here. Further elaboration of the extent of this communicative interaction is an avenue for further experimentation.

Some of the challenges of this project were the re-lichenization process itself, which is often very slow and occurs over the course of months, and contamination from molds and bacteria in the relichenized cultures. It is often difficult to discern which one of the many fungal

or algal partners relevant to the lichen symbiosis is the one present in the developing culture. Though there were challenging aspects, the success of the relichenized *Physciaceae* cultures shows their resilience as a potential model candidate for future experimentation.

The qualitative nature of these experiments makes tracking changes and understanding the extent of these patterns as indicative of any larger interaction framework challenging. Previous experimentation involving lichen imaging has been similarly qualitative, which is a problem that has historically challenged microscopy studies. A general argument of early assumptions since the work of Stahl (1877) that is readily argued here, however, is the assumption that the role of the photobiont on morphogenesis is irrelevant, and though it stimulates lichen development its specificity is not critical to the physical form of the lichen. The experiments performed here can eventually be pieced together with similar data from previous studies to create a full picture of lichenization by including morphological data, nutrient requirements, comparative species analysis, and gene expression level data. For their piece of the lichen development puzzle, these preliminary findings show resulting trends in phenotypic variation between phylogenetically distanced fungal species and show promise for being used as indicators of symbiotic interaction between various photobiont and mycobiont pairings. These experiments are therefore presented as preliminary findings for what could be a broader scale experiment with more controlled conditions, for true comparisons of growth and gene expression during lichen development to follow secondary metabolite production and development of the lichen thallus.

Future directions could be used to confirm the patterns found in these findings via use of Environmental Scanning Electron Microscopy for even more accurate imagery and calculations via usage of a gaseous environment to image the same plate sections continuously over the

course of the experiment rather than requiring removal of an individual section for imaging each session. Another avenue of future research would be determining gene expression levels at each of the previously described stages and doing so with both the experimentally combined samples in the methods used here along with plating the samples together without being mixed. This could allow for fungal and algal growth to occur in the direction of the other symbiont, which would also indicate further levels of interaction beyond those being forced in this experiment. These further avenues for analysis can also incorporate full genome sequencing which has not yet been performed for some of the listed species. Much of the culture methods developed in this procedure and fine-tuned imaging techniques should also be incorporated into further analysis and can be expanded to include testing of samples on fungal growth media rather than algal BBM or water agar to look for shifts in patterning, as well as plating of algal cultures after fungal cultures have been well established on media as described by Ahmadjian (1980). Expansion of the experimentation methods and investigation of the extent of symbiont interaction found here can place this project into a wider analysis of lichenization, and develop a deeper understanding of this unique symbiotic interaction critical to the global ecosystem.

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figure in my research thus far, and I look forward to keeping the knowledge I have gained from him as well as his joyful demeanor with me for the rest of my career and life.

VIII. Figures

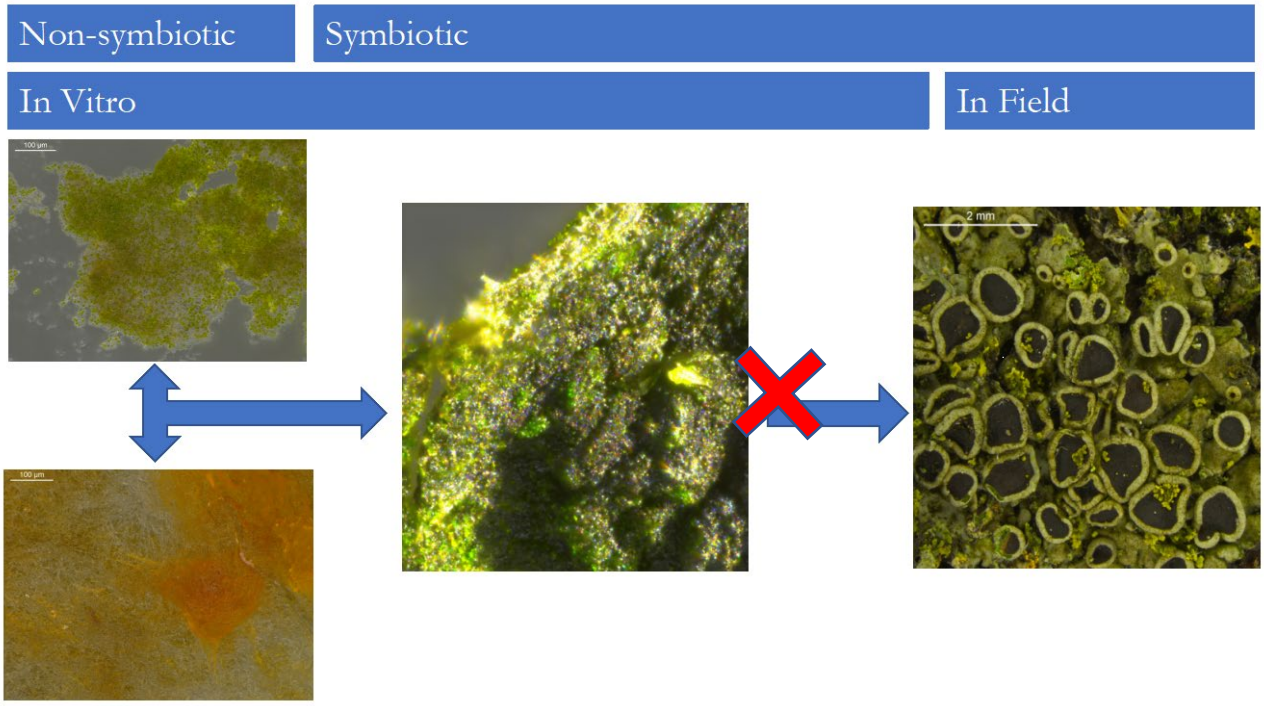

Figure 1: *Resynthesis schematic* – Depiction of morphological difference between co-cultured resynthesis of fungal and algal cultures *in vitro* in comparison to the naturally occurring lichen *in vitro* the fungal culture was isolated from. The first column represents the individual fungal and algal cultures before relichenization. The image of the symbiotic association between cultures was taken one month after co-inoculation.

