Metagenome sequencing of the lichen species *Flavopunctelia flaventior* and *Parmotrema tinctorum* from Gauteng, South Africa.

by

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Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Master of Science Degree at the University of the Witwatersrand. It has not been submitted before for any degree or examination at any other University.

(Signature of candidate)

27 of June 2024 at 33 Oosterland Avenue, Bloubosrand, Randburg, 2188

Preface

Lichens are defined as a mutualistic association between fungi (mycobiont) and an algal and/or cyanobacterial photobiont. Increasing evidence suggests that lichens comprise more diverse microorganisms than initially thought, where lichens represent an interaction between archaea, bacteria, filamentous fungi, green algae, yeasts, and viruses. Not many comprehensive studies have been done of South African lichen species. The present study employed metagenome sequencing to investigate the lichen microbiomes of *Flavopunctelia flaventior* and *Parmotrema tinctorum* sampled from Bryanston, Gauteng province, South Africa. Furthermore, the roles played by the members of the lichen microbiome within symbioses were also studied by functionally annotating the assembled metagenomes of the two lichen species. This study sets the groundwork for future studies on South African lichen species.

In Chapter 1, an extensive literature review on lichens, their ecology, taxonomy and biology is discussed. Furthermore, it delves into the existence and shape of the microbiome beyond the mycobiont and the photobiont. Additionally, possible roles that the lichen microbiome may play in sustaining the lichen symbiosis is also discussed.

In Chapter 2, the metagenomes of two lichen species were sequenced, the quality of the reads were assessed, and taxonomic classification was performed to elucidate the composition of microorganisms associated with each lichen species. Both microbiomes were dominated by bacteria, with limited fungi, viruses, and archaea. The majority of the identified phyla and genera were found to be common between the two lichen species. Similarities in the core microbiome was accounted for by the fact that *F. flaventior* and *P. tinctorum* were sampled from the same location and they are both members of the *Parmeliaceae* family.

In Chapter 3, the metagenomic reads were assembled and functionally annotated using various bioinformatics tools. We demonstrate that the members of the lichen microbiome are involved in the cycling of nutrients such as carbon and nitrogen. We also found differences in carbon fixation pathways, which were attributed to the accessory microbiome.

Finally, a summary highlights key results and recommendations on future work that could be undertaken to further provide insight into biological pathways essential to sustain the lichen symbiosis.

Dedicated to my late grandfather Solomon Poeta Ditshego Katane

My late great-grandmother Joie Tshwenyego Rasogo

My mother Gladys Thale Katane,

And

My father Daniel Ramokgopang Katane,

All of you have been my pillar of strength,

Thank you for your support.

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3-hydroxypropionate (3HP)

3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB)

4-hydroxyburyrate/3-hydroxypropionate (HB/HP)

16S ribosomal RNA (16S rRNA)

Bayesian Re-estimation of Abundance after Classification with Kraken (Bracken)

C4-dicarboxylic acid (DC/HB)

Cadmium (Cd)

Candidate Phyla Radiation (CPR)

Carbohydrates-Active enZymes (CAZymes)

Carbon dioxide (CO_2)

Carboxylesterases (CE)

Chrysothrix chrysovirus 1 (CcCV1)

Cluster of Orthologous Groups (COGs)

Dissimilatory nitrate reduction to ammonia (DNRA)

Double-stranded DNA (dsDNA)

Double-stranded DNA bacteriophages (dsDNA phages)

Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG)

Glycosyl hydrolases (GH)

Genome Taxonomy Database Toolkit (GTDB-tk)

Glycosyltransferase (GT)

Hidden Markov model (HMM)

Horizontal gene transfer (HGT)

Internal transcribed spacer (ITS)

Kyoto Encyclopedia of Genes and Genomes (KEGG)

Lead (Pb)

Lepraria chrysovirus (LiCV1)

METabolic And BiogeOchemistry anaLyses In miCrobes (METABOLIC)

Metagenome assembled genomes (MAGs)

Mercury (Hb)

Metagenomic DNA (mDNA)

Minimal set of Pathways (MinPath)

Multi-Draft based Scaffolder (MeDuSa)

N-acetylglucosamine (NAG)

N-acetylmuramic acid (NAM)

Nucleo-Cytoplasmic Large DNA Viruses (NCLDVs)

Photosystem II (PSII)

Polymerase chain reaction (PCR)

Polysaccharide lyases (PL)

Ribulose 1,5-bisphosphate (RuBP)

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)

TYpe Genome Server (TYGS)

Ultraviolet (UV)

UniProt Knowledgebase (UniProtKB)

Chapter 1: Literature Review

1.1. Lichens

Lichens are obligatory, stable mutualistic associations between fungi (the mycobiont) and microalgae and/or cyanobacteria (collectively termed the photobiont) (Turetsky, 2003). A lichen consists of thalli, which are an integration of mycobiont tissues, as well as cells of the photobiont (Ahmadjian, 1993). The lichen thallus comprises the cortex and the medulla, which are made up of fungal tissues and a layer of fungal hyphae, respectively (Figure 1). These structures envelop the photobiont (algal zone). The cortex provides the photobiont with protection against excessive drying and light, while the medulla facilitates gaseous exchange. Within this symbiosis, the fungi provide structural support to the photobiont and allow for the efficient absorbance of water and nutrients (Turetsky, 2003). The photobiont uses the absorbed water, nutrients, and regulated sunlight for photosynthesis, providing the fungi with carbohydrates in return (Turetsky, 2003). In the case of cyanolichens (comprising a fungus and cyanobacterial partner), the cyanobacteria also provide the mycobiont with a source of nitrogen, through nitrogen fixation (Nash, 1996).

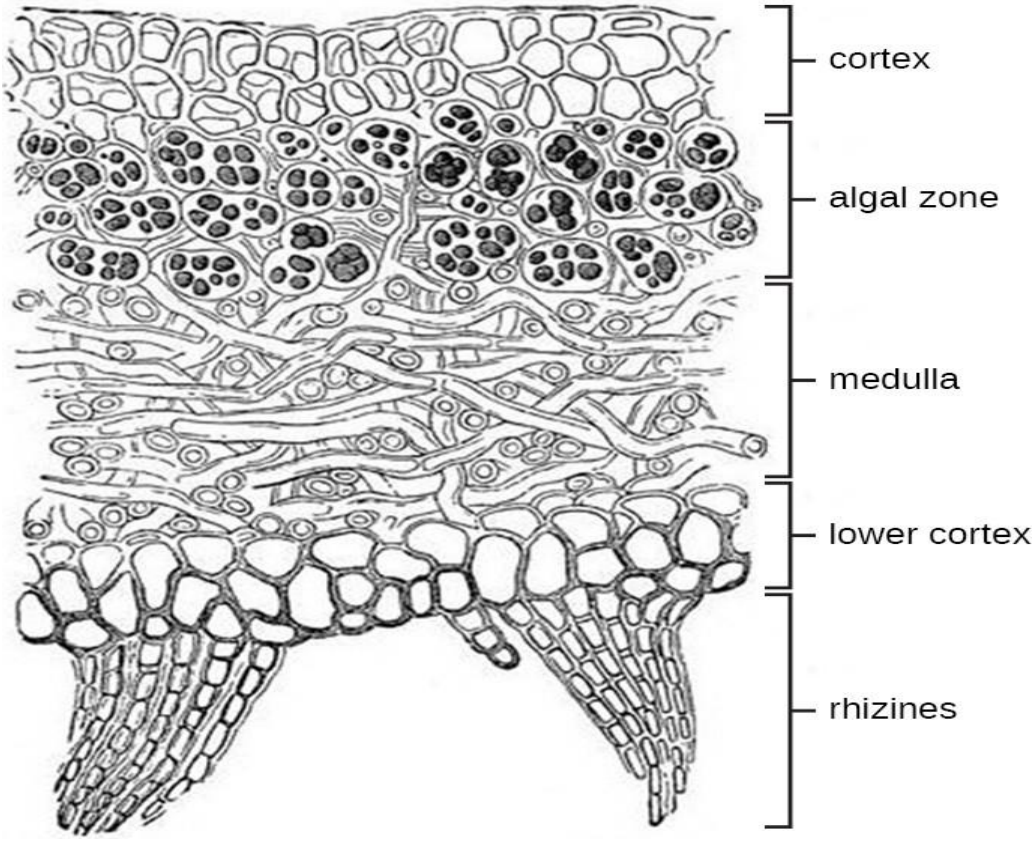

Figure 1: A cross-section showing the interaction between the mycobiont and the photobiont, within the lichen association. Adapted from Ahmadjian (1993).

While a lichen association is generally defined as a mutualistic relationship (Garty, 2001; Brunialti and Frati, 2014; Yousuf *et al*., 2014), some researchers have considered it as a form of controlled parasitism, as the mycobiont appears to benefit more than the photobiont (Ahmadjian, 1993; Nash, 1996). This idea is supported by the fact that the photobionts grow faster when they are free-living compared to when they are found within the lichen symbiosis (Garty, 2001; Brunialti and Frati, 2014; Yousuf *et al*., 2014). Additionally, no comparable nutrient flux from the fungi to the photobiont has been demonstrated other than the poorly investigated ability of the mycobiont to store inorganic nutrients for the photobiont (Ahmadjian, 1993; Nash, 1996). The observation of a lichen being a mutualistic association is, however, supported by the fact that both partners are equally important during the vegetative reproduction and growth of lichens (Watson, 1929). During vegetative reproduction, fungal filaments are wrapped around the algal cells to produce soredia, which are the reproductive structures of lichens (Ahmadjian, 1993; Nash, 1996). In the case where fungal reproduction occurs through spores, the mycobiont produces the spores while the photobiont supplies food and provides a conducive environment for the spore-bearing partner (Garty, 2001; Brunialti and Frati, 2014; Yousuf *et al*., 2014).

1.2. Lichen taxonomy

Lichens can be classified according to morphology, geographical location, the type of substrate on which they grow and the type of fungal partner they possess (Clerc and Herrera-Campos, 1997; Spribille *et al.*, 2008; Armstrong and Bradwell, 2011; Thell *et al.*, 2012). By evaluating their external feature, lichens are traditionally classified into three morphological groups, namely fruticose, crustose and foliose (Ahmadjian, 1993; Armstrong and Bradwell, 2011). Foliose lichens are those that appear leafy and have both a lower and upper cortex. They also have thread-like rhizines that facilitate their loose attachment to the substrate (Armstrong and Bradwell, 2011). Conversely, crustose lichens lack the lower cortex and attach closely to the substrate (Ahmadjian, 1993). They appear crust-like and thin on the surface of the substrate (Notov, 2014). Fruticose lichens may appear cup-like or hairlike and can hang down or be upright on the substrate (Notov, 2014).

On the basis of habitat, lichens are classified as corticolous, freshwater, lignicolous, marine, saxicolous and terricolous (Clerc and Herrera-Campos, 1997; Spribille *et al.*, 2008; Shukla *et al.*, 2013; Bilovitz *et al.*, 2014; Krzewicka *et al.*, 2017; West *et al.*, 2018). The lignicolous

lichens are those that inhabit the wood and bark of fallen tree branches (Spribille *et al.*, 2008), while corticolous lichens inhabit barks of healthy intact trees (Shukla *et al.*, 2013). Marine and freshwater lichens grow and occupy water bodies and may prefer areas that have a solid substrate for attachment (Krzewicka *et al.*, 2017; West *et al.*, 2018). Saxicolous lichens are rock inhabiting lichens (Clerc and Herrera-Campos, 1997), while terricolous lichens occupy the terrestrial environment and grow directly on soil (Bilovitz *et al.*, 2014).

With the development of modern taxonomic methodologies such as molecular approaches, a more comprehensive picture of lichen diversity has been developed (Mitchell, 2007: Hawksworth and Grube, 2020). It has been estimated that there are over 25,000 lichen species with Ascomycota as the predominant fungal partner (Yousuf *et al*., 2014). Approximately 40% of the members of the Ascomycota are lichenised fungi (Zedda and Rambold, 2015; Tripathi and Joshi, 2019). The taxonomy of lichens is founded on the mycobiont counterpart of the symbiosis and lichens are being integrated into fungal taxonomic schemes (Yousuf *et al*., 2014).

1.2.1. The lichen mycobiont

The lichen mycobiont can be a fungus of either the phylum Basidiomycota or Ascomycota (Zedda and Rambold, 2015; Tripathi and Joshi, 2019). Lichens incorporating Ascomycota are termed ascolichens and are classified into 60 lichenised orders (Thell *et al.*, 2012). Most of the ascolichens are classified under the order Lecanorales, which has around 20 families (Hafellner *et al.*, 1994). Among these, the family *Parmeliaceae* is the largest with 79 genera and over 2,700 species identified (Gómez-Serranillos *et al.*, 2014). This family comprises seven clades, with the Parmelioid clade being the largest (27 genera, 1,854 species), followed by the Usneoid (one genus, 350 species) and Cetrarioid (17 genera, 97 species) clades (Thell *et al.*, 2012; Divakar *et al.*, 2015).

Lichens whose mycobiont is from the phylum Basidiomycota are referred to as basidiolichens (Zhong *et al.*, 2020). They form a small proportion (<1%) of lichens and are distributed among the orders Hymenochaetales (Larsson *et al.*, 2006), Lepidostromatales (Hodkinson *et al.*, 2014), Agaricales (Zhao *et al.*, 2008), and Cantharellales (Wartchow *et al.*, 2012). The majority of the basidiolichens are found in the order Agaricales (350 genera, over 9,000 species) (Matheny *et al.*, 2006), which are further divided into two clades namely, Dictyonema *sensu*

lato (five genera, 148 species) and Lichenomphalia *sensu lato* (Lawrey *et al.*, 2009) and classified under the family *Hygrophoraceae* (Dal-Forno *et al.*, 2019).

1.2.2. The lichen photobiont

Photobionts are lichen partners who provide the mycobiont with sugars through photosynthesis and they also have their own taxonomic classification system (Friedl and Büdel, 2008). Green algae (also termed phycobionts) are the primary photobiont (90%) of most lichens while the remainder incorporate cyanobionts (cyanobacterial photobionts) (Weber and Büdel, 2011; Honegger, 2012; Zedda and Rambold, 2015). A limited number of lichen species have both phycobiont and cyanobiont associated with lichens, where the phycobiont is the main partner and the cyanobiont is secondary (Zedda and Rambold, 2015).

An estimated 50 genera and over 120 species of photoautotrophic partners of lichens, usually cyanobacteria or green algae, have been identified so far (Honegger, 2012). However, there are still some lichen phyco- and photobionts which have not been classified at the species level (Honegger, 2012; De Carolis *et al*., 2022). The most prevalent lichen phycobionts (green algae) belong to the genus *Trebouxia* (Kosecka *et al.*, 2022; De Carolis *et al*., 2022). The fact that many of the species within the genus *Trebouxia* are unculturable in media limits understanding of their species diversity (Nyati *et al.*, 2014). However, some species have been identified as being associated with lichens and they include *Trebouxia corticola* (clade C), *Trebouxia gigantea* (clade A) and *Trebouxia suecica* (clade S) (Xu *et al.*, 2020). Numerous cyanobacterial species, including members of the orders Chroococcales, Nostocales, Pleurocapsales and Stigonematales have been shown to be lichen cyanobionts (Friedl and Büdel, 2008). *Nostoc* is the most prevalent genus for cyanobacteria (Máguas *et al.*, 1993).

1.3. Lichen ecology

Lichens have been found in almost all known ecological niches on Earth, including terrestrial, fresh water and marine ecosystems (Pankratov *et al.*, 2017). In terrestrial niches, lichens can be found attached to different substrates including on trees, rocks, and undisturbed man-made surfaces such as bricks (Yousuf *et al*., 2014). Lichens can also be found in freshwater environments wherever there is a solid substratum, alkaline water, sufficient light and low to moderate silting (Nascimbene *et al.*, 2013). The species diversity and richness of freshwater

lichen ecosystems are influenced by ecological variables, such as water availability, substrate stability and eutrophication (Nascimbene *et al.*, 2007). Seashore lichens are most noticeable on rocks where they can cover the surface both completely and permanently (Sonina and Androsova, 2020). In contrast to freshwater lichens, little is known about the species diversity of marine lichens (Sonina and Androsova, 2020).

Lichens are adapted to grow in different climatic conditions, including those that are generally considered to be hostile to plants (Yousuf *et al*., 2014; Grimm *et al.*, 2021). Thus, they have been found in environments with limited light availability, extremely low and high temperatures, areas affected by drought, soils with high saline concentrations, areas with limited nutrients and even sites that are heavily polluted (Yousuf *et al*., 2014). Lichens, such as those of the genera *Peltigera*, and *Pseudocyphellaria*, survive in wet forests, where light is a major limiting factor. As a result, the lichen morphology frequently changes depending on the availability of light (Benson and Coxson, 2002). The thalli exposed to less light tend to be less pigmented and have fewer apothecia, a thinner cortex, and a greater number of soredia (Benson and Coxson, 2002; Armstrong, 2017).

Lichens found in alpine locations have been used as models to study the effect of environmental gradients at elevation (Armstrong, 2017; Leavitt *et al.*, 2021). Altitude may have an impact on a broad range of distinct lichens by reducing the spectrum of algal partners that the fungal partner has access to (Piercey-Normore and Deduke, 2011). These lichens display various adaptations in response to the progressively harsher conditions encountered at higher elevations (Armstrong, 2017), e.g. at higher elevations, *Cetraria nivalis* retains usnic acid in the upper cortex for the absorption of sunlight (Armstrong, 2017; Leavitt *et al.*, 2021). Since cell membranes comprise mainly of saturated fatty acids for stability, a decrease in saturation levels with altitude may indicate modifications in the composition of the cellular membrane (Armstrong, 2017). Lichens subjected to environmental stressors such as long severe winters and low heat output may have to undergo morphological adaptation in response to intense radiation, low temperatures, limited periods of metabolic activity, drought or wet conditions, persistent snow cover, or wind erosion (Thomson, 1995; Armstrong, 2017).

Lichens can also be recovered from a variety of chemically rich habitats, such as highly eutrophicated areas and environments affected by pollution, heavy metals and high salt concentrations (Armstrong, 2017). Lichens can acquire heavy metals from a range of sources, such as metal-enriched rocks, industrially contaminated areas, metallurgical facilities and

landfills (Anderson *et al.*, 2022). The ability of lichens to tolerate high metal concentrations may be attributed to the fact that they can immobilise the metals and facilitate the movement of their ions outside the cell wall and membrane (Armstrong, 2017; Anderson *et al.*, 2022).

1.4. Lichens and their roles in the environment

Lichens play several important roles in the environment, including the cycling of minerals, the fixation of nitrogen and the maintenance of soil integrity (Bates *et al*., 2011; Yousuf *et al*., 2014; Sierra *et al*., 2020). Nitrogen fixation involves the conversion of nitrogen from the atmosphere to ammonia and related nitrogenous compounds and is usually carried out by cyanobacteria in lichens (Máguas *et al.*, 1993; Zedda and Rambold, 2015).

The ability of lichens to fix atmospheric carbon dioxide (CO_2) enables them to contribute substantially to the flow of carbon (Bates *et al*., 2011; Yousuf *et al*., 2014; Sierra *et al*., 2020). They do this by facilitating the transfer of carbon from the atmosphere to the terrestrial ecosystems through respiration and photosynthesis (Nimis *et al.*, 2002). Cyanobacteria and/or the phycobiont (green algae) fix CO_2 and convert it to organic sugars via the Calvin cycle, using a series of light dependent and independent pathways (Pribil and Leister, 2017). In this cycle, ribulose 1,5-bisphosphate (RuBP), which accepts CO_2 is carboxylated in a reaction catalysed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Yang *et al.*, 2017).

In addition to the contribution made by lichens to the cycling of nutrients, they can bind together soil particles and provide aggregate stability to soils, thus shielding soil surfaces from wind and water erosion (Zedda and Rambold, 2015; Ghiloufi *et al.*, 2023). The stabilisation of the soil surface is substantially enhanced by the presence of rhizines and crustose lichen thalli (Jackson, 2015; Zedda and Rambold, 2015). The structure of the soil surface can be altered by lichen crusts, rendering it rougher and facilitating the retention of water and organic matter (Zedda and Rambold, 2015).

The surface of the thalli allows for the direct absorption of both nutrients and contaminants (Forbes *et al.*, 2009). As such, lichens are susceptible to numerous types of environmental toxins (Rola, 2020). They have therefore been extensively employed as bioindicators of environmental heavy metal pollution levels (Kłos *et al.*, 2018). Multiple factors such as the species, altitude, temperature, and moisture affect the accumulation of metals by lichens (Conti and Cecchetti, 2001; Backor and Loppi, 2009; Kłos *et al.*, 2018). Studies investigating the

accumulation of cadmium (Cd), mercury (Hb), and lead (Pb) in the thalli of *Hypogymnia physodes*, observed a correlation with altitude (Conti and Cecchetti, 2001; Backor and Loppi, 2009) indicating that the concentrations of Pb and Cd accumulated by the lichen rose with increasing altitude (Conti and Cecchetti, 2001; Backor and Loppi, 2009; Forbes *et al.*, 2009). Moreover, increased hydration was also associated with higher heavy metal accumulation (Conti and Cecchetti, 2001; Backor and Loppi, 2009; Kłos *et al.*, 2018). *Neophuscelia pulla* and *Xanthoparmelia taractica* have been used to study the emission of copper dust from mines (Conti and Cecchetti, 2001; Frank-Kamenetskaya *et al.*, 2021). A significant correlation was observed between the copper content in the lichen thalli and the soil (Frank-Kamenetskaya *et al.*, 2021).

Lichens do not have a stoma or cuticle to control the rate of gaseous exchange (Nash and Gries, 1991). As a result, contaminants are absorbed on the surface of the lichen, making them ideal bioindicators of air quality (Van der Wat and Forbes, 2015). One study indicated that there is a positive correlation between atmospheric sulphur dioxide and the sulphur content of lichens (Conti and Cecchetti, 2001). *Lobaria pulmonaria* is considered highly sensitive to sulphur dioxide, with an average concentration of 30 $\mu g\ m^{-3}$ during winter (Conti and Cecchetti, 2001). Another study has also shown that the concentration of chlorophylls *a*+*b* within *Usnea* spp. are directly proportional to automotive traffic emissions (sulphur oxide and nitrogen oxide) (Carreras, 1998). As traffic emissions increase, so too does the concentration of chlorophylls *a*+*b* in lichens (Carreras, 1998).

Lichens have an essential function in filtering impurities and eliminating pollutants from water and their different growth substrates (Ahmadjian, 1993; Zedda and Rambold, 2015). This is due to their capacity to retain water and atmospheric nutrients in the thallus (Forbes *et al.*, 2009). Lichens have effective systems for accumulating major and minor necessary elements by exchanging cations and absorbing soluble cations over the entire thallus surface (Ahmadjian, 1993; Zedda and Rambold, 2015). The process entails the reversible attachment of negatively charged areas of the cell wall to the surface of the plasma membrane (Cecconi *et al.*, 2021). Total element concentrations in the thallus are influenced by soil or atmospheric particles that adhere to its surface or become lodged in the hyphal weft (a loose layer of tissue in the medulla) (Ahmadjian, 1993; Zedda and Rambold, 2015; Cecconi *et al.*, 2021).

1.5. The lichen microbiome

The study of lichen biology has primarily focused on the mutualistic relationship between the photobiont and mycobiont. In recent years, however, it has been realised that the lichen association incorporates other microorganisms, including non-lichenised fungi, bacteria, archaea and viruses, which contribute to the functioning and biology of lichens (Grube and Berg, 2009). The microbiome is defined as a collection of the microorganisms and their gene and metabolic products found in a certain environment, such as soil or the digestive tract of humans (Knight, 2016; Taneja, 2017; Setubal and Dias-Neto, 2022). The lichen microbiome is considered one of the most diverse part of the lichen association, harbouring different microorganisms that coexist together to form a lichen (Grimm *et al.*, 2021).

Research has shown that the lichen microbiome has both core (shared between different lichen species) and specific microbial communities that contribute to the lichen association (Ahmadjian, 1993; Pankratov *et al.*, 2017; Grimm *et al.*, 2021). The discovery of the core and specific microbial communities led to an interest in establishing the roles of the lichen microbiome within the mutualistic association and studying the effects of the environment in shaping the composition of this microbiome (Hawksworth and Grube, 2020; Yang *et al.*, 2023).The lichen symbiotic notion must account for the broad array of microorganisms which form part of the association (Hawksworth and Grube, 2020). It can thus be considered a holobiont, with a fungus that dominates and an integrated microbiome (Simon *et al.*, 2019).

The process that establishes the microbial community of lichen associations is not well understood. One study has suggested that the diversity of the lichen microbiome was determined by either the photobiont or the environment (Sierra *et al*., 2020). Another study assessed the role of geography on the microbial composition of lichens and identified temperature and nutrient availability as important selection factors for the bacteria associated with the lichen (Pankratov *et al.*, 2017). Understanding the biotic and abiotic factors that affect the composition and diversity of the lichen microbiome may provide insights into what drives the development and diversity of the microbiomes associated with lichen species.

1.6. The lichen bacteriome

Prokaryotic abundance and taxonomic diversity in lichens have been the subject of integrative research over the past two decades (Pankratov *et al.*, 2017). The first evidence of bacteria within lichen associations was observed when the deltaproteobacterium *Melittangium*

lichenicola was found in lichens in 1892 (Thaxter, 1892). A more recent study used nutrient media which was selective for *Actinomycetes* to isolate and characterise individuals from eight families within the order *Actinomycetales* from saxicolous (rock inhabiting) lichens (González *et al.*, 2005). The bacterial genera that predominated these lichens were observed to be *Micromonospora* and *Streptomyces* (González *et al.*, 2005). A similar technique using media selective for nitrogen fixing bacteria was employed by Brazilian researchers to separate several bacterial cultures from the cyanobacterial lichens *Canoparmelia* and *Parmotrema* (Liba *et al.*, 2006). The isolated bacteria were identified using 16S rRNA sequencing and members of Gammaproteobacteria including isolates from the genera *Acinetobacter, Pantoea, Pseudomonas, Serratia*, and *Stenotrophomonas* were identified (Liba *et al.*, 2006).

Using culture media as a means of identifying prokaryotes associated with lichens has drawbacks, such as the absence of standardisation in the conditions and duration of incubation, the type of media used and the prevalence of unculturable microbial taxa (Lee *et al.*, 2013; Zapka *et al.*, 2017). More in depth insights into the complexity of the bacterial community associated with lichens has been facilitated through the use of molecular genetic techniques (Pankratov *et al.*, 2017). Fluorescent *in situ* hybridisation utilising targeted primers in conjunction with scanning confocal laser microscopy demonstrated the dominance of the class Alphaproteobacteria, and in particular the family *Acetobacteriaceae*, known to thrive in nutrient-deficient conditions, in the lichen *Cladonia arbuscula* (Cardinale *et al.*, 2006). By employing the same methods to analyse the microbial communities of the lichen species *Umbilicaria cilindrica*, *Lecanora polytropa*, and *C. arbuscula*, the dispersion of bacteria throughout the thallus and the dominance of Alphaproteobacteria were further confirmed (Grube and Berg, 2009).

The evolutionary structure that defines lichen microbial populations is influenced by the type of substrate, climate, and lichen species (Pankratov *et al.*, 2017). Alphaproteobacteria likely predominate in lichens for the same reasons they do in other acidic and nutrient-deficient substrates, such as sphagnum peat, soils with reduced organic matter content and freshwater aquatic habitats (Pankratov *et al.*, 2017). Extracellular polysaccharides, which are produced by the majority of Alphaproteobacteria, may be involved in the growth and maintenance of lichen thalli (Pankratov *et al.*, 2017).

1.6.1. The role of the bacteriome in lichen associations

The bacteria in lichen associations facilitate various processes including the uptake of phosphorus, the cycling of nitrogen and detoxification by breaking down toxic organic compounds (Grube and Berg, 2009; Grube *et al*., 2009; Bates *et al*., 2011). Nitrogen is delivered to the symbiotic partners by nitrogen-fixing taxa (Grube and Berg, 2009). Both bacteria residing within the thalli and those in substrate regions impacted or accessed by mycobiont hyphae (the hypothallosphere) may be involved in this process (Almendras *et al.*, 2018). The nitrogen budget of lichens may be improved by the release of nitrogenated substances such as amino acids (Liba *et al.*, 2006). This may be especially pertinent for lichens lacking cyanobacterial connections, as they would otherwise be forced to rely on nitrogenated substances either from their substrate or from external sources such as air, bird droppings and water (Grube and Berg, 2009; Grube *et al*., 2009; Bates *et al*., 2011).

Bacteria have demonstrated the ability to be major players involved in glucanolytic, cellulolytic and proteolytic activity within the lichen symbiosis (Grube and Berg, 2009; Pankratov *et al.*, 2017). As fungal cell walls are intricate blends of polysaccharides and proteins, these bacteria may be implicated in the destruction of lichen sections that are senescent (Pankratov *et al.*, 2017). In order to encourage growth in other crucial portions of the lichen, degraded lichen material might be mobilised again in the thallus structures (Grube and Berg, 2009). Bacteria further play a role in lichen defense against pathogens and feeders (Grube and Berg, 2009). Actinobacteria, which are commonly associated with lichens, are especially important due to their ability to produce a broad range of antimicrobial substances (González *et al.*, 2005).

1.6.2. The lichen archaeome

The thalli of rock inhabiting lichens were also demonstrated to contain members of the domain Archaea using molecular techniques, providing the first indication of archaeal presence in lichens (Bjelland *et al.*, 2011). A proteomic study of *Lobaria pulmonaria* supported these findings (Schneider *et al.*, 2011). Archaea of the phylum Thaumarchaeota have been found associated with the lichens *Hydropunctaria maura*, *Ophioparma ventosa*, *Pertusaria corallina* and *Rhizocarpon geographicum*, although in considerably lower quantities than bacteria (Bjelland *et al.*, 2011). To date, no study has focused on the complex community structure of archaea, instead focusing primarily on the investigation of the bacterial community (Pankratov *et al.*, 2017). More research on archaea in relation to the lichen microbiome is required to shed

light on the diversity and roles of members of this domain in lichens. Within the lichen symbiosis, members of the phylum Thaumarchaeota may be implicated in the cycling of nitrogen through the oxidation of ammonia as well as being able to fix carbon through the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle (Stieglmeier *et al.*, 2014; Nelkner *et al.*, 2023).

1.7. The lichen mycobiome

The notion that the fungal partner is the only factor that determines the characteristics of a lichen has been called into question recently by a hidden component in the symbiotic relationship of lichens (Hawksworth and Grube, 2020). This component includes basidiomycetous yeasts of the class *Cystobasidiomycetes* and sub-phylum *Pucciniomycotina* that were observed in the thallus cortex of pendent lichens (Spribille *et al.*, 2016; Pankratov *et al.*, 2017). By utilising a PCR assay and highly specific primers to find members of these yeast lineages in a range of lichens, it was demonstrated that these yeasts were an essential part of the top cortex (Spribille *et al.*, 2016). Some yeasts were shown to produce indole-3-acetic acid, which may be responsible for promoting the growth of the lichen thalli, as well as increasing water absorption and biomass of lichens (Tang *et al.*, 2023). The notion that yeasts may have a unique regulatory role within mutualistic associations of lichens was supported by the identification of basidiomycetous yeasts in the lichen cortex (Pankratov *et al.*, 2017).