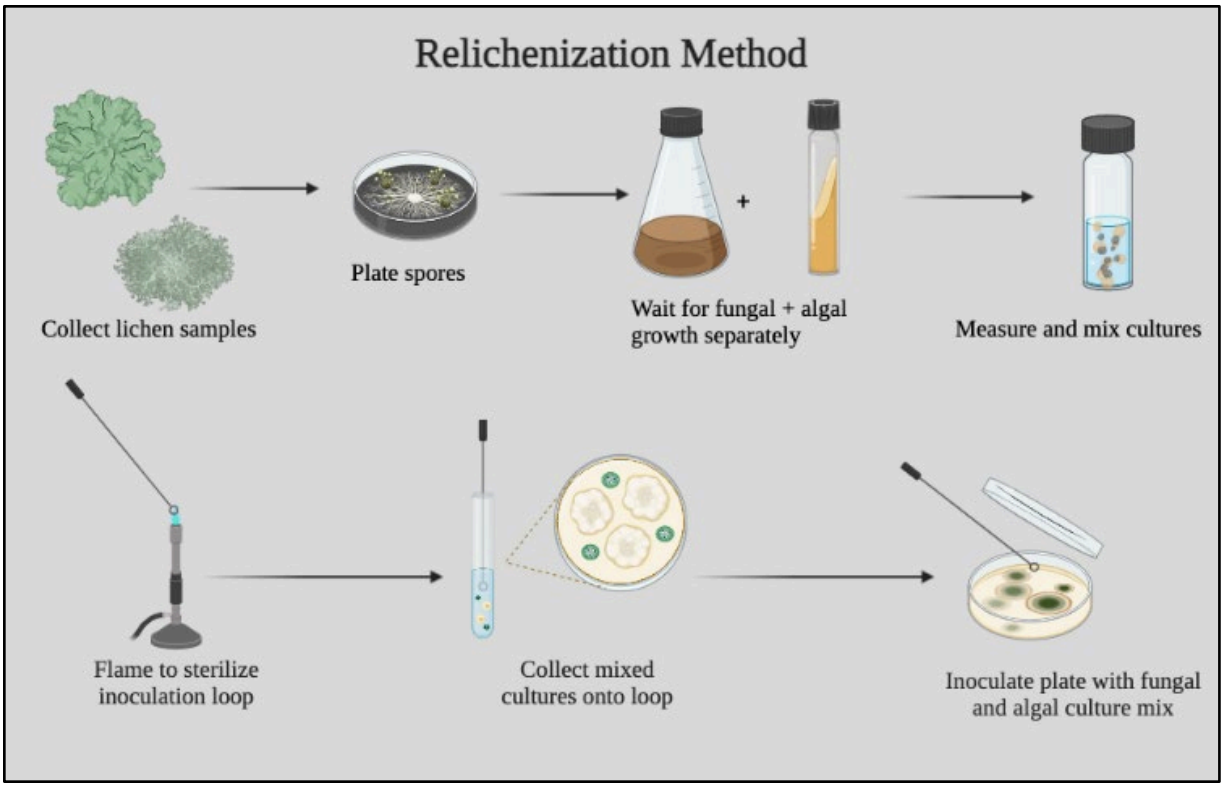

Figure 2: *Relichenization Method* - Schematic of the culture relichenization process. The process begins with sample collection and plating of fungal spores. Selected algal cultures were purchased from culture collections and allowed to grow separately from fungal cultures with continuous replating every three months until mixing. Once mixed, cultures were shaken and plated onto either BBM or water agar, depending on the experiment.

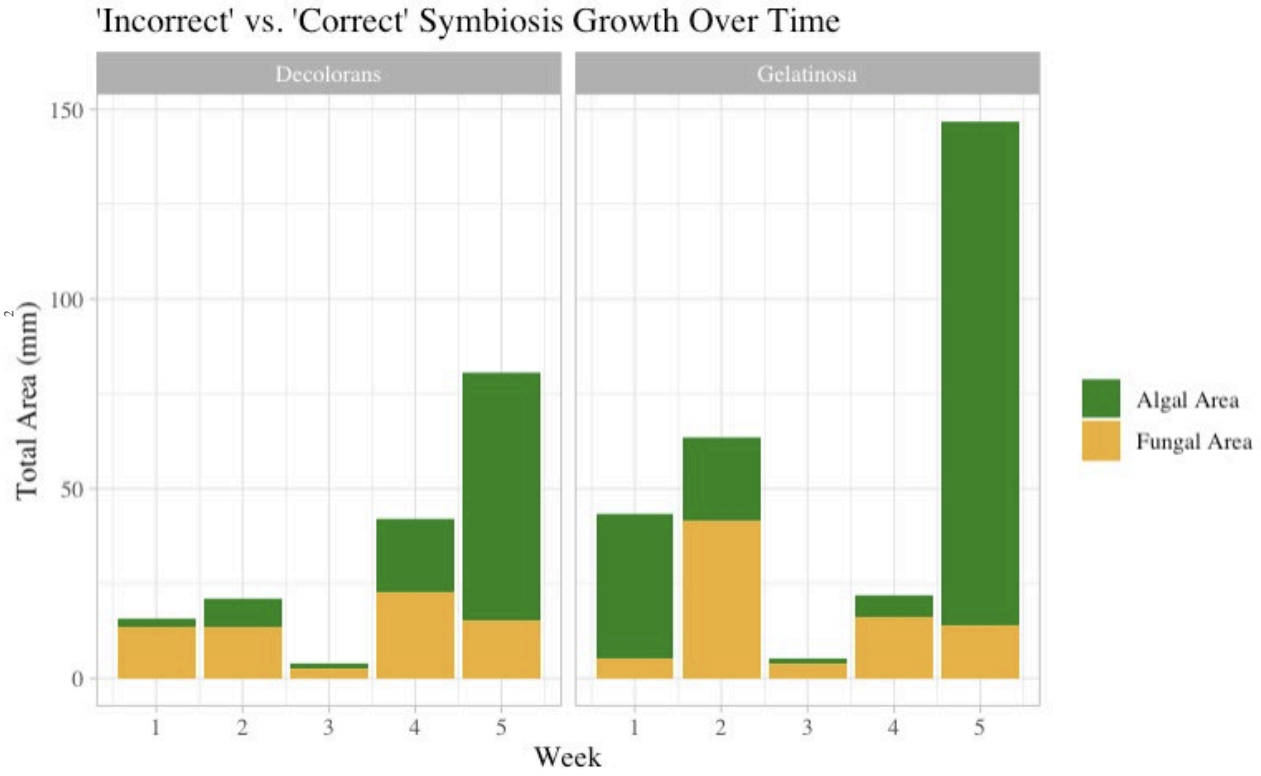

Figure 3: *Growth Over Time* - Comparative algal and fungal growth over the course of five weeks in the preliminary experiment. *Physcia millegrana* with non-naturally occurring algal symbiont of *Trebouxia decolorans* is shown on the left with algal area colored in green and fungal area colored in yellow. Growth of the fungus *P. millegrana* with the naturally occurring symbiont *T. gelatinosa* is shown on the right with the same schematic. Area calculations are taken from images collected with Fiji ImageJ. Figure was created using the R package ggplot2.