A major component of the lichen mycobiome is contributed by the endolichenicolous and pathotropic (causes harm to host to acquire carbon) lichenicolous fungi (Lawrey and Diederich, 2003; Muggia *et al.*, 2011; Yang *et al.*, 2023). The majority of the studies that have been conducted so far on endolichenicolous were focused on the different metabolites they produce within the lichen association, most of which have antimicrobial properties (Shrestha and Clair, 2013; Suzuki *et al.*, 2016; Goga *et al.*, 2020). These lichen-derived secondary metabolites have been shown to provide the lichen with protection against opportunistic pathogens and contribute to structuring the composition of the lichen bacteriome (Kosanić and Ranković, 2019; Goga *et al.*, 2020). Other secondary metabolites are involved in forming fungal galls which are important habitats for members of the lichen mycobiome (Muggia *et al.*, 2017). Additionally, some secondary metabolites have been shown to facilitate the decomposition of the lichen thalli, thus catering for saprotrophic (feed on decaying organic matter) fungi within the symbiosis (Aptroot and Alstrup, 1999). More studies are required to comprehensively study

the roles ascomycetous fungi play within the lichen symbiosis as they constitute the majority of fungi found in lichens (Yang *et al.*, 2023).

Aside from providing structural support to the photobiont, the mycobiome can contribute to lichen symbioses in different ways (Grube and Berg, 2009; Grimm *et al.*, 2021; Tagirdzhanova *et al.*, 2021). A recent study suggested that the mycobiome produces polysaccharides that contribute to the formation of the extracellular matrix, within the lichen association (Tagirdzhanova *et al.*, 2021). The mycobiome could also play a role in offering the mycobiont protection against other pathogenic fungi, through the production of secondary metabolites that have antifungal properties (Goga *et al.*, 2020). Additionally, fungal taxa in lichens may be involved in producing ethanol, which may act as a carbon source for other microorganisms within the lichen symbiosis (Lin and Tanaka, 2006). Furthermore, the antiseptic properties of ethanol at higher concentrations may contribute to shaping the community of microorganisms forming part of the lichen (Lin and Tanaka, 2006; Kalra *et al.*, 2021).

1.8. The lichen virome

In addition to a complex bacteriome and mycobiome associated with lichens, there is increasing evidence that lichens harbour a viral community, or virome (Merges *et al*., 2021; Grimm *et al.*, 2021). Plant viruses such as *Cytorhabdovirus* and Apple mosaic virus have been observed in lichens (Petrzik *et al.*, 2014). Another study on *Chrysothrix chlorina* and *Lepraria incana* lichens sampled in the Czech Republic reported the close association of *Chrysothrix chrysovirus* 1 (CcCV1) and *Lepraria chrysovirus* (LiCV1) with the mycobionts in these lichens (Petrzik *et al.*, 2019).

Bacteriophages from the families *Myoviridae*, *Podoviridae* and *Siphoviridae* (Caudovirales) were reported as being associated, as prophages, with bacteria within the microbiome of the lichen *Peltigera* (Garg *et al.*, 2016). A metagenome sequencing study found viruses from the families *Caulimoviridae*, *Myoviridae*, *Podovidae*, and *Siphoviridae* in the lichen *Umbilicaria phaea* (Merges *et al*., 2021; Grimm *et al.*, 2021). It was further determined that the green algae photobiont *Trebouxia* of the lichen species *U. phaea* harboured a representative of the genus *Caulimovirus* (Merges *et al*., 2021). Additionally, in a study that was reviewing the symbiotic relationship between *Cladonia grayi* and its photobiont *Asterochloris glomerata* using genomic analysis, the genome of the lichen photobiont showed evidence of remnants of a double-

stranded DNA (dsDNA) virus classified under the family *Phycodnaviridae* integrated as viral insertions (Armaleo *et al.*, 2019).

Bacteriophages infecting archaea and bacteria within the lichen association may benefit the symbiosis through their involvement in horizontal gene transfer (HGT), which can occur directly via transduction resulting in a prophage, or indirectly via transformation of DNA from lysed cells (Roossinck and Bazán, 2017). Additionally, by killing opportunistic pathogens, viruses may protect the members of the lichen microbiome (Merges *et al.*, 2021). Metagenomic studies aimed at exploring the diversity and composition of the lichen virome are essential to further understand the potential roles of the virome in sustaining the symbiosis.

1.9. Aims and objectives

This study aimed to sequence, assemble, and annotate the metagenomes of two lichen species, *Flavopunctelia flaventior* and *Parmotrema tinctorum* from South Africa. This was done with the overarching aim to gain insights into the complete microbiome associated with these lichen species as well as how these microorganisms may contribute to the overall lichen symbiosis.

1.9.1. Objectives

1. Sampling of *F. flaventior* and *P. tinctorum* from Bryanston, Gauteng, South Africa.

2. Metagenomic DNA (mDNA) extraction, library preparation and metagenome sequencing.

3. Performing high coverage metagenome assemblies of the two lichen microbiomes.

4. Taxonomic profiling of the lichen microbial communities.

5. Contrasting the metagenomes of the *F. flaventior* and *P. tinctorum* lichen microbiomes and predicting the potential roles of the lichen associated microorganisms to the lichen symbiosis.

1.10. References

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Chapter 2: Metagenomic diversity analysis of *Flavopunctelia flaventior* and *Parmotrema tinctorum* lichen microbiomes

Abstract

The symbiotic interactions between fungi with one or more photosynthetic partners has been thought of as the definition of lichens. It has been demonstrated that lichen symbioses are much more sophisticated, involving other collaborating organisms, such as non-photosynthetic bacteria, auxiliary fungi, viruses, and algae. In the present study, the metagenome reads of *Flavopunctelia flaventior* and *Parmotrema tinctorum* were analysed using Kraken and Braken2, to characterise the lichen microbiomes. At domain level, bacteria accounted for more metagenomic reads than the fungal counterpart, with archaea and viruses contributing fewer reads. Actinomycetota and Pseudomonadota were the most prevalent bacterial phyla within the two lichen bacteriomes. *Streptomyces* and *Nacordioides* of the Actinomycetota and *Methylobacterium*, *Sphingomonas*, *Bradyrhizobium*, and *Pseudomonas* of the Pseudomonadota were the most abundant bacterial genera. The most prevalent mycobiome phylum was Ascomycota, with Basidiomycota being less abundant. *Aspergillus* and *Fusarium* were the most abundant fungal genera across the mycobiome in both lichen species. Archaea were also identified, with the phylum Euryarchaeota accounting for 99% of archaeal reads. The dominating archaeal genera were *Haloquadratum* and *Halarchaeum*. Uroviricota was the most abundant viral phylum with a relative abundance of 98.25 and 96.89% for *P. tinctorum* and *F. flaventior*, respectively. *Alphabaculovirus*, *Pandoravirus*, *Timquatrovirus* and *Fromavirus* were the most abundant viral genera. The core microbiome observed between the *P. tinctorum* and *F. flaventior* lichens may be a result of shared geography and climatic conditions, as both lichen species were isolated from the same location. Alternatively, the shared microbiome may be inherent of the taxonomy of the lichen species, as both are representative of the family *Parmeliaceae*. More research has to be done to decipher the roles that the lichen microbiomes play within the symbioses.

2.1. Introduction

Lichens are among the oldest and most diversified symbioses on Earth (Yuan *et al.*, 2005). Lichens are obligatory, stable mutualistic associations between fungi (the mycobiont) and microalgae and/or cyanobacteria (collectively termed the photobiont) (Hale, 1979; Ahmadjian, 1993; Nash, 1996; Bačkor and Loppi, 2009; Printzen, 2010; Armstrong and Bradwell, 2011; Notov, 2014; Sierra *et al.*, 2020; Grimm *et al.*, 2021). However, lichen associations have been shown to be far more complex when it comes to species composition and symbiotic associations.

Metagenomic studies have revealed that lichens, in addition to the photobiont and mycobiont, include a broad spectrum of bacteria, fungi and viruses, all of which contribute to the functioning of these complex ecosystems (Sierra *et al.*, 2020; Merges *et al*., 2021; Grimm *et al.*, 2021). The predominant bacterial phyla within the lichen microbiome are Acidobacteriota, Actinomycetota, Bacteroidota, Cyanobacteriota, Pseudomonadota, and Verrucomicrobiota (Bates *et al*., 2011; Sierra *et al*., 2020). Fungi that associate with lichen include members of the phyla Ascomycota, Basidiomycota, Chytridiomycota and Glomeromycota and are generally found in both lower abundance and diversity than their bacterial counterparts (Smith *et al*., 2020). To date, the viruses reportedly in close association with the lichen mycobiont are predominantly *Chrysothrix chrysovirus* 1 (CcCV1) and *Lepraria chrysovirus* (LiCV1), while the lichen photobionts have remnants of a double-stranded DNA (dsDNA) virus insertion, classified under the family *Phycodnaviridae* (Merges *et al*., 2021).

The lichen microbiome is considered to be one of the most diverse, structurally integrated, and abundant aspects of the lichen association (Grimm *et al*., 2021). Studies have shown that the lichen microbiome is not merely an extension of prokaryotic microorganisms that reside in close proximity to the lichens but instead have both the core and specific microbial communities that contribute to the lichen association (Ahmadjian, 1993; Pankratov *et al*., 2017; Grimm *et al*., 2021). Core microbiomes are microbial communities that are persistently linked to a particular host or present in a sizable portion of samples from a specific environment and have been discovered and categorised in sponges, corals, insects, plant roots, lichens and mammals (Shade and Handelsman, 2012; Sanders *et al.*, 2014; Ainsworth *et al.*, 2015; Yeoh *et al.*, 2017; Sierra *et al.*, 2020). The accessory microbiome consists mainly of, but is not limited to, microorganisms that do not affect the fitness of the host (Vos, 2023). Some specific microbiomes can have pathogenic effects, while some may be beneficial to the host (Grimm *et al.*, 2021; Vos, 2023). To fully understand the ecology of microbial consortia, it is imperative

to identify the core species, as it has been suggested that these frequently occurring organisms, are probably essential to the operation of that type of community (Ochman *et al.*, 2010; Shapira, 2016; Sierra *et al.*, 2020).

Given the intricacy of the symbiotic lichen structures, it is crucial to study lichen microbiomes in order to comprehend their ecological function (Sierra *et al.*, 2020). In this study, the complete metagenomes of two South African lichen species, *Flavopunctelia flaventior* and *Parmotrema tinctorum*, were sequenced and their microbial community composition analysed *in silico.* These lichen species were selected because they are readily accessible, can be found on the majority of trees within the study area (Adams and Gottardo, 2012). These lichens are also easy to remove from their substrate. In South Africa, a total of 32 and two species have been identified within the genera *Parmotrema* and *Flavopunctelia*, respectively (Fryday, 2015). Members of the lichen genus *Parmotrema* has been implemented in biomonitoring studies of lead and manganese pollution in Pretoria (Forbes *et al.*, 2009). Additionally, the species within this genus have also been applied to monitor pollution of trace elements in the atmosphere in the Western Cape (Ndlovu *et al.*, 2019). Similarly, species within the genus *Flavopunctelia* have been used to monitor atmospheric pollution of trace elements in Mexico (Rangel-Osornio *et al.*, 2022). To date, limited studies have been performed on these two lichen genera in South Africa.

Our analysis revealed diverse and distinct communities of microorganisms associated with the two lichen species, dominated by bacteria, but also comprising diverse fungal, archaeal, and viral taxa. Furthermore, we highlight the common taxa as well as those specific to the two lichen species, despite them occupying the same ecological niche. More research is required to further understand the roles the members of the lichen microbiomes play within the lichen symbioses.

2.2. Methodology

2.2.1 Lichen sampling

Two lichen species, namely *F. flaventior* and *P. tinctorum*, were sampled (in triplicate) from the bark of *Melia azedarach* (Syringa tree, native to Indomalaya and Australasia) from Hamilton Park in Bryanston, Gauteng (-26.047399, 28.04250) in February of 2022. These lichen species were selected for this study because they are ubiquitous across the study region,

and they are easy to separate from their substrate (Adams and Gottardo, 2012). The base part of the lichen was removed from the tree bark using sterile forceps and stored in sterile glass jars. Between sample collections, forceps were sterilised using 70 % (v/v) ethanol to avoid cross contamination. The lichen samples were then aseptically cleaned of dirt, insects, and bark material in the laboratory and were processed on the same day as collection.

2.2.2. Identification of the lichen samples

A morphological analysis was performed to classify the two lichen species. This was achieved by assessing the physical characteristics of the lichen specimens, which included the thallus appearance, rootlet type, the colour of the upper and lower surfaces, the surface pattern, and the presence or absence of fruiting bodies (Notov, 2014). The interactive lichen identification guide was used to compare the physical characteristics and to confirm the genera of the lichen species (ISpot Keys | Foliose Lichens on Trees (v. 1.0), 2021). The Lichens of South Africa checklist was then used to confirm the geography of the sampled lichens (Fryday, 2015).

2.2.3. Metagenomic DNA extraction and metagenome sequencing

Metagenomic DNA (mDNA) was extracted from 100 mg of three replicate lichen samples of each species using the ZymoBIOMICS DNA Miniprep kit (Zymo Research, United States of America) according to the protocol of the manufacturer. The lichen samples were added to 750 µl ZymoBIOMICS lysis buffer. In the subsequent lysis step, the Disruptor Genie (Scientific Industries Inc, United States of America) was used to bead beat the samples for eight minutes. Subsequently the samples were centrifuged at 14,000 x g for two minutes. The supernatant (400 µl) was then transferred to a Zymo-spin filter placed in a collection tube and centrifuged at 10,000 x g for one minute. After this, 1,200 µl of the DNA binding buffer was added to the collection tube and mixed with the filtrate by pipetting up and down. The resulting mixture (800 µl) was transferred to a spin column and centrifuged at 10,000 x g for one minute. The liquid collected was discarded and the spin column was centrifuged again at 10,000 x g for one minute.

Three DNA washes were performed, first by transferring the spin column to a clean column and adding 400 µl of DNA wash buffer one. During the second and third wash, 700 µl and 200 µl of DNA wash buffer two was used, respectively. At each wash step, the spin column

was centrifugation at 10,000 x g for one minute and the flow through discarded. Then 50 µl of DNase/RNase-free water was used to elute the mDNA from the spin column by centrifuging at 10,000 x g for one minute. The concentration and quality of the mDNA (Table A1) were analysed using a Nanodrop 1000 spectrophotometer (ThermoFisher, United States of America).

To assess the efficacy of mDNA extraction, polymerase chain reaction (PCR) was performed using the ITS (internal transcribed spacer) 1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 primers (5'-TGTGTTCTTCATCGATG-3') using the Bio-Rad T100 Thermocycler (Bio-Rad Laboratories, United States of America) (Gardes and Bruns, 1993; Andreev *et al.*, 1998). PCR cycling was performed as follows: initial denaturation at 95 ^{o}C for 30 seconds; 32 cycles of: denaturation at 95 ^{o}C for 30 seconds, annealing at 55 ^{o}C for 30 seconds, and elongation at 68 ^{o}C for 1 minute; final elongation at 68 ^{o}C for 5 minutes. The amplified mDNA was electrophoresed on 0.8% (w/v) agarose gel supplemented with 10 µg/µl ethidium bromide and viewed under ultraviolet (UV) light.

The extracted mDNA samples with the highest concentrations were selected for metagenome sequencing at Molecular Research LP (MR DNA, Clearwater, Texas, United States of America). Sequencing libraries were prepared by MR DNA by fragmenting the mDNA and ligating Nextera adaptors at the end of the fragments (Illumina Adapter Sequences, 2021). The Illumina NovaSeq 6000 platform was used for sequencing using the 2 x 250bp paired-end sequencing configuration for an expected yield of ten million reads.

2.2.4. Metagenomic sequence quality control

The quality of the raw metagenomic sequence reads was assessed using FastQC (Andrews, 2010; Ewels *et al*., 2016). The raw paired-end reads were submitted to the cloud-based cluster of InsideDNA (InsideDNA, 2017) and bad tiles, representing reads in the provided tile with lower quality reads than in other tiles, were removed using the RemoveBadTiles tool (InsideDNA, 2017). The resultant reads were then trimmed using Trimmomatic (Bolger *et al.*, 2014). Trimmomatic is an optimised preprocessing tool that filters poor quality reads and removes adapters for improved downstream analyses (Bolger *et al.*, 2014; Chen *et al*., 2018). Nucleotides with bad quality ends were trimmed, using nucleotide position 232 as the cropping point (CROP:232). Overrepresented sequences were removed by setting the HEADCROP parameter to 15 nucleotide position (HEADCROP:15). The Nextera-PE adapters dataset was used (ILLUMINACLIP:Nextera-PE.fa) to remove adapters and possible contaminating

sequences. The SLIDINGWINDOW:4:30 command was used to trim both ends of the reads that had a Phred (Q) score below 30, setting the window size to four nucleotides. Lastly, the (MINLEN:150) command was used to remove all read pairs that were < 150 bp in length.

2.2.5. Taxonomic assessment of metagenomic datasets

The trimmed and curated reads were subjected to Kraken v1.1.1 (Lu *et al.*, 2022). Kraken is a classification predictive tool that makes use of exact-match database searches of k-mers, rather than sequence alignment, which often includes imperfect matches, thereby reducing computing time (Mande *et al*., 2012; Wood and Salzberg, 2014). It outperforms conventional classifiers and abundance estimating programs in terms of speed, while maintaining accuracy on a level with the most effective sequence classification techniques (Wood *et al.*, 2019; Lu *et al.*, 2022).

The analysis was undertaken in accordance with the following workflow: a custom Kraken database was generated using *kraken-build* (Sierra *et al.*, 2022). The database was generated from the bacterial, archaeal, and viral RefSeq databases, along with complete plasmid genomes and UniVec Core (a database used to find nucleic acid sequence regions that might be vector-derived) from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). Furthermore, the entire collection of protozoan genomes, algal plant genomes and all fungal genomes from the NCBI database were included in the database (Lu *et al.*, 2022; Sierra *et al.*, 2022). The metagenomic read datasets for both lichen species were classified by comparing against the database using Kraken v1.1.1 with default settings (k = 31).

Bracken2 (Bayesian Re-estimation of Abundance after Classification with Kraken) was further used for taxonomic classification (Lu *et al.*, 2017). Bracken2 is a highly reliable statistical approach for calculating species abundance in mDNA sequences (Lu *et al.*, 2022). The program estimates abundance at species, genus, or higher taxonomic levels by providing an estimate of the number of reads per classification rank, using taxonomic assignments made by Kraken v1.1.1. The kreportmpa.py script was used to convert Bracken2 reports into the MetaPhlAn format (MetaPhlAn: Metagenomic Phylogenetic Analysis, 2023), and the resultant files were used for taxonomic profiling.

2.3. Results and discussion

2.3.1. Lichen identification

Based on morphology and the interactive lichen identification guide, the two lichen species sampled in this study were identified as *Flavopunctelia flaventior* and *Parmotrema tinctorum* (Figure 2.1 and Figure 2.2). The *F. flaventior* lichen species (Figure 2.1), commonly known as speckled greenshield, belongs to the genus *Flavopunctelia* and the family *Parmeliaceae* (Brodo *et al.*, 2001). This lichen was first recognised as a distinct *Parmotrema* species in 1877 by James Stirton, prior to its transfer to the subgenus *Flavopunctelia* by Hildur Krog (Krog, 1982). Subsequently, the subgenus was raised to genus status, with *F. flaventior* as the type species (Hale, 1989). This species occurs in East and Southern Africa, Asia, Europe and both North and South America (Nash *et al*., 2004; Sohrabi and Jamshidikia, 2007). It is corticolous, growing on tree trunks and has large foliose thalli that are closely attached to the bark, crowded broad sub-irregular lobes, wavy margins, and an upper surface that is yellowish green to grey in colour. It also has a dark brown to black lower surface, with rhizines acting as a basal attachment to the tree trunk.

Figure 2.1: *Flavopunctelia flaventior* species sampled from Bryanston in Gauteng province, South Africa. Photo by Pascale Naude, 2 February 2022.

The lichen species *P. tinctorum* (Figure 2.2), commonly known as Palm Ruffle, belongs to the family *Parmeliaceae* and has a near-global distribution. (Hale, 1979; Nash *et al*., 2002; Nash *et al*., 2004). It grows on trees, having foliose thalli that are loosely attached to the substrate, imbricate elongated sub-irregular lobes, entire margins, and a grey smooth upper surface. It

also has a black lower surface, with rhizines serving to aid attachment to tree trunks (Nash *et al*., 2002; Nash *et al*., 2004).

Figure 2.2: *Parmotrema tinctorum* species sampled from Bryanston in Gauteng province, South Africa. Photo by Pascale Naude, 2 February 2022.

2.3.2. Lichen metagenome sequences

High quality mDNA was extracted from both lichen species (Appendix A1). Metagenome sequencing with the Illumina® HiSeq6000 platform yielded a total of 23,300,359 and 30,311,225 paired-end reads for *P. tinctorum* and *F. flaventior*, respectively. Quality control of the raw reads with FastQC showed that the datasets were of good quality, with Phred (Q) scores >25 and lower quality tails in longer sequences (Figure 2.3A and C). The observed quality decline at the ends of the reads could be attributed to signal decay (due to degrading fluorophores or a proportion of strands not being elongated) and phasing (failure of nucleotides to incorporate due to incomplete removal of the 3' terminators and fluorophores) (Khetani, 2018). The trimmed reads had a Q-score >34 (Figure 2.3B and D), which correlates with a base call accuracy of > 99.95%.

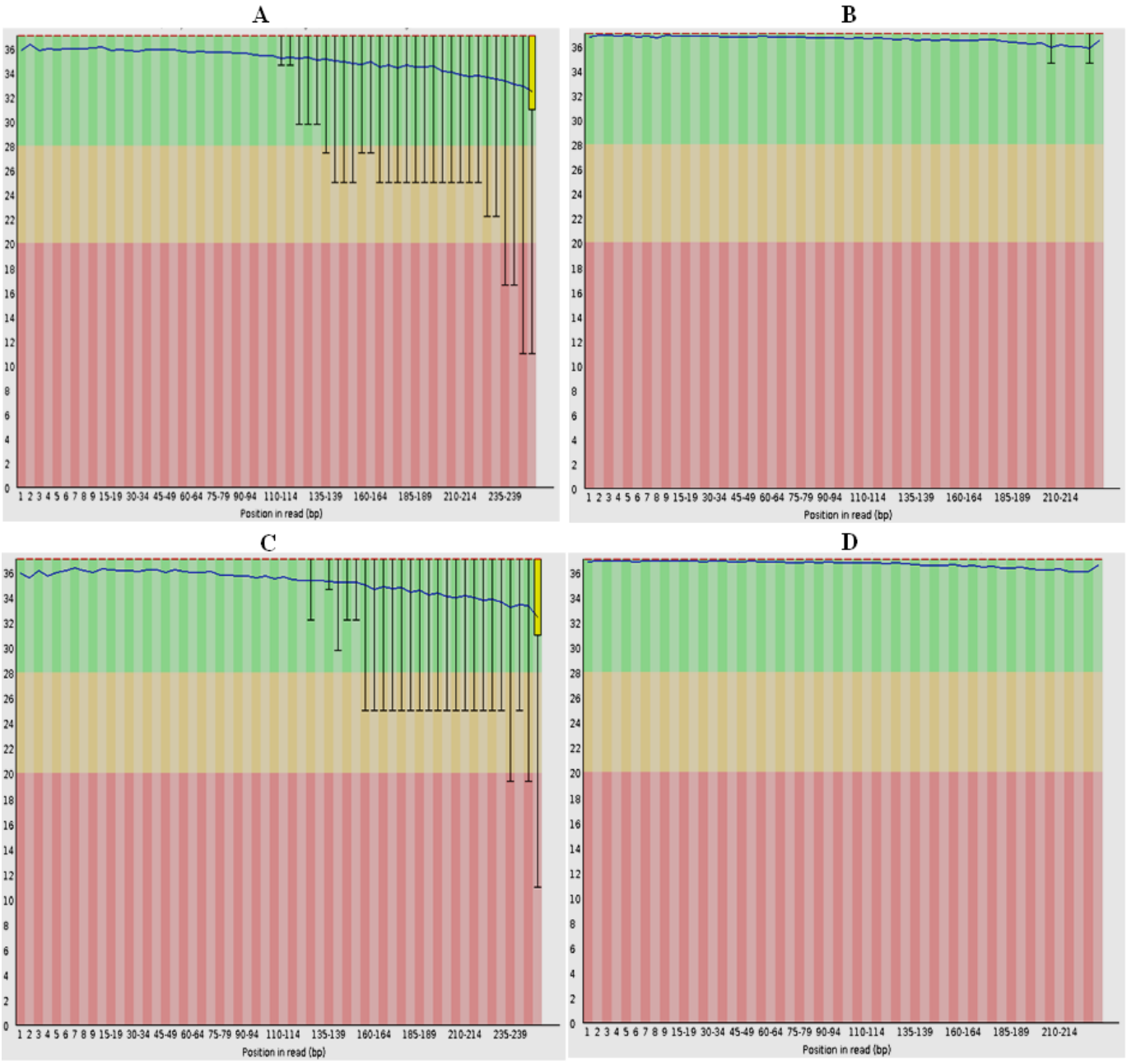


Figure 2.3: Per base sequence quality plot of *Parmotrema tinctorum* (**A** and **B**) and *Flavopunctelia flaventior* (**C** and **D**) metagenome reads. A and C = data before quality trimming and B and D = data after quality trimming. Green = high quality scores, Yellow = medium quality scores and Red = low quality scores. Blue line represents the average quality score for the nucleotides.

This study aimed to use only high-quality data and thus applied the most stringent trimming checks on Trimmomatic (Bolger *et al.*, 2014). During trimming, a total of 5,317,204 *P. tinctorum* and 6,407,869 *F. flaventior* reads were removed, while 17,983,155 and 23,903,356 reads were retained, respectively (Table 2.1). Particularly for sequence alignment

investigations, losing reads could have an adverse effect on the downstream analysis of metagenome data (Maran and Davis, 2022). The quantity of reads preserved can be considerably reduced by performing quality trimming first before merging the data. Merging paired-end reads directly before trimming can therefore avoid the possibility of removing informative reads that do not pass the trimming stringent checks of Trimmomatic (Maran and Davis, 2022).

Table 2.1: A summary of the metagenomic reads of *Parmotrema tinctorum* and *Flavopunctelia flaventior* before and after quality trimming with Trimmomatic (Bolger *et al.*, 2014).

	Parmotrema tinctorum		*Flavopunctelia flaventior*	
	Pre-trimming	Post-trimming	Pre-trimming	Post-trimming
Total sequence	23,300,359	17,983,155	30,311,225	23,903,356
Sequences flagged as poor quality	0	0	0	0
Sequence length	35-251	200-232	35-251	200-232
% GC	58	58	56	57

2.3.3. An overall perspective of the *F. flaventior* and *P. tinctorum* lichen microbiomes

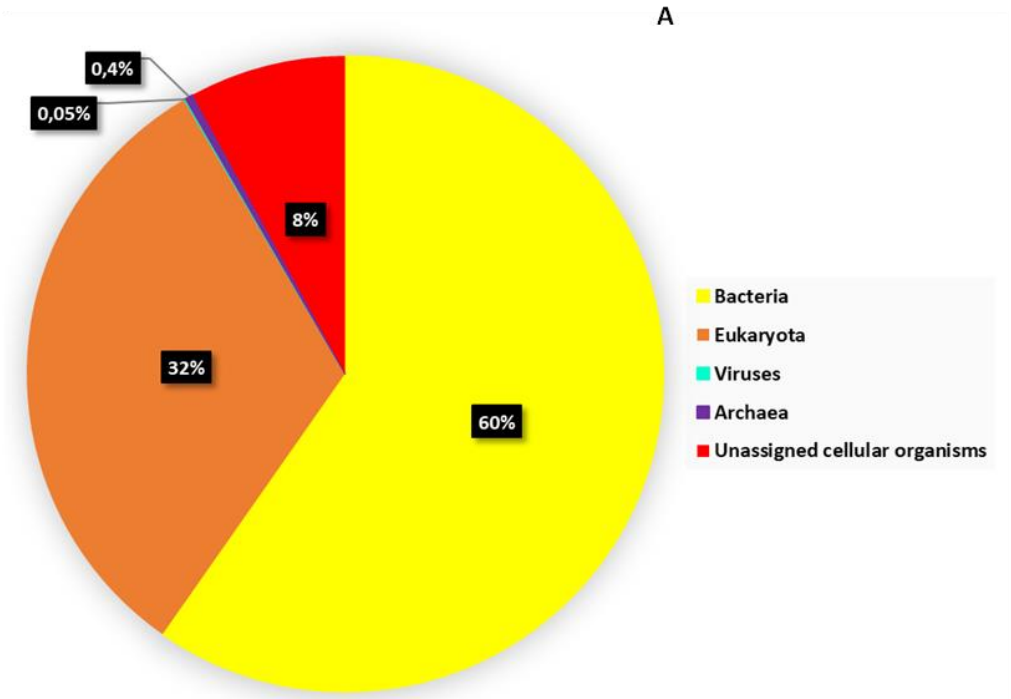
A major disadvantage to metagenomics where a considerable number of reads are either not assembled or linked to a specific phylogenetic marker for a definitive taxonomic classification of microorganisms informed the decision to use metagenomic reads for taxonomic profiling of the microorganisms (Luo and Moran, 2013).

Taxonomic delineation of the lichen metagenomic reads at the domain level showed that bacterial reads accounted for 60 and 57%; while the fungal counterpart accounted for 32 and 37% of the *P. tinctorum* and *F. flaventior* microbiomes, respectively (Figure 2.4). A similar trend was observed in the lichens *Lobaria pulmonaria* and *Cladonia arbuscula,* where the surfaces of the lichens were predominantly populated by diverse bacteria (Grimm *et al.*, 2021). Similarly, in a metagenome derived from the lichen microbial community of *Peltigera hymenina* (dog or pelt lichen) the microbial community was observed to comprise primarily of bacteria (80.9%, while 5.2% were cyanobacteria), with smaller proportions of fungi (18.8%) and archaea (0.001%) (Garg *et al.*, 2016).

The *F. flaventior* and *P. tinctorum* microbiome also incorporate members of the domain Archaea at a very low abundance of ~ 0.4% of the total reads (*P. tinctorum*: 71,932 reads and *F. flaventior*: 95,613 reads) in both lichen species (Figure 2.4). Archaea have previously been observed in the rock-dwelling lichens *Hydropunctaria maura* and *Ophioparma ventosa*, but also at much lower numbers relative (~ 1.88%) to bacteria (Bjelland *et al.*, 2011).

Reads representing dsDNA viruses were also mapped for the lichen metagenomes, albeit at very low abundance (~ 0.06% of the total reads), with 10,789 and 14,342 reads for *P. tinctorum* and *F. flaventior*, respectively. In the present study RNA extraction was not performed, as a result not all viruses were represented (Yin *et al.*, 2022). Terricolous (ground-dwelling), corticolous (bark-dwelling) and saxicolous (rock-dwelling) lichens sampled from Antarctica, Czech Republic, Greece, Norway, and United States of America, also indicated the presence of these viruses within their microbiomes (Petrzik *et al.*, 2014).

In our study, unassigned cellular microorganisms represented 7 and 8% of the total reads for *P. tinctorum* and *F. flaventior*, respectively. (Figure 2.4). These are prokaryotes and eukaryotes that have not been assigned any taxonomic status by the Kraken databases.