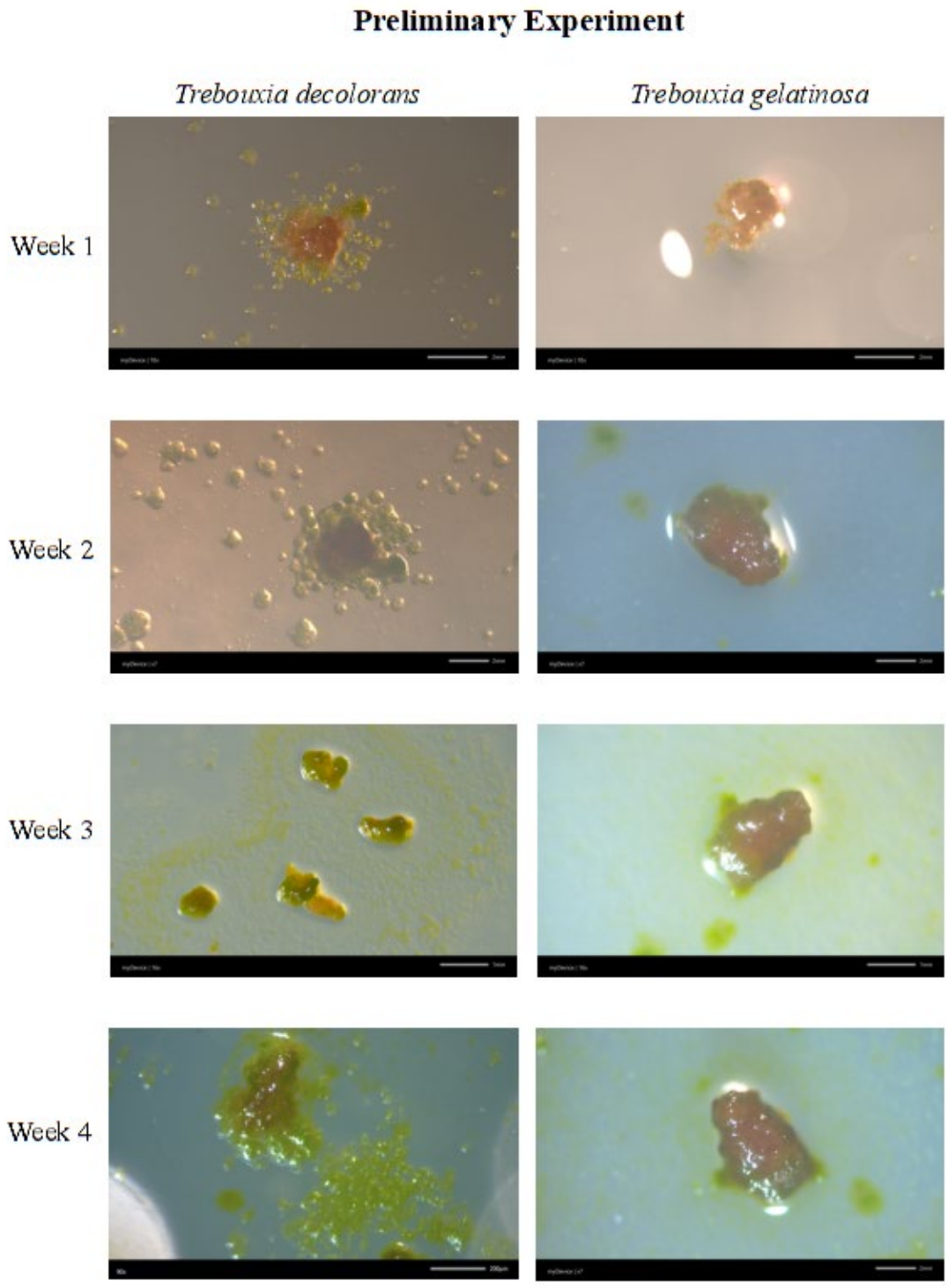

Figure 4: *Preliminary Experiment* - Weekly images collected of the fungus *Physcia millegrana* plated after mixture with algal symbionts *T. decolorans* and *T. gelatinosa*, respectively. Fungus is yellow-brown in color and algae is green in color. Later images have fungus darker in color as algae grows over top. Scale bar in bottom right-hand corner reads 2 mm.

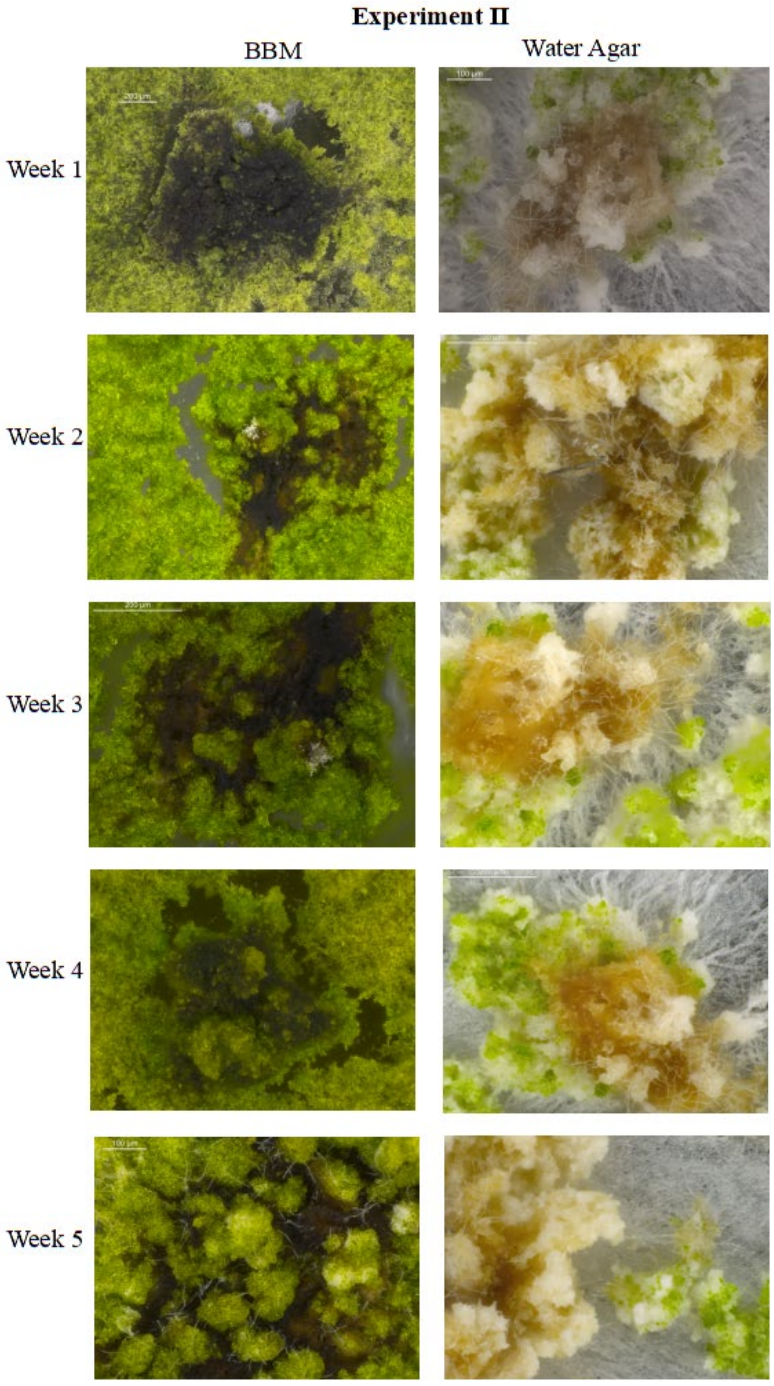

Figure 5: *Experiment II* – Images collected of *Physcia millegrana* fungal culture with naturally occurring photobiont *T. gelatinosa* plated on Bold's Basal Media and water agar, respectively. Scale bars in top left-hand corner read 200 µm. Each week's image is a unique section removed from the original culture and not replaced. Algal cultures are green in color and fungal cultures are dark or light brown in color. Hyphal structures are white filaments protruding from the fungal cluster in the right-hand column, indicating growth in the water agar samples starting from Week 1 and the BBM samples in Week 5.

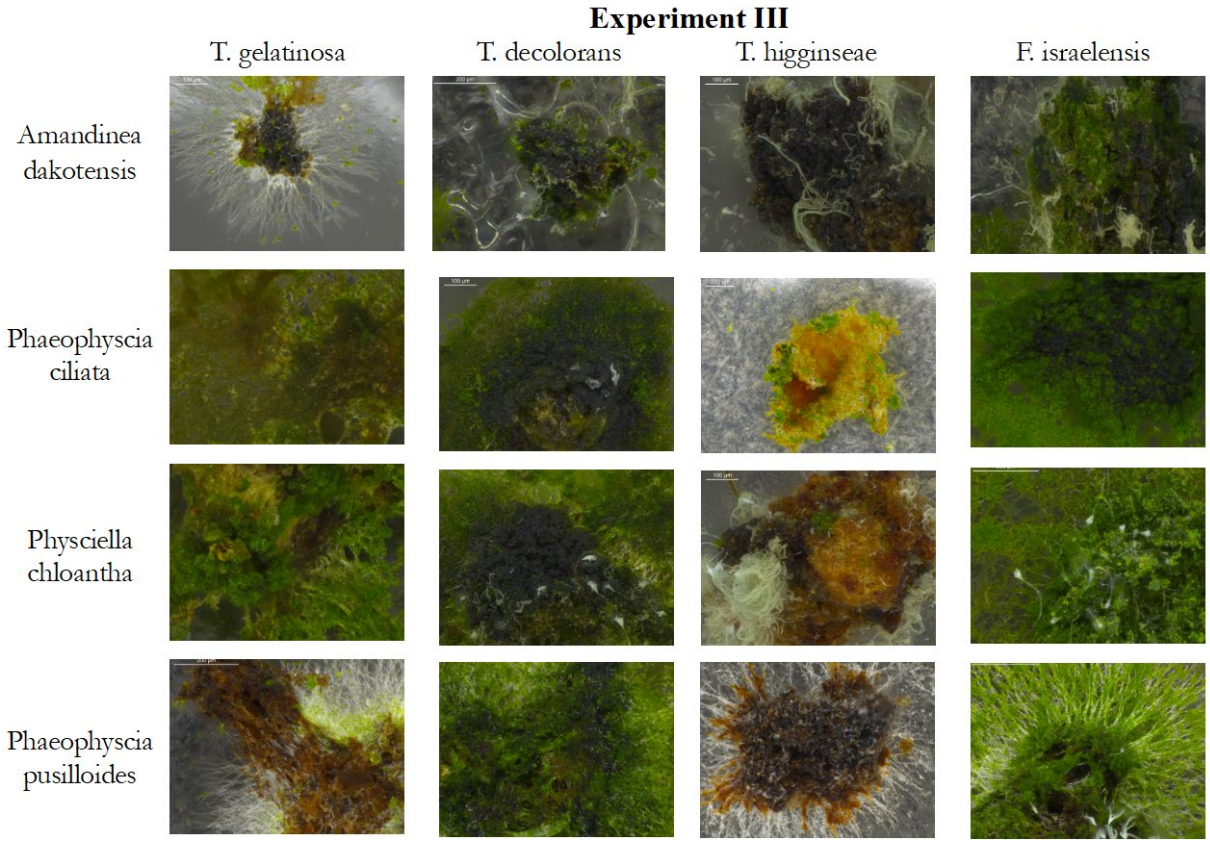

Figure 6: *Experiment III*- Images collected of various fungal cultures (including *Amandindea dakotensis*, *Phaeophyscia ciliata*, *Physciella chloantha,* and *Phaeophyscia pusilloides*, respectively) with naturally occurring photobiont *T. gelatinosa* on the right, followed by *T. decolorans*, *T. higginsiae*, and *F. israelensis*. All were plated on Bold's Basal Media. Scale bars in top left-hand corner read 100 µm. Each week's image is a unique section removed from the original culture and not replaced. This figure shows final images taken one month after inoculation. Algal cultures are green in color, fungal cultures are dark or light brown.