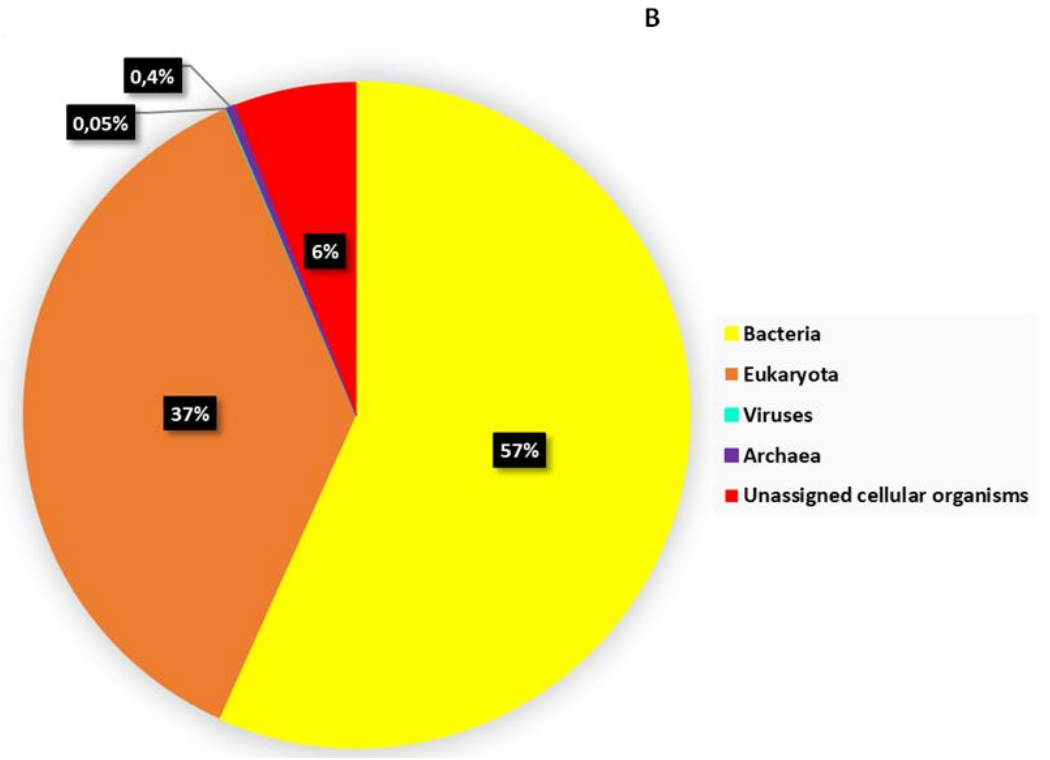


Figure 2.4.: Relative abundances of reads mapped at the domain level with Kraken for the lichens *Parmotrema tinctorum* (**A**) and *Flavopunctelia flaventior* (**B**).

2.3.3. The *F. flaventior* and *P. tinctorum* lichens support a highly versatile bacteriome

Classification of the metagenome-derived lichen-associated bacteria at the phylum level showed that the bacteriome comprises members of 38 distinct phyla. Of these, ~96% were core to both lichen species, while two phyla (Atribacterota and Candidatus Absconditabacteria) were unique to *F. flaventior* and one (Caldisericota) to *P. tinctorum*. Atribacterota are frequently found in methane-rich anoxic sediments (Carr *et al.*, 2015). The heterotrophic metabolism of these bacteria produces acetate, carbon dioxide, and ethanol (Carr *et al.*, 2015). Candidatus Absconditabacteria is a phylum of Candidate Phyla Radiation (CPR) bacteria which are described as obligate anaerobes capable of fermentation (Moreira *et al.*, 2021). Caldisericota, unique to *P. tinctorum*, comprises anaerobic heterotrophs that are predicted to have an acetyl-CoA to acetate fermentative metabolism that may produce either ethanol or propanoate (Martinez *et al.*, 2019).

For both lichen species, the bacteriome is predominated by members of the phyla Pseudomonadota (~ 59% of total reads) and Actinomycetota (~ 31% of total reads) (Figure 2.5). Pseudomonadota is a phylum of Gram-negative bacteria that incorporates a diverse range

of pathogenic taxa as well as nitrogen fixers (Maheshwari and Sankar, 2023). Members of the phylum Actinomycetota harbour Gram-positive bacteria and have been researched for secondary metabolite production, while some of the taxa in this phylum have an opportunistic predatory role (Hoshino *et al.*, 2015; Barka *et al.*, 2016; Ouchari *et al.*, 2018). Most Actinomycetota are aerobic chemotrophs, rendering them able to use different nutrient sources including complex carbohydrates such as starch (Hoshino *et al.*, 2015; Barka *et al.*, 2016).

Pseudomonadota and Actinomycetota form part of the bacteriome of all lichen associations that have been studied thus far (Ahmadjian, 1993; Grube and Berg, 2009; Bjelland *et al.*, 2011; Eymann *et al.*, 2017; Pankratov *et al.*, 2017; Armaleo *et al.*, 2019; Sierra *et al.*, 2022; Grimm *et al.*, 2021). In our study, these two phyla contributed 94% of the total bacteriome for both *P. tinctorum* and *F. flaventior*.

A previous study of the bacteriome associated with the studied lichens using 16S rRNA amplicon sequencing approaches identified ten distinct phyla post rarefication (Naude, 2023). The latter study likewise identified the phylum Pseudomonadota as the most abundant. While in our metagenomic analysis the phylum Acidobacteriota contributed 2 and 3% for *P. tinctorum* and *F. flaventior*, respectively, amplicon sequencing identified it as the second most dominant phylum (18% of total reads) (Naude, 2023). Additionally, the phylum Actinomycetota was identified as the least abundant while in our metagenomic study it was the second most abundant. Similar observations were made in a study that assessed the relative abundance of the human faecal microbial communities, where 16S rRNA amplicon sequencing attributed 0.4% of reads to the phylum Actinomycetota, while metagenome sequencing of the same dataset placed the relative abundance of this phylum at 7% (Ranjan *et al.*, 2016).

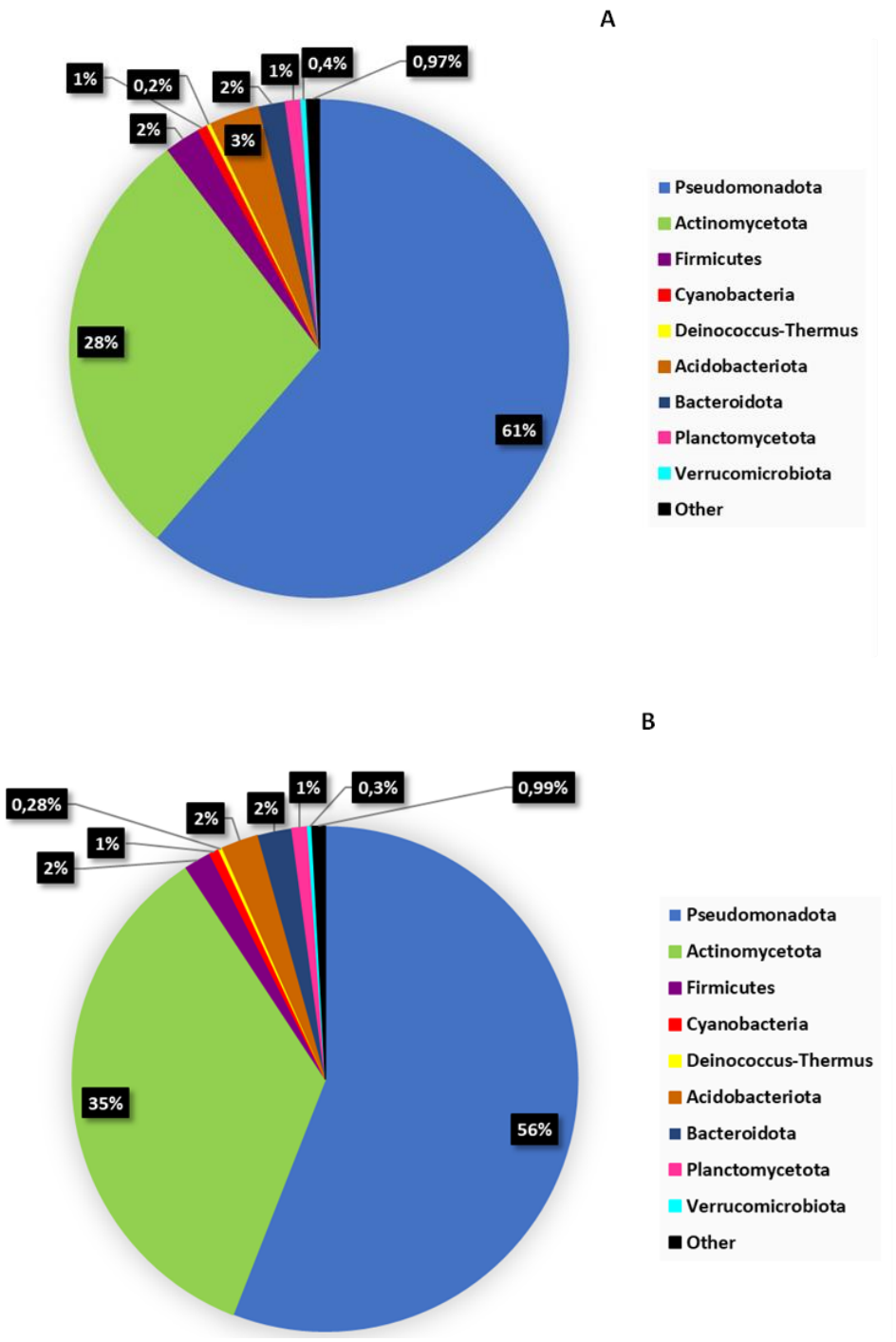


Figure 2.5: The lichen bacteriome of *Flavopunctelia flaventior* (**A**) and *Parmotrema tinctorum* (**B**) at phylum level.

A combined total of 1,622 distinct bacterial genera were identified from the metagenomic datasets of *F. flaventior* and *P. tinctorum*. Of these, 83 and 37 genera were specific to *P. tinctorum* and *F. flaventior*, respectively. The core bacteriome for both lichen species accounted for ~ 96% of the total genera identified. At the genus level, *Streptomyces* and *Nocardioides* of the phylum Actinomycetota and *Methylobacterium*, *Sphingomonas*,

Bradyrhizobium and *Pseudomonas* of the phylum Pseudomonadota were identified as the dominant genera (Figure 2.6). *Methylobacterium*, *Streptomyces* and *Sphingomonas* were the top three most abundant genera, contributing ~ 6, 7 and 5% of the total bacteriome, respectively (Figure 2.6).

Streptomyces spp. form protective mutualistic symbioses, wherein the host provides nutrients and protection for the bacteria in exchange for antimicrobial metabolites that shield the host from infections (Seipke *et al.*, 2012). The genus *Sphingomonas* is comprised of strict aerobes that can break down organic and xenobiotically generated compounds, including cellulose, carbazole chlorinated phenols and estradiol (Asaf *et al.*, 2020; Sorouri *et al.*, 2023). Both *Streptomyces* and *Sphingomonas* species have been observed in multiple lichen species, as part of the core bacteriome (Cardinale *et al.*, 2006; Grube *et al.*, 2015; Asaf *et al.*, 2020; Grimm *et al.*, 2021; Sorouri *et al.*, 2023). In a study on six *Cladonia*, two *Hypogymnia*, and one *Pseudevernia*, lichen species sampled from Austria and two *Roccella* lichen species sampled from France, the genera *Streptomyces* and *Sphingomonas* were observed as part of the bacteriome composition (Cardinale *et al.*, 2006). Additionally, *Streptomyces* was shown to be involved in the degradation of lichen associated tissues and chitin in all 11 lichen species (Cardinale *et al.*, 2006). Another study that focused solely on culturable Actinomycetota from different lichen species from tropical and cold regions, found *Micromonospora* and *Streptomyces* to be the most prevalent genera (Aschenbrenner *et al.*, 2016).

Within lichen symbioses, the genus *Sphingomonas* may be involved in the uptake of amino acids, iron and xylose (Grimm *et al.*, 2021). This genus has also been shown to be important in the production of vitamins, which are involved in processes such as photosynthesis, gene regulation and the breakdown of carbohydrates to support the growth of the mycobiont (Bates *et al.*, 2011). *Sphingomonas* members may also provide symbiotic partners with protection against abiotic stressors, however, mechanisms detailing how they achieve this is currently unclear (Grube *et al.*, 2015).

Methylobacterium spp. are facultative methylotrophs, implying they may oxidise single-carbon molecules including formaldehyde, methylamine, and methanol, or they can use conventional carbon sources such as carbohydrates (Palberg *et al.*, 2022). Members of this genus have also been characterised as part of the microbiome of six lichen genera from Antarctica (Noh *et al.*, 2021). Within the lichen symbiosis, *Methylobacterium* spp. have shown to protect members of the mutualistic relationship against the accumulation of methanol and other toxic single carbon

metabolites (Grube *et al.*, 2015). These species perform oxidation of methanol and single carbon metabolites, catalysed by formaldehyde activating enzymes, into formaldehyde and carbon dioxide (Grimm *et al.*, 2021). Additionally, *Methylobacterium* isolated from *L. pulmonaria*, showed evidence of carbon monoxide dehydrogenase, thus suggesting that they might be involved in the oxidation of carbon monoxide, protecting symbiotic partners from its toxic effects (Eymann *et al.*, 2017).

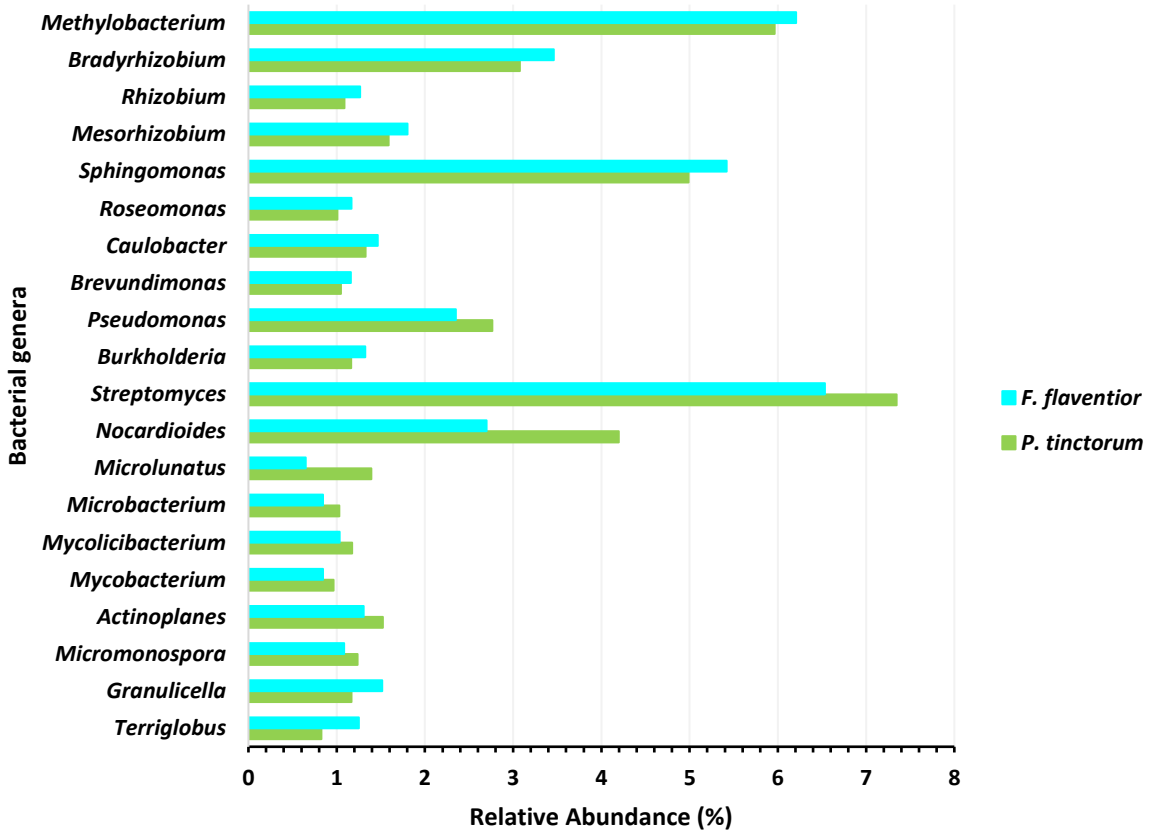


Figure 2.6: Relative proportions of 20 most abundant bacterial genera in the studied lichen taxa, *Parmotrema tinctorum* (**green**) and *Flavopunctelia flaventior* (**blue**).