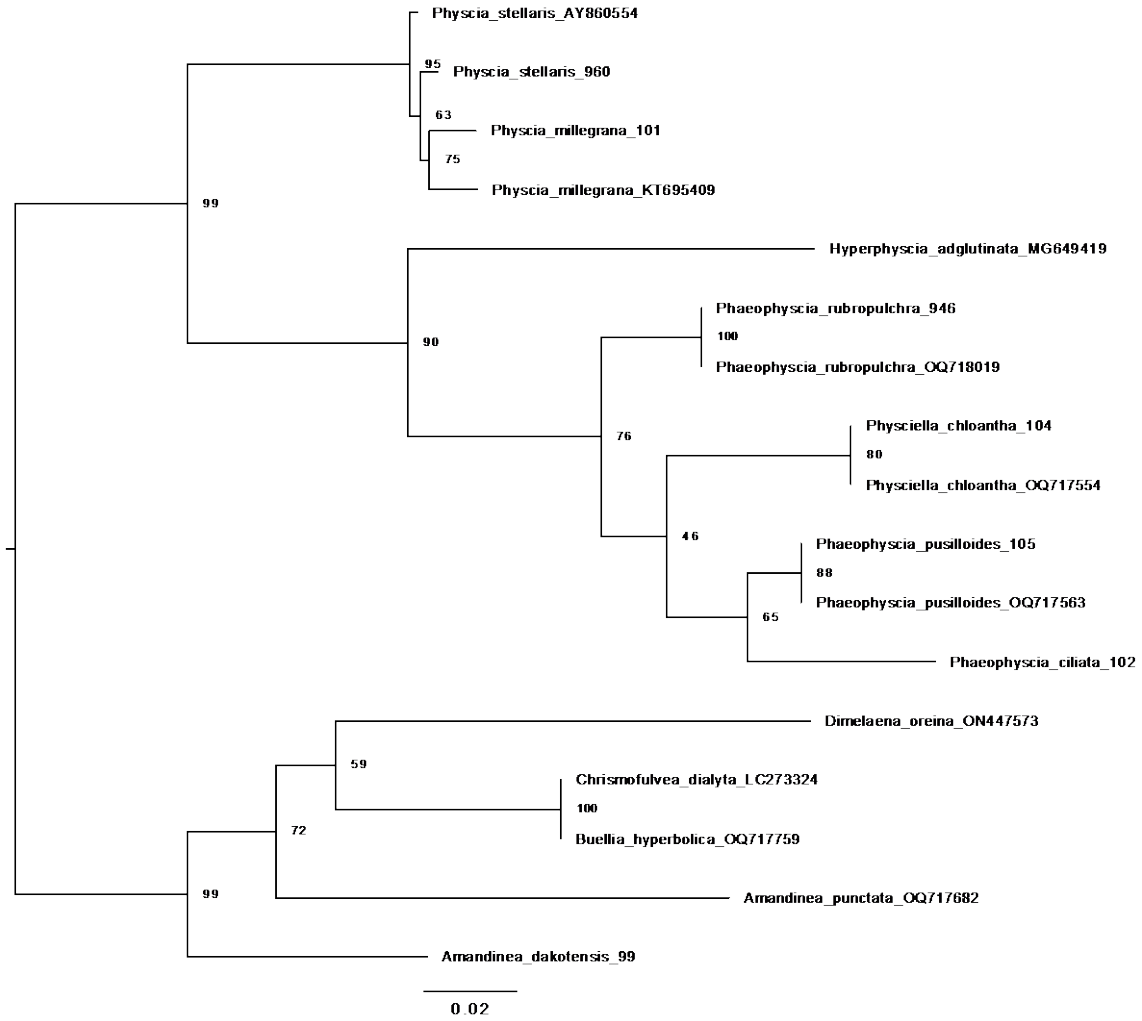

Figure 7: *Phylogenetic Tree* - Fungal ITS sequences used include both extracted DNA from collected lichen thallus samples as well as from dried fungal culture material of the collections used in this experiment. Species used in the experimental procedures are labeled by their species name and sample collection number (eg. 99, 102). Other sequences have been added from GenBank and are identified by species name and accession number. This maximum likelihood tree was made from FASTA files using the CIPRES portal and displayed through the FigTree program application as outlined in Methods. The scale bar is representative of substitutions per site.

Table 1: Phenotypic Data for Fungal and Algal Symbiont Pairings

Algae	Fungi	Fungal Weight (g)	Fungal Color (2/27)	Fungal Color (3/27)	Algal Growth	Algal Spread	Hyphal Growth
Treb. gelatinosa	Amandinea dakotensis	0.0198	Dark	Dark	High	Close	Yes
Treb. decolorans	Amandinea dakotensis	0.022	Dark	Dark	High	Broad	No
Treb. higginseae	Amandinea dakotensis	0.0225	Dark	Dark	Low	Close	No
Frie. israeliensis	Amandinea dakotensis	0.0132	Dark	Dark	High	Broad	Yes
Treb. gelatinosa	Phaeophyscia ciliata	0.0345	Light	Dark	High	Close	No
Treb. decolorans	Phaeophyscia ciliata	0.0148	Light	Dark	High	Broad	No
Treb. higginseae	Phaeophyscia ciliata	0.0254	Light	Light	Low	Close	No
Frie. israeliensis	Phaeophyscia ciliata	0.0199	Light	Dark	High	Broad	No
Treb. gelatinosa	Physciella chloantha	0.0123	Light	Dark	High	Close	Yes
Treb. decolorans	Physciella chloantha	0.0656	Light	Dark	High	Broad	No
Treb. higginseae	Physciella chloantha	0.0242	Light	Light	Low	Close	No
Frie. israeliensis	Physciella chloantha	0.0285	Light	Dark	High	Broad	Yes
Treb. gelatinosa	Phaeophyscia pusilloides	0.0108	Dark	Dark	High	Close	Yes
Treb. decolorans	Phaeophyscia pusilloides	0.0064	Light	Dark	High	Broad	No
Treb. higginseae	Phaeophyscia pusilloides	0.0205	Dark	Dark	Low	Close	No
Frie. israeliensis	Phaeophyscia pusilloides	0.0197	Dark	Dark	High	Broad	Yes

Table 1: *Phenotypic Data* - Table of phenotypic data for each of sixteen fungal and algal symbiont mixtures on BBM plates. Data is collected from images shown in Figure 6. Categories include Fungal Weight, Fungal Color (start of experiment and end of experiment), Algal Growth, Algal Spread, and Hyphal Growth. The dates listed in the Fungal Color columns are the beginning and end dates of the experiment. Fungal weight is in grams, listed exactly as accurately as the scale used.

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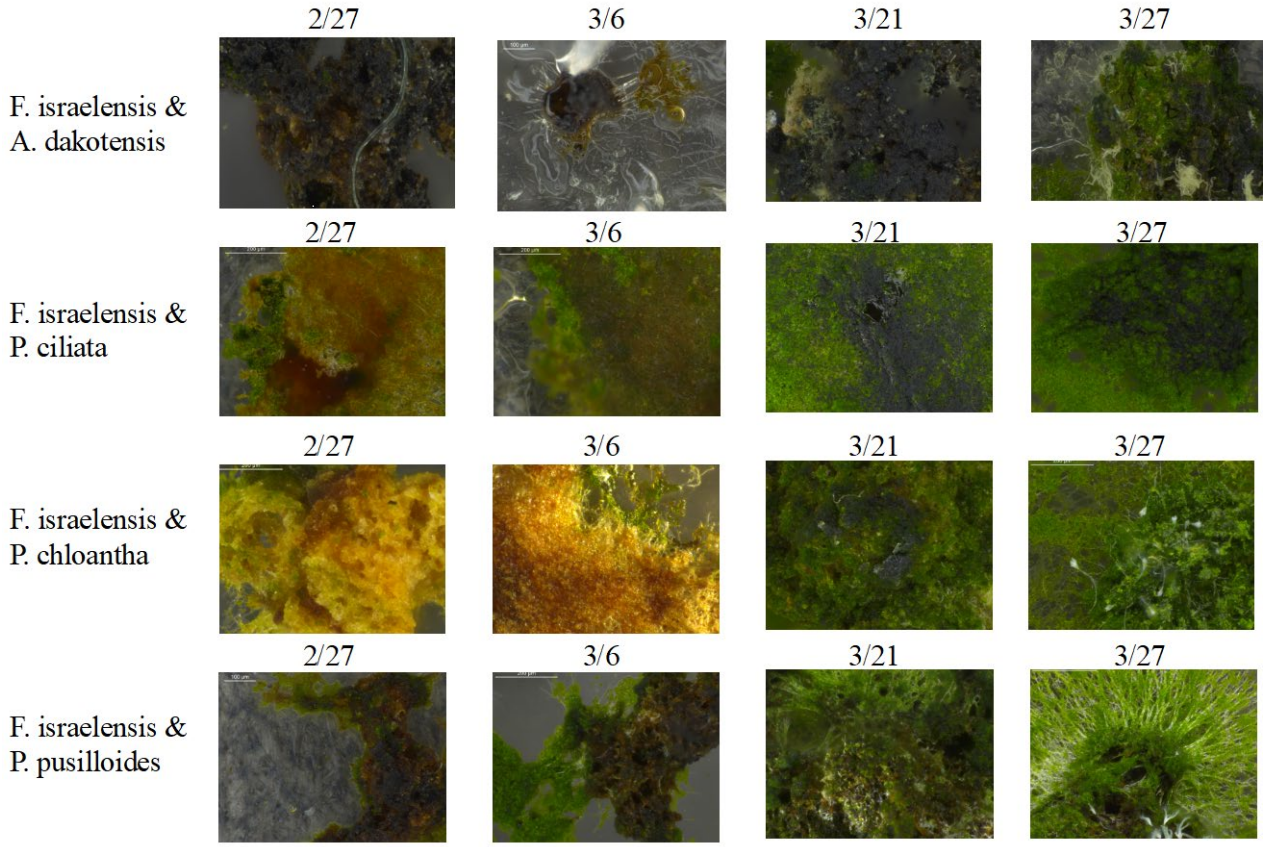
X: Supplemental Figures

Algal Sample	Wavelength (540 nm)	Wavelength (260 nm)	Wavelength (800 nm)
Treb. gelatinosa	0.031	0.725	0.032
Treb. decolorans	0.086	0.15	0.148
Treb. higginseae	0.029	0.413	0.028
Fried. israeliensis	0.032	0.268	0.039

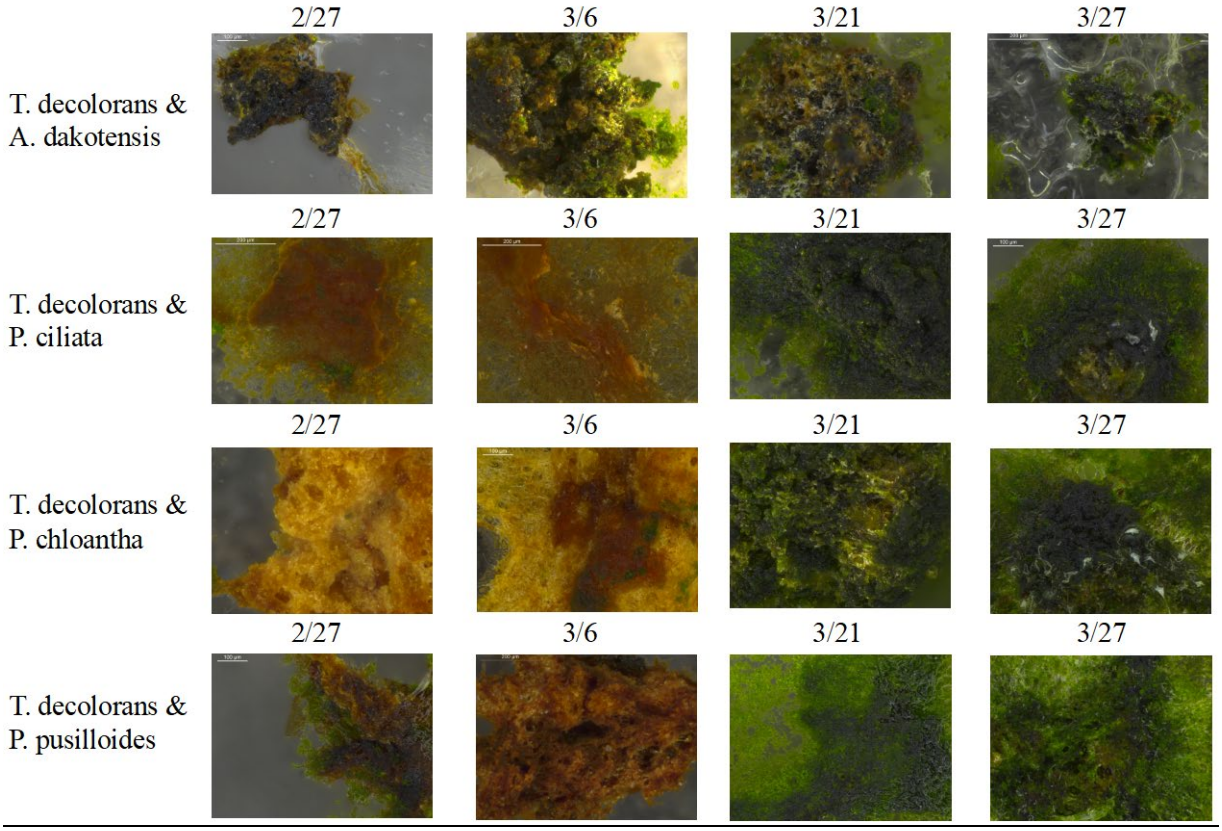
Supplemental Figure 1: *Algal Concentrations* – inoculated 1 mL water with algal culture and transferred into a sterile cuvette filled for the DU Series 700 UV/Vis Scanning Spectrophotometer. Wavelength values of each algal sample, used as a proxy for algal concentration, are listed. Intended to standardize amount of algal culture added to each plate for Experiment III.