A 16S rRNA amplicon sequencing study of the *F. flaventior* and *P. tinctorum* lichen microbiome identified 93 distinct genera where the Pseudomonadota genera *Methylosinus*, *Sphingomonas*, *Acidisphaera*, and *Methylobacterium* and Acidobacteriota genera *Granulicella* and *Terriglobus* represented the most abundant bacterial genera (Naude, 2023). Only the genera *Granulicella*, *Methylobacterium*, *Sphingomonas* and *Terriglobus* were shared among the 20 most abundant genera of the *F. flaventior* and *P. tinctorum* bacteriome using both metagenomic approaches.

2.3.4. The mycobiome of *F. flaventior* and *P. tinctorum*

Metagenome sequencing indicated that the mycobiome of *F. flaventior* and *P. tinctorum* comprises of three distinct fungal phyla, Ascomycota, Basidiomycota, and Microsporidia, all of which were core to both lichens. Members of the phylum Ascomycota dominated the lichen mycobiome, contributing ~ 96% of the total fungal reads (Figure 2.7). The phylum Ascomycota is the most species-rich and diversified in the kingdom Fungi (Wijayawardene *et al.*, 2021). It consists of endophytic (form endosymbiotic associations with plants), saprobic (break down dead and decaying organic matter) and pathogenic fungi (Wijayawardene *et al.*, 2021). Members of the phylum Basidiomycota, accounted for ~ 4% of the total fungal reads (Figure 2.7). The phylum Basidiomycota is made up of approximately 35,000 species that are classed into three sub-phyla: the Pucciniomycotina (rusts), Agaricomycotina (mushrooms) and Ustilaginomycotina (smuts and allies) (Nagy and Szöllősi, 2017). The phylum Microsporidia was also present within the *F. flaventior* and *P. tinctorum* mycobiomes at very low abundances of ~ 0.00044% for *P. tinctorum* and 0.00049% for *F. flaventior*. Microsporidia are obligatory, intracellular, eukaryotic pathogens that infect insects and mammals (Fadhilah *et al.*, 2023).

ITS tagged amplicon sequencing also identified Ascomycota as the predominant phylum, accounting for ~ 99% of the mycobiome of *F. flaventior* and *P. tinctorum*, while Basidiomycota accounted for ~ 0.75% of the mycobiomes (Naude, 2023). The observations from metagenome and ITS sequencing are further supported by a study on the lichens *Umbilicaria pustulata*, *Rhizoplaca melanophthalma*, *Xanthoparmelia* cf. *mexicana*, *Rhizoplaca arbuscula*, *Xanthoparmelia* aff. *chlorochroa*, *Bryoria fremontii*, *Physcia biziana*, and *Rinodina olivaceobrunnea* sampled from western North America (Smith *et al.*, 2020). In another study on *L. pulmonaria*, Ascomycota comprised the majority of fungi (98.9%), while only a minor part of the fungal spectrum belonged to the Basidiomycota, primarily in the form of yeasts (Schneider *et al.*, 2011).

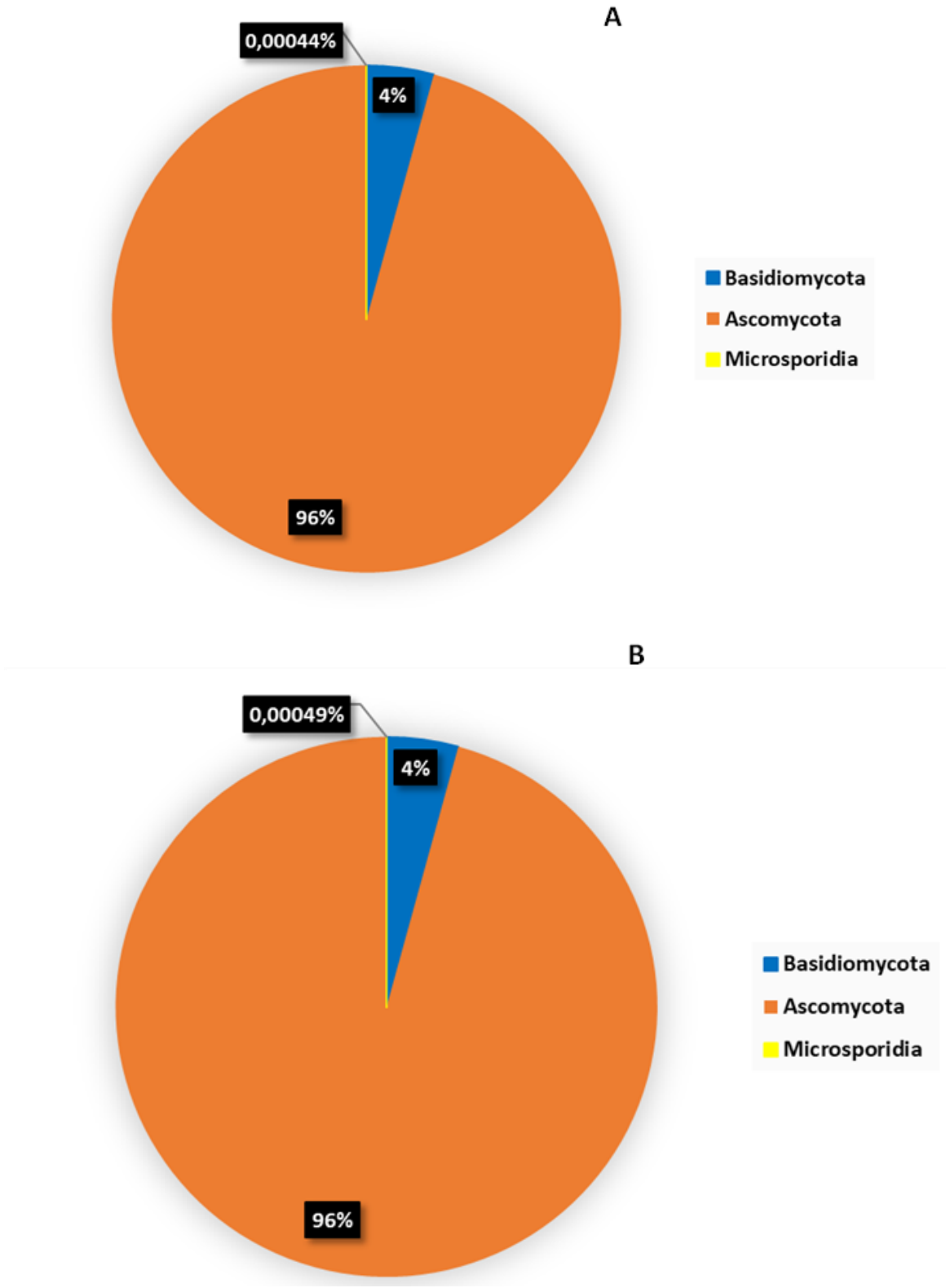


Figure 2.7: The lichen mycobiome of *Parmotrema tinctorum* (**A**) and *Flavopunctelia flaventior* (**B**) at phylum level.

The mycobiome of *F. flaventior* and *P. tinctorum* comprised of 43 distinct fungal genera that were all core to both lichen species. The genera *Aspergillus*, *Fusarium*, *Pyricularia*, *Colletotrichum* and *Botrytis* were the most abundant in both lichen species (Figure 2.8). In contrast, ITS tagged amplicon sequencing identified 111 distinct fungal genera, and the genera *Curreya*, *Cladophialophora*, *Knufia*, *Microcera*, *Autroafricana*, *Physconia* and *Penidiella* were the most abundant (Naude, 2023).

Aspergillus spp. and other moulds are proficient at recycling celluloses, starches, hemicelluloses, and pectins (Mousavi *et al.*, 2016). Certain *Aspergillus* spp. have the ability to break down more refractory molecules such as chitin, lipids, keratin, and oils and can also be pathogenic to both plant and animal hosts (Mousavi *et al.*, 2016). Members of this genus are usually found inside the thallus of a lichen (Agrawal *et al.*, 2020). The lichen species *Usnea longissima* Ach. harbours *Aspergillus quandricinctus*, which is capable of preventing biofilm formation by *Pseudomonas aeruginosa* (Prateeksha *et al.*, 2020). The lichen genera *Cetrelia*, *Lobaria*, *Parmotrema*, and *Peltigera* are also considered hosts for species of the *Aspergillus* genus (Zhao *et al.*, 2014; Li *et al.*, 2015; Chen *et al.*, 2019; Padhi *et al.*, 2020). Within the lichen symbiosis, *Aspergillus* spp. are responsible for the synthesis of secondary metabolites such as terpenes, steroids and peptides, which have antibacterial and antifungal properties (Agrawal *et al.*, 2020), thus protecting the symbiotic partners against pathogens.

With a global distribution, the filamentous fungal genus *Fusarium* comprises the most economically damaging plant pathogens, infecting nearly all commercially valuable plants and incurring annual losses to the agricultural sector amounting to billions of dollars (Dongzhen *et al.*, 2020). *Fusarium* has been found in lichens of the genera *Flavoparmelia* and *Lasallia* sampled from Maryland and Virginia, respectively, and are suggested to be pathogenic to the mycobionts (Lawrey *et al.*, 1999). This is because *Fusarium* was found to break-down the protective secondary metabolites, which allows the fugus *Marchandiomyces corallinus* to colonize the lichens and degrade them (Lawrey *et al.*, 1999).

The genera *Botrytis*, *Colletotrichum* and *Pyricularia* comprise pathogenic fungi found in tropical and subtropical regions and several well-known species also impact crops in temperate regions (Cannon *et al.*, 2012; Murata *et al.*, 2014; Garfinkel *et al.*, 2019). Members of the genera *Botrytis* and *Colletotrichum* have been linked with *L. pulmonaria* (Grimm *et al.*, 2021), *Lecanora argentata* from Korea, *Nephromopsis pallescens* and *Parmelia simplicior* from China (Wei *et al.*, 2008) as pathogens targeting the mycobiont.

It is worth noting that the mycobionts *F. flaventior* and *P. tinctorum* and the photobiont *Trebouxia* were not identified among all the genera in the present study. This is likely due to the fact that the databases that were used for taxonomic classification of the metagenomic reads did not incorporate reference genomes of the mycobionts and photobiont (Peterson *et al.*, 2021).

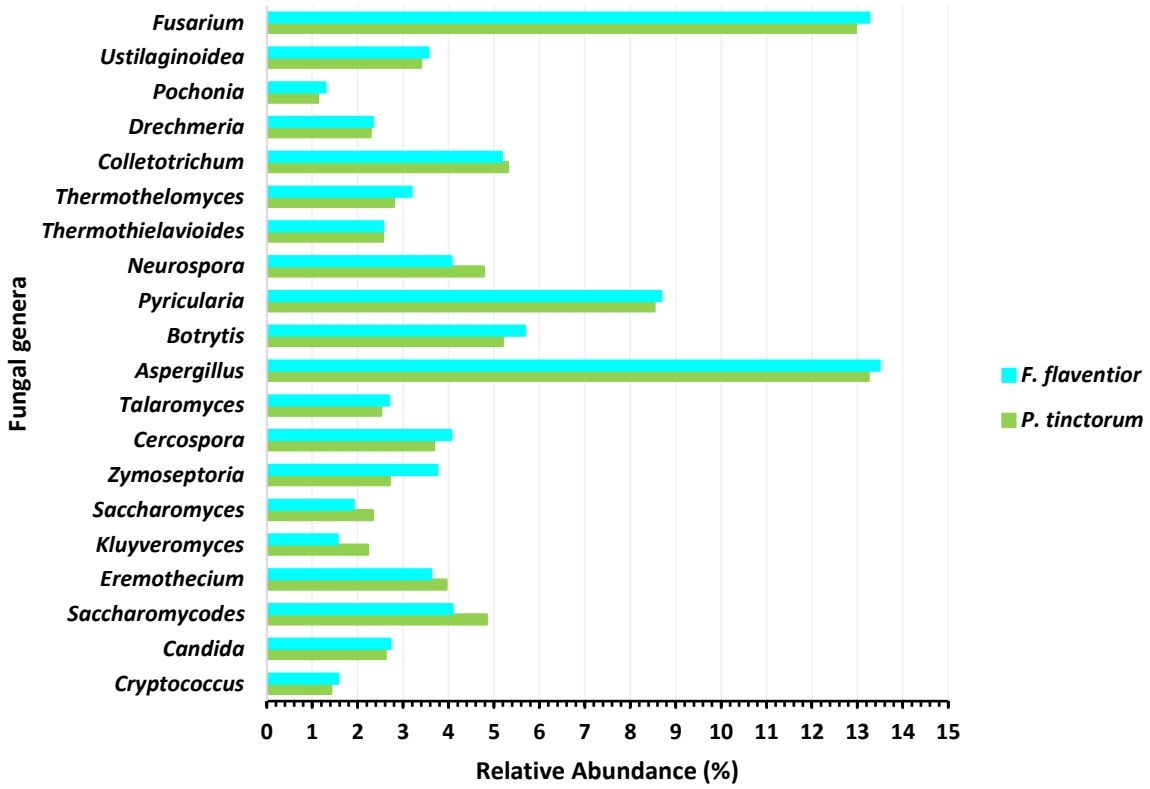


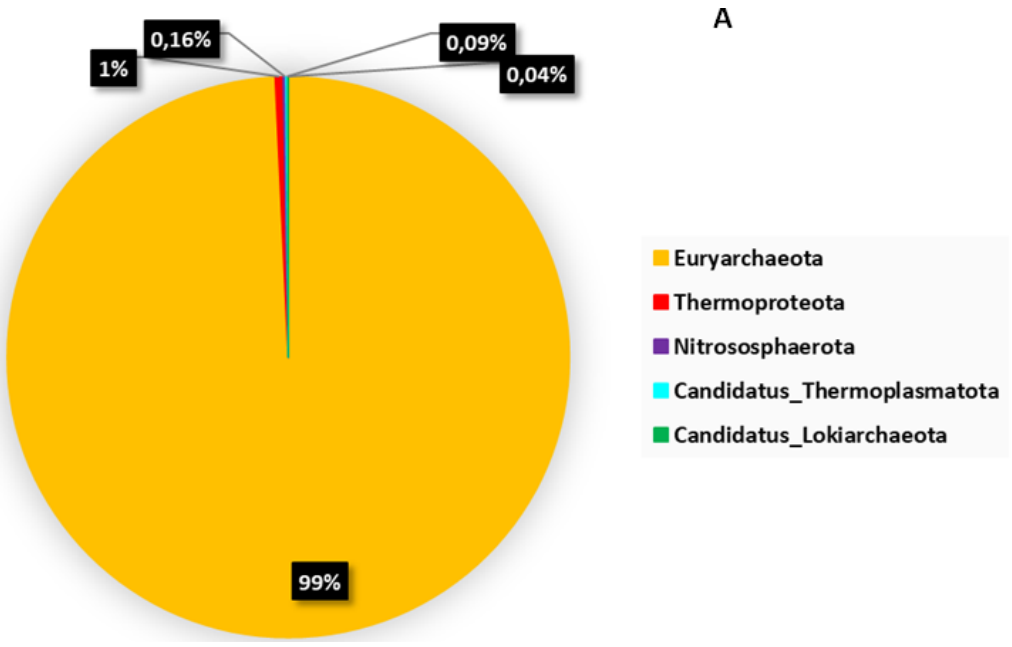
Figure 2.8: Relative proportions of 20 most abundant fungal genera in the studied lichen taxa, *Parmotrema tinctorum* (**green**) and *Flavopunctelia flaventior* (**blue**).

2.3.5. Archaea associated with *F. flaventior* and *P. tinctorum*

The presence of members of the domain Archaea in the lichen microbiome was first reported in 2011 in the lichen species *H. maura*, *O. ventosa*, *Pertusaria corallina* and *Rhizocarpon geographicum* (Bjelland *et al.*, 2011). The archaeal component of the microbiome of *F. flaventior* and *P. tinctorum* in the present study included five core phyla, namely, Candidatus Lokiarchaeota, Nitrososphaerota, Thermoproteota, Candidatus Thermoplasmatota, and Euryarchaeota. Of the five phyla, Euryarchaeota was dominant, accounting for ~ 99% of the

total archaeal reads (Figure 2.9). This phylum consists of methanogens, which are strict anaerobes that convert single-carbon compounds to methane (Castro-Fernandez *et al.*, 2017). Methane can be oxidised further to produce carbon dioxide, which can be readily available to microorganisms (Thauer *et al.*, 2008). As such, within the lichen symbioses, it can be proposed that methanogens may be responsible for increasing the supply of carbon dioxide to the members of the microbiome. Acidophilic and thermophilic species are also found in this phylum (Leigh and Whitman, 2013; Castro-Fernandez *et al.*, 2017).