Week	Cluster	Fungi	Algae	Symbiont	Total Area (mm^2)	Fungal Area (mm^2)	Algal Area (mm^2)	Algal Distribution	Ratio Fungal Area	Ratio Algal Area	Radius
1	1	Physcia millegrana	Treb. gelatinosa	Yes	43.51	5.171	38.339	central	0.119	0.881	2.566
1	2	Physcia millegrana	Treb. decolorans	No	15.864	13.623	2.241	central	0.859	0.141	4.165
1	3	Physcia millegrana	Treb. gelatinosa	Yes	4.264	3.731	0.533	central	0.875	0.125	2.178
2	2	Physcia millegrana	Treb. decolorans	No	20.834	13.561	7.273	central	0.651	0.349	4.155
2	1	Physcia millegrana	Treb. gelatinosa	Yes	63.424	41.62	21.804	spread	0.656	0.344	7.279
3	1	Physcia millegrana	Treb. gelatinosa	Yes	5.148	3.886	1.262	spread	0.755	0.245	2.224
3	2	Physcia millegrana	Treb. decolorans	No	3.857	2.689	1.168	spread	0.697	0.303	1.851
4	2	Physcia millegrana	Treb. decolorans	No	42.144	22.728	19.416	very spread	0.539	0.461	5.379
4	1	Physcia millegrana	Treb. gelatinosa	Yes	21.825	16.194	5.631	spread	0.742	0.258	4.541
4	3	Physcia millegrana	Treb. gelatinosa	Yes	19.697	12.037	7.66	spread	0.611	0.389	3.915
5	2	Physcia millegrana	Treb. decolorans	No	80.482	15.4916	64.991	expanded	0.192	0.808	4.441
5	1	Physcia millegrana	Treb. gelatinosa	Yes	146.919	13.885	133.034	expanded	0.095	0.905	4.205

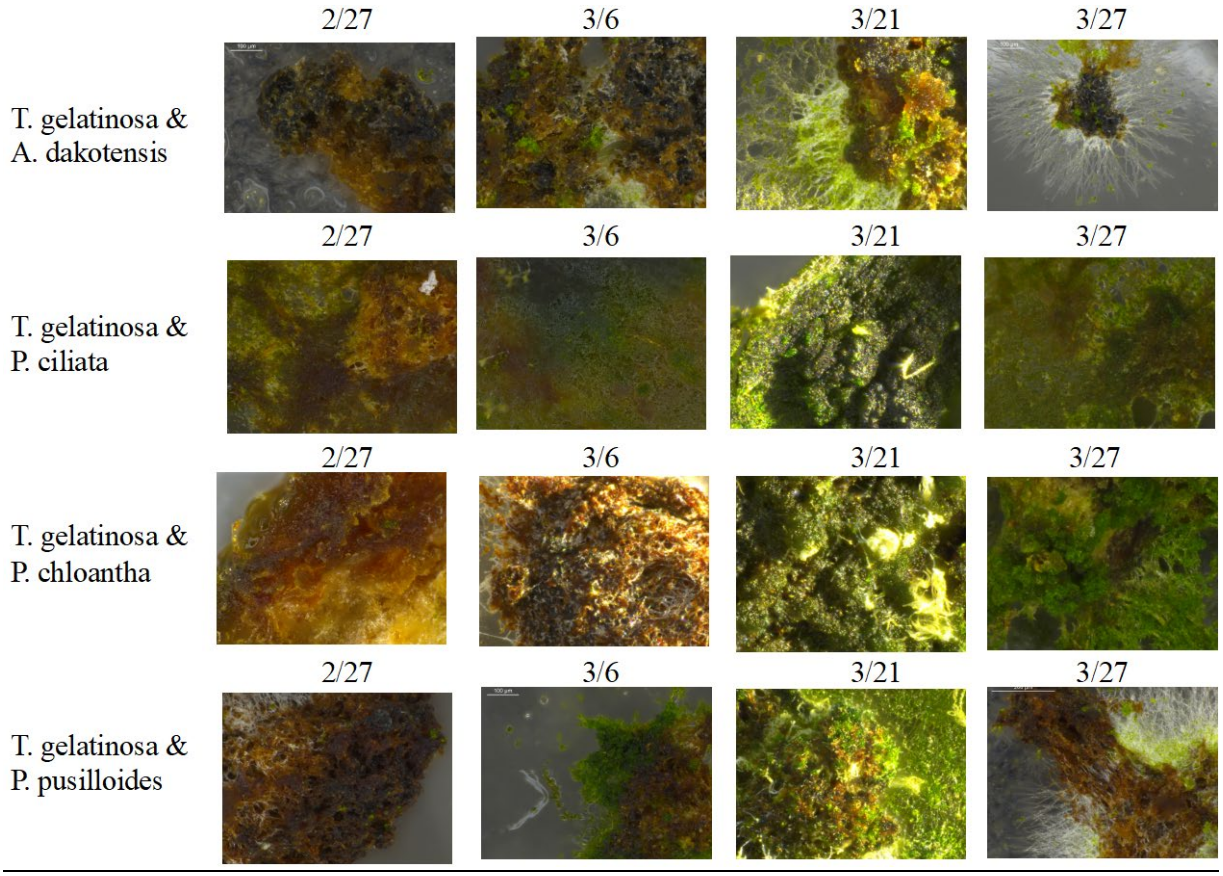
Supplemental Table 1: *Preliminary Experiment Data* - Dataset collected from *Physcia millegrana* cultures plated with naturally occurring photobiont *T. gelatinosa* and non-naturally occurring photobiont *T. decolorans*. Data was then tidied into an Excel spreadsheet and used for creation of Figure 3 in R. Categorical variables include "cluster" (the region of the plate used to keep track of smaller sections of fungal culture), "symbiosis" (whether this pairing was the 'correct' symbiosis seen in natural settings), and "algal distribution" (description of the algal spread over fungal area). The calculations done for area and growth rates, along with radius, were done using Fiji ImageJ. All area calculations are in mm^2.



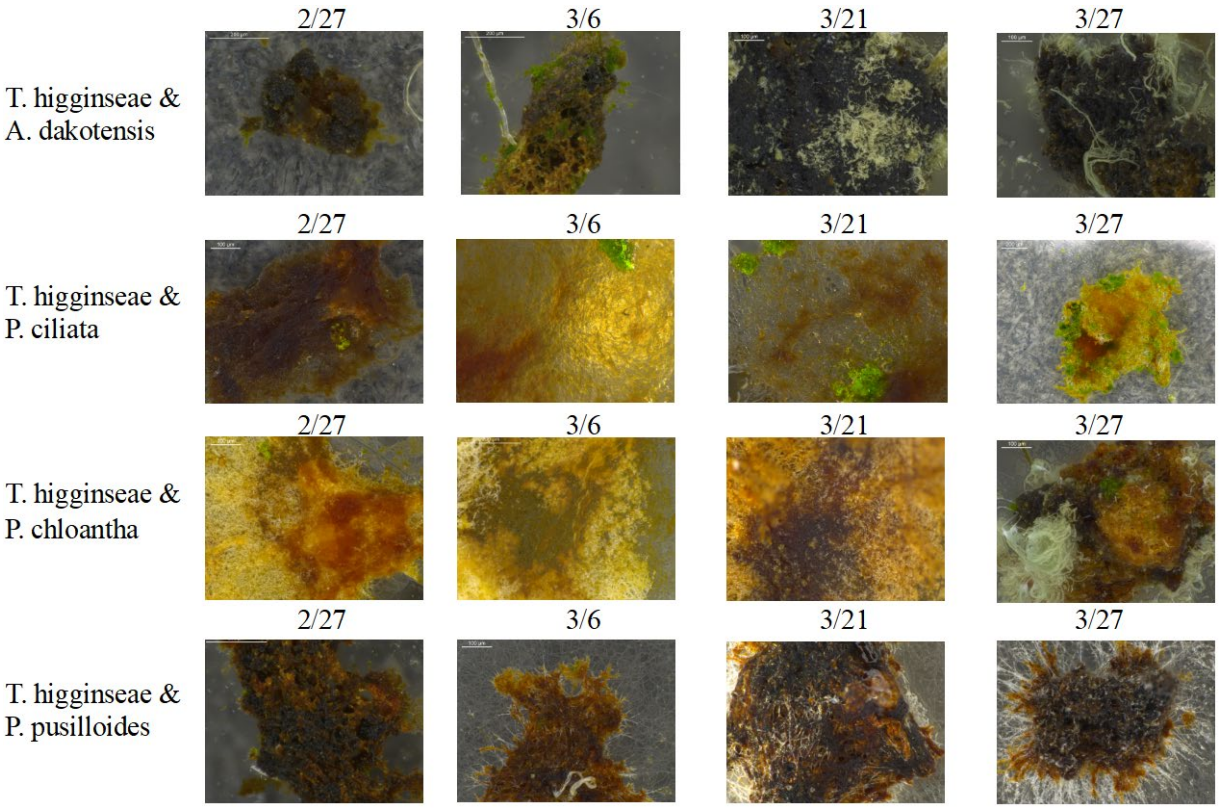
Supplemental Figure 2: *F. israelensis* - Images collected of various fungal cultures (including *Amandindea dakotensis*, *Phaeophyscia ciliata*, *Physciella chloantha,* and *Phaeophyscia pusilloides*, respectively) with non-naturally occurring photobiont *F. israelensis*. All were plated on Bold's Basal Media. Scale bars in top left-hand corner read 100 µm. Dates are listed above for each week. Each week's image is a unique section removed from the original culture and not replaced. Algal cultures are green in color, fungal cultures are dark or light brown. This pairing shows significant algal growth in nearly all samples for each week and minimal hyphal growth.



Supplemental Figure 3: *T. decolorans* - Images collected of various fungal cultures (including *Amandindea dakotensis*, *Phaeophyscia ciliata*, *Physciella chloantha,* and *Phaeophyscia pusilloides*, respectively) with non-naturally occurring photobiont *T. decolorans*. All were plated on Bold's Basal Media. Scale bars in top left-hand corner read 100 µm. Dates are listed above for each week. Each week's image is a unique section removed from the original culture and not replaced. Algal cultures are green in color, fungal cultures are dark or light brown. This pairing shows significant algal growth in nearly all samples for each week, and no hyphal growth.



Supplemental Figure 4: *T. gelatinosa* - Images collected of various fungal cultures (including *Amandindea dakotensis*, *Phaeophyscia ciliata*, *Physciella chloantha,* and *Phaeophyscia pusilloides*, respectively) with naturally occurring photobiont *T. gelatinosa*. All were plated on Bold's Basal Media. Scale bars in top left-hand corner read 100 µm. Dates are listed above for each week. Each week's image is a unique section removed from the original culture and not replaced. Algal cultures are green in color, fungal cultures are dark or light brown. This pairing shows significant algal growth in nearly all samples for each week, as well as hyphal growth in multiple pairings.



Supplemental Figure 5: *T. higginseae*- Images collected of various fungal cultures (including *Amandindea dakotensis*, *Phaeophyscia ciliata*, *Physciella chloantha,* and *Phaeophyscia pusilloides*, respectively) with non-naturally occurring photobiont *T. higginseae*. All were plated on Bold's Basal Media. Scale bars in top left-hand corner read 100 µm. Dates are listed above for each week. Each week's image is a unique section removed from the original culture and not replaced. Algal cultures are green in color, fungal cultures are dark or light brown. This pairing shows minimal algal growth in nearly all samples for each week.