Members of the phylum Candidatus Lokiarchaeota (Lokiarchaeon) are capable of fermentation and producing acetate through the reduction of carbon dioxide (Orsi *et al.*, 2020), while the Nitrososphaerota consists of ammonia-oxidizing microorganisms found in marine environments, soil and hot springs (Stieglmeier *et al.*, 2014; Nelkner *et al.*, 2023). Although some of members of the Nitrososphaerota depend on the presence of additional microorganisms or trace amounts of organic substances, they are autotrophic microorganisms that fix carbon dioxide. Thermoproteota comprises predominantly anaerobic microorganisms that use sulphur as an electron acceptor during respiration (Leigh and Whitman, 2013). This phylum was found to predominate in the rock-inhabiting lichen *H. maura* (Bjelland *et al.*, 2011).



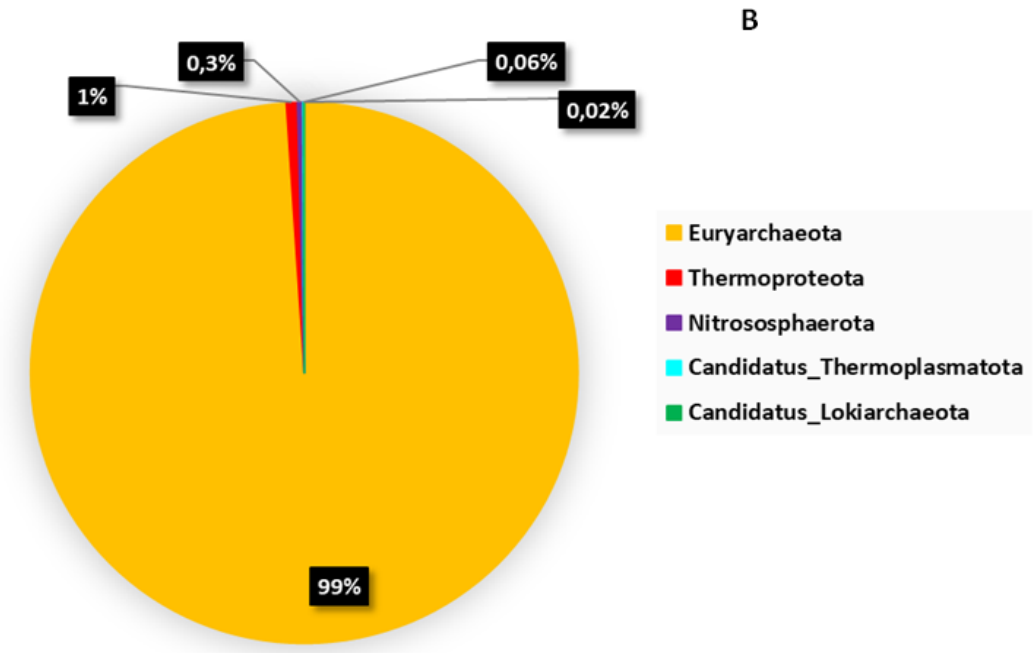


Figure 2.9: The lichen archaeal composition of *Parmotrema tinctorum* (**A**) and *Flavopunctelia flaventior* (**B**) at phylum level.

A total of 89 distinct archaeal genera were identified as part of the *F. flaventior* and *P. tinctorum* microbiome. Of these, ~ 93% were core to both lichens while seven (*Ignicoccus*, *Desulfurococcus*, *Vulcanisaeta*, *Stygiolobus*, *Methanohalophilus*, *Methanomethylovorans* and *Methanospirillum*) and six (*Picrophilus*, *Candidatus Methanomethylophilus*, *Methanomassiliicoccus*, *Infirmifilum*, *Fervidicoccus*, and *Sulfuracidifex*) genera, were specific to the *F. flaventior* and *P. tinctorum* microbiomes, respectively.

The genera *Desulfurococcus* and *Stygiolobus* (unique to the *P. tinctorum* microbiome) comprise of anaerobic chemoheterotrophic microorganisms that perform the reduction of elemental sulphur in the presence of dihydrogen gas to hydrogen sulfide (Fauque and Barton, 2012; Huber and Stetter, 2015). The lichen *Parmelia sulcata* demonstrated reduced rates of carbon fixation and biomass when exposed to higher concentrations of hydrogen sulfide (Tretiach and Baruffo, 2001). Additionally, a study that assessed the effects of hydrogen sulfide on Photosystem II (PSII) of the photobionts of *Peltigera praetextata*, *P. sulcata*, *Pseudevernia furfuracea*, *Flavoparmelia caperata* and *Parmotrema perlatum*, also supported the negative implications of hydrogen sulfide on lichens (Bertuzzi and Tretiach, 2013).

The genera *Haloquadratum* (~63% of total archaeal reads) and *Halarchaeum* (~24% of total archaeal reads) predominated the archaeal counterpart of the microbiomes of both lichens (Figure 2.10). Typically, *Haloquadratum* and *Halarchaeum* species are aerobic heterotrophs (Andrei *et al.*, 2012). As methanogens (Thauer *et al.*, 2008), they may also contribute to increasing the supply and availability of carbon dioxide to the photobiont, which will in return produce glucose for the mycobiont and other microorganisms (Grimm *et al.*, 2021).

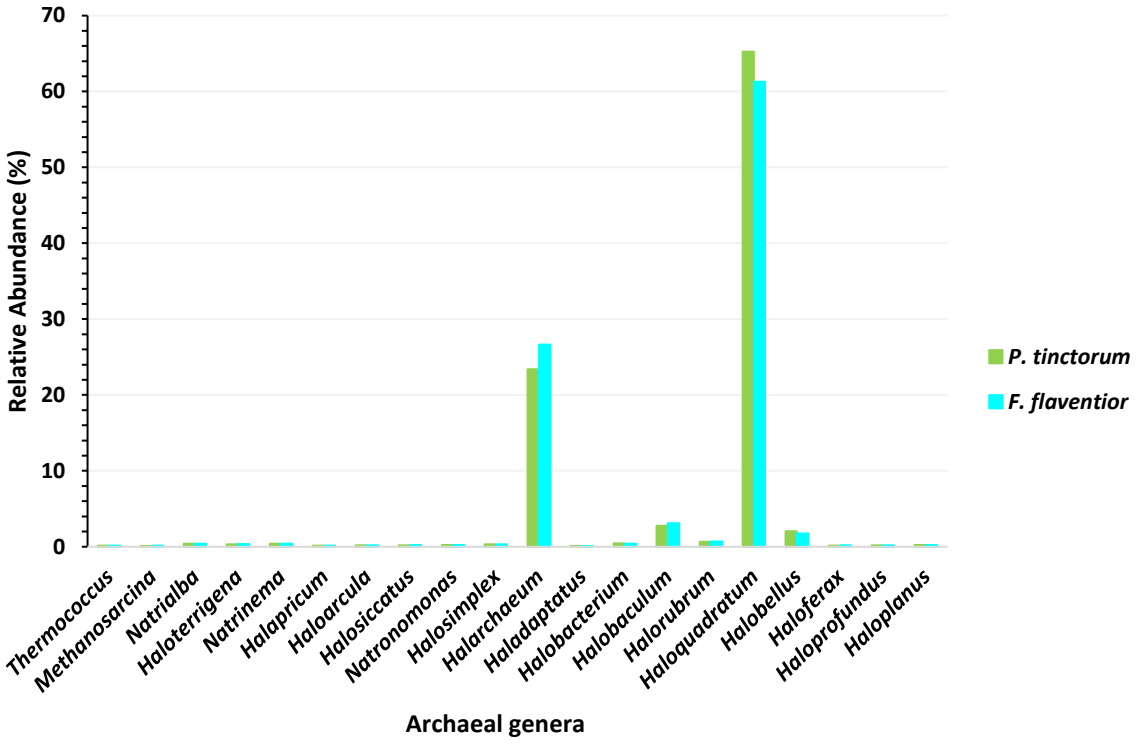


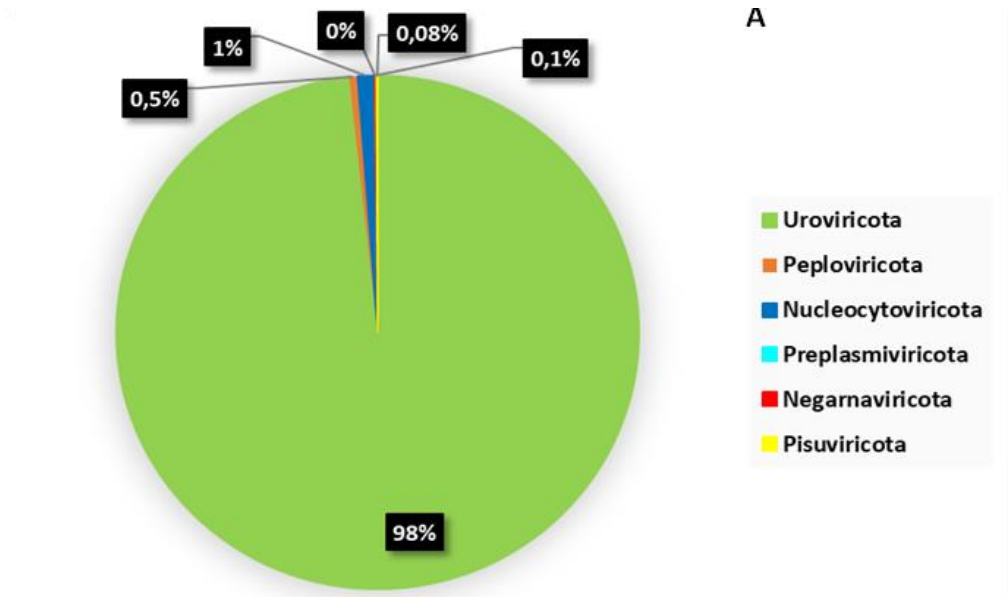
Figure 2.10: Relative proportions of 20 most abundant archaeal genera in the studied lichen taxa, *Parmotrema tinctorum* (**green**) and *Flavopunctelia flaventior* (**blue**).

2.2.6. Evidence of viral presence within the *F. flaventior* and *P. tinctorum* microbiomes

Viral diversity connected to lichens is poorly understood (Merges *et al.*, 2021). A total of 1,279 *P. tinctorum* and 1,372 *F. flaventior* viral reads were found within the respective lichen metagenomes. The viromes of *F. flaventior* and *P. tinctorum* are comprised of six distinct viral phyla namely Pisuviricota, Negarnaviricota, Preplasmiviricota, Nucleocytoviricota, Peploviricota, and Uroviricota. One phylum (Preplasmiviricota) was not found in the *P. tinctorum* metagenome while the remaining five phyla were core.

Members of the phylum Uroviricota dominated the lichen species, contributing ~97% of the total viral reads (Figure 2.11). This phylum is made up of tailed double-stranded DNA bacteriophages (dsDNA phages) (Benler *et al.*, 2021). In both lichens, the representative viruses in this phylum fall under the class Caudoviricetes.

Viruses from the phylum Pisuviricota are positive stranded dsRNA viruses capable of infecting fungi (Chang *et al.*, 2023), while Negarnaviricota is comprised of negative-strand RNA viruses, that use arthropods as their host (Kuhn *et al.*, 2021). Nucleo-Cytoplasmic Large DNA Viruses (NCLDVs) in the phylum Nucleocytoviricota are limited to infecting eukaryotes, such protozoa, invertebrates and alga, while Preplasmiviricota infect bacteria, archaea and eukaryotes (Woo *et al.*, 2021). Additionally, the dsDNA viruses that belong to the phylum Peploviricota are known for their animal hosts and their icosahedral capsids, which are encased in a lipid envelope that contains glycoproteins (Gaïa *et al.*, 2023).



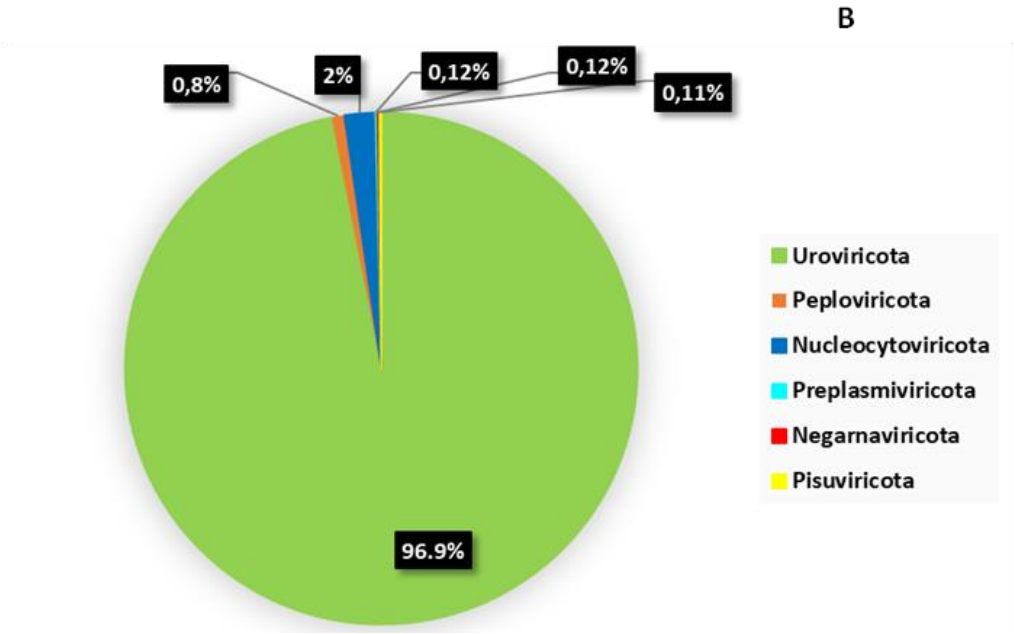


Figure 2.11: The lichen virome of *Parmotrema tinctorum* (**A**) and *Flavopunctelia flaventior* (**B**) at phylum level.

A total of 24 distinct viral genera were identified as part of the *F. flaventior* and *P. tinctorum* viromes. Of these, a total of seven and six genera were specific to *F. flaventior* and *P. tinctorum*, respectively, while ~ 72% of the identified genera were core to both lichen species. *Alphabaculovirus*, *Pandoravirus*, *Timquatrovirus* and *Fromavirus* were the predominant viral genera (Figure 2.12). The genus *Alphabaculovirus* belongs to the *Baculoviridae*, a family of viruses with dsDNA genomes that infect invertebrates such as insects (Passarelli, 2021). The genus *Pandoravirus* incorporates dsDNA icosahedral viruses that infect protists (Abergel *et al.*, 2015). The genera *Timquatrovirus* and *Fromanvirus* of class Caudoviricetes consists of bacteriophages that infect bacteria and archaea (Duda, 2021).

The genera *Prasinovirus*, *Tequatrovirus*, and *Brizovirus* were unique to the *P. tinctorum* virome (Figure 2.12). *Prasinoviruses* are nucleocytoplasmic giant DNA viruses that infect the *Mamiellophyceae*, a common and abundant family of phytoplankton (Clerissi *et al.*, 2014). Members of the genera *Tequatrovirus* and *Brizovirus* are dsDNA bacteriophages of class Caudoviricetes that infect Gram negative bacteria such as *Escherichia coli* and some archaea (Duda, 2021).

For the *F. flaventior* virome, the genera *Marseillevirus*, *Alphanudivirus*, and *Phikzvirus* were unique (Figure 2.12). The *Marseillevirus* genus consists of giant viruses with dsDNA genomes of ~763 kilobases (kb), and belongs to the class Megaviricetes and phylum Nucleocytoviricota

(Sahmi-Bounsiar *et al.*, 2021). *Alphanudivirus* comprise large nuclear circular dsDNA viruses that infect arthropods and is one of the two genera under the family *Nudiviridae* (Chao *et al*., 2020), while *Phikzvirus* (PhiKZ-like viruses) consist of dsDNA bacteriophages of the class Caudoviricetes (Duda, 2021).

As mentioned previously, viral diversity connected to lichens is poorly understood. Evidence of bacteriophages has been found within the metagenomes of the lichens *L. pulmonaria*, *Peltigera* sp and *U. phaea*, while the genome of the lichen photobionts *Asterochloris* and *Trebouxia* were shown to contain remnants of viral insertion by a large dsDNA virus and representative of the genus *Caulimovirus*, respectively (Garg *et al.*, 2016; Eymann *et al.*, 2017; Armaleo *et al.*, 2019; Merges *et al.*, 2021). Plant viruses such as Cauliflower mosaic virus, *Cytorhabdovirus* and Apple mosaic virus have also been observed in the lichen species *C. arbuscula*, *Usnea* sp., and *Xanthoria parietina* and in the photobiont *Trebouxia* (Petrzik *et al.*, 2014; Petrzik *et al.*, 2015). Additionally, another study on *Chrysothrix chlorina* and *Lepraria incana* sampled in the Czech Republic, reported *Chrysothrix chrysovirus* 1 (CcCV1) and *Lepraria chrysovirus* (LiCV1) to be in close association with the lichen mycobionts (Petrzik *et al.*, 2019). While there is increasing evidence of a lichen associated viruses, the role they play in shaping the symbiosis remains to be explored.

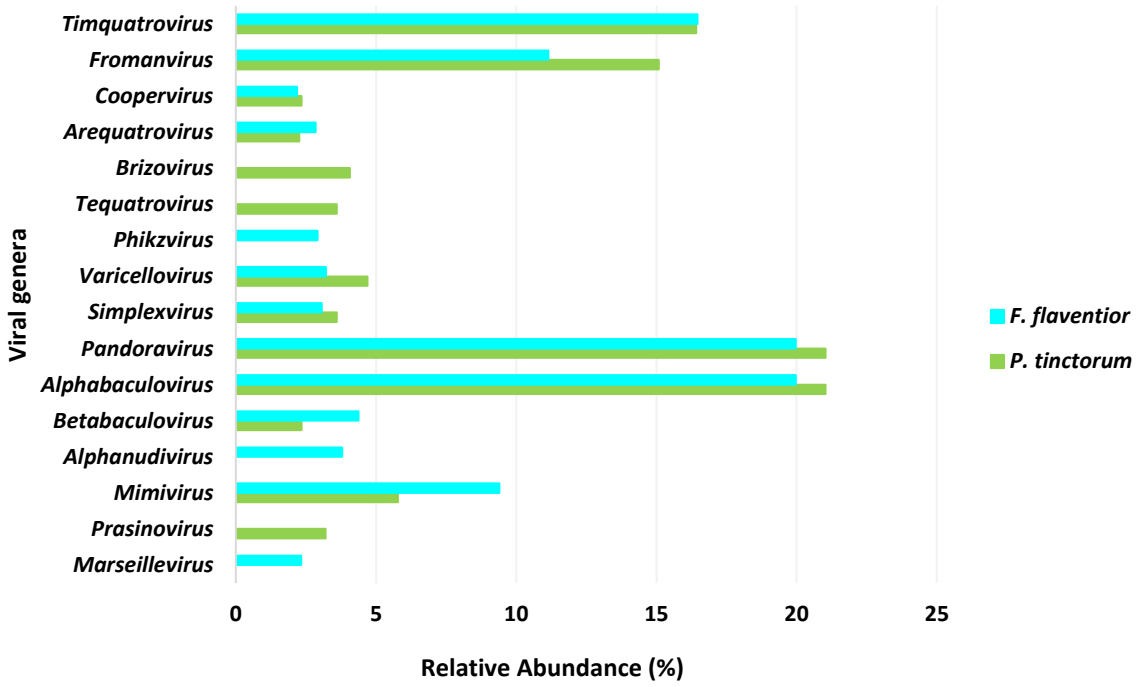


Figure 2.12: Relative proportions of 16 most abundant viral genera in the studied lichen taxa *Parmotrema tinctorum* (**green**) and *Flavopunctelia flaventior* (**blue**).

2.4. Conclusions

The metagenomes of two South African lichen species, namely *F. flaventior* and *P. tinctorum*, were sequenced and reads were taxonomically classified. In both *P. tinctorum* and *F. flaventior*, bacteria represented the overarching majority of taxa within the lichen microbiome, followed by fungi, while viruses and archaea contributed minorly. Within the microbiomes of *P. tinctorum* and *F. flaventior*, a total of 1,700 genera were identified as core. The shared microbiome may be a result of shared geography and climatic conditions, as both lichen species were isolated form the same location. Alternatively, the shared microbiome may be inherent of the taxonomy of the lichen species, as both are representative of the family *Parmeliaceae*. Some, but limited overlap was found between this study using metagenome sequencing, and a previous study based on 16S rRNA and ITS amplicon sequencing of *P. tinctorum* and *F. flaventior*. Factors such as the availability of full microbial genomes on databases and primer efficacy contribute to the disparities observed between the different sequencing methods. Future studies may include characterising other South African lichen species sampled from different locations, to investigate the factors that can contribute to the formation of the respective lichen microbiomes. In addition, studying the effects of climate, growth substrate and pH on the composition of the lichen microbiome may shed insight on what shapes this pertinent symbiosis.

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Chapter 3: Metagenomic insights into the functioning of the lichen microbiome of *Parmotrema tinctorum* and *Flavopunctelia flaventior*.

Abstract

There is increasing evidence that lichens present a complex mutualistic relationship, harbouring microorganisms such as fungi, archaea, bacteria, and viruses, thus extending the conventional definition of a mycobiont-photobiont lichen symbiosis. While functional roles have previously been allocated to the mycobiont and photobiont, the roles of other microorganisms within the symbioses remain to be explored. The present study employed metagenome sequencing, assembly and functional annotation to further describe the roles the members of the *Parmotrema tinctorum* and *Flavopunctelia flaventior* microbiomes play in sustaining the lichen symbioses. The *P. tinctorum* and *F. flaventior* lichen microbiomes were shown to be involved in the cycling of nitrogen and carbon, supported by the presence of peptidases and carbohydrate-active enzymes, along with a broader range of other metabolic and regulatory functions. Despite having been isolated from the same geographic location, a large number of distinct proteins and pathways were identified in the two lichen species, which can be ascribed to differences in their microbiome. The *P. tinctorum* lichen microbiome encoded almost twice as many proteins involved in information storage and processing compared to *F. flaventior*. In addition, only the *P. tinctorum* lichen metagenome was shown to also fix carbon through the HB/HP, 3HP bi-cycle, and DC/HB cycles. Further analyses, using meta-transcriptomics and meta-proteomics, will shed further light on the true functions of the lichen microbiomes in these intriguing symbioses.

3.1 Introduction

Metagenomics is a field of study that focuses on the high throughput sequencing and in-depth analysis of integrated genomic DNA from biological samples (Lapidus and Korobeynikov, 2021; Delgado and Andersson, 2022). With this approach, complex microbial communities can be characterised in great detail without the requirement for cultivation (Delgado and Andersson, 2022). While the molecular analysis of taxonomic markers from a community of microorganisms can be used to assess the diversity of the sampled community, extended genomic regions or metagenome-derived genomes must be analysed to reveal the genetic potential of said community (Ayling *et al.*, 2020; Lapidus and Korobeynikov, 2021; Delgado and Andersson, 2022). Metagenomes can also shed light on the role that undiscovered microorganisms play in the metabolism of microbial communities in the environment (Yue *et al.*, 2020).

Lichens are useful models for studying symbiosis and possible sources of biosynthetic genes for biotechnological uses (Greshake Tzovaras *et al.*, 2020). The conventional definition of lichens suggests they are a mutualistic interaction between an autotrophic photobiont, phycobiont and a heterotrophic mycobiont (Armaleo *et al.*, 2019; Grimm *et al.*, 2021). However, there is increasing evidence to suggest that lichens are far more complex than it was initially thought, harbouring a diverse community of microorganisms. Lichens can therefore be thought of as fungi that combine with algae, bacteria, archaea, and viruses to generate self-sustaining life forms (Petrzik *et al.*, 2014; Petrzik *et al.*, 2015; Garg *et al.*, 2016; Pankratov *et al.*, 2017; Armaleo *et al.*, 2019; Petrzik *et al.*, 2019; Grimm *et al.*, 2021; Merges *et al*., 2021). Within a symbiotic relationship, the microbiome may support the production of vitamins and auxins, fix nitrogen, provide nutrients, regulate the growth of symbiotic partners, and offer defence against biotic and abiotic stresses (Bates *et al*., 2011; Yousuf *et al*., 2014; Zedda and Rambold, 2015; Sierra *et al*., 2020).

In the present study, metagenomes of two South African lichen species, namely *Parmotrema tinctorum* and *Flavopunctelia flaventior*, were sequenced, assembled, and used to predict the gene repertoires of the two lichen microbiomes. The assembled metagenomes were then used to compare the gene repertoires of the two lichen microbiomes and to predict the potential contributions of the microbiome to the lichen symbiosis.

3.2. Methodology

3.2.1. Metagenome assembly

Metagenomic DNA was extracted from *P. tinctorum* and *F. flaventior* and complete metagenomes were sequenced using Illumina NovaSeq 6000 in the paired-end configuration (Chapter 2 section 2.2.3) and the read datasets were subsequently quality trimmed (Chapter 2 section 2.2.4). SqueezeMeta (Tamames and Puente-Sánchez, 2019) was used for further analysis of the curated metagenomic reads. The metagenome reads were assembled using the SqueezeMeta reference assembler MegaHit v1.2.9 (Li *et al.*, 2015; Li *et al*., 2016) as well as metaSPAdes (Nurk *et al.*, 2017). MegaHit allows the rapid and computationally efficient assembly of large and complex metagenomic datasets (Li *et al.*, 2015; Li *et al*., 2016) by using the relationships between k-mer substrings to construct a de Bruijn graph (Ghurye *et al*., 2016; Ayling *et al*., 2020). The k-mers are arranged in a graph shape, with k-1 prefixes and suffixes acting as the nodes and the k-mers acting as the edges. This approach infers read overlaps from shared k-mers rather than explicitly aligning them (Ghurye *et al*., 2016; Ayling *et al*., 2020). The option *--presets meta-sensitive* was selected for MegaHit assembly.

metaSPAdes first generates a de Bruijn graph of all reads and then converts it into the assembly graph using several graph simplification techniques (Nurk *et al.*, 2017). metaSPAdes v3.13.0 was applied to both paired-end metagenomic read datasets with the following assembly parameters: k: [21, 33, 55], repeat resolution enabled, mismatch careful mode turned off, coverage cut-off turned off, threads: 30 and memory limit: 75 Gb. MetaQUAST (Mikheenko *et al.*, 2016) was used to assess the quality of the MegaHit and metaSPAdes metagenome assemblies. Based on the assembly statistics, the MegaHit assembly was used for all further downstream analyses.

3.2.2. Metagenome contig classification

The tools Whokaryote (Pronk and Medema, 2022) and Tiara v 1.0.2 (Karlicki *et al.*, 2022) were used to differentiate between prokaryotic and eukaryotic metagenome contigs, on the basis of gene structure. Whokaryote determines if a particular metagenomic contig belongs to a prokaryote or a eukaryote using gene density, intergenic distance, and other distinguishing genomic traits (Pronk and Medema, 2022). A triangle distribution with the lower left limit at 5,000, the mode at 10,000 and the upper right limit at 100,000 bp were used to divide genomes into non-overlapping metagenome contigs with a random length ranging between 5,000 and

100,000 bp. To avoid an overrepresentation of eukaryotic contigs, which often have very large genomes compared to prokaryotes, a maximum of 500 contigs per genome were employed. Genes were predicted for each prokaryotic contig using the prokaryotic gene prediction tool Prodigal v 2.6.3 (Hyatt *et al.*, 2010) with the metagenomic setting (*--meta*). Only contigs for which ≥ 2 genes were predicted were considered in the analysis.

Tiara v 1.0.2 categorises assembled DNA sequences into classes representing distinct genome origins (Pronk and Medema, 2022). The first classification step categorises sequences into six classes: three for prokaryotes (Archaea, Bacteria, and prokaryote sequences not distinguishable as being of bacterial or archaeal origin), two for eukaryotes (nuclear genomes and organelles such as the mitochondrial or plastidial genomes), and one for sequences that could not be precisely classified. This was done using the following parameters: (k: 6, hidden 1: 2048, hidden 2: 1024, dropout: 0.2, probability cutoff: 0.65, fragment length: 5000, dim out: 5). The organellar sequences are further categorised in the second stage into classes representing plastidial genomes, mitochondrial genomes, and unknowns for unclassified sequences. This was achieved using the following parameters: (k: 7, hidden 1: 128, hidden 2: 64, dropout: 0.2, probability cutoff: 0.65, fragment length: 5000, dim out: 3). The output was converted into features by converting predictions into numbers: 0 (eukarya contigs), 1 (prokarya contigs) and 2 (unknown and organelle contigs).

3.2.3. Metagenome binning

Metagenomic binning involves the classification and clustering of reads into genomes of individual taxa or bins of closely related taxa on the basis of alignment similarities (Nissen *et al.*, 2021) and compositional features (Maguire *et al.*, 2020). The automated binning programs MaxBin 2 v 2.1.1 (Wu *et al.*, 2016), MetaBAT v 0.25.4 (Kang *et al.*, 2019), and CONCOCT v 0.4.0 (Sieber *et al.*, 2018) were used individually to create bin sets from the MegaHit assembled metagenome contigs. Different tools were used as some metagenome binners are better at reconstructing high abundance taxa than others. MaxBin 2 relies on the expectation-maximisation method and uses gene markers (sequences whose location on the chromosome are known), tetranucleotides frequencies (measures of variability in the genomes of microorganisms) and differential coverage (measures abundance of microorganisms) to dissociate metagenomic contigs into bins (Wu *et al.*, 2016; Sieber *et al.*, 2018). MetaBAT employs k-medoid clustering (uses actual data points as representatives of clusters to improve

accuracy) on differential coverage and tetranucleotide frequencies to distinguish bins (Kang *et al.*, 2015; Kang *et al.*, 2019; Sieber *et al.*, 2018). Tetranucleotide frequencies with differential coverage are also used in CONCOCT, in conjunction with Gaussian mixture models (Alneberg *et al.*, 2014; Sieber *et al.*, 2018).

The binning tools were run using default settings and the output bin datasets were submitted to the DAS tool (Sieber *et al.*, 2018) to obtain consensus bins. DAS proposes single-copy genes in each of the submitted bin sets, aggregates bins from various binning predictions, and then extracts a more comprehensive consensus bin from every aggregate so that it contains the greatest number of single-copy genes, while also having a manageable number of duplicate genes. The completeness of the bins is greatly enhanced by this collapsing method. CheckM (Parks *et al.*, 2015) was then used to estimate the completeness of the MAGs (metagenome assembled genomes) represented by each of the bins. CheckM assesses bin completeness on the basis of the presence of lineage specific gene markers.

To further assemble and polish the genome bins, sequences were randomly selected from each bin and subjected to BLASTn (Altschul *et al.*, 1990) against the NCBI non-redundant nucleotide dataset (https://www.ncbi.nlm.nih.gov/refseq/). Further, the bin sequence sets were submitted to the TYpe Genome Server (TYGS) (https://tygs.dsmz.de/user_requests/new) (Meier-Kolthoff and Göker, 2019). This allowed for the identification of the nearest phylogenetic relative for which a genome sequence is available. The genome sequences of these related microorganisms were obtained from the NCBI database (Pruitt *et al.*, 2007) and subsequently used as the reference genome on the MeDuSa (Multi-Draft based Scaffolder) (http://150.217.159.17/medusa/) web server (Bosi *et al.*, 2015) to scaffold the bins. For taxonomic classification of the refined bins, the GTDB-tk (Genome Taxonomy Database Toolkit) was used (Chaumeil *et al.*, 2020). Using domain-specific, connected protein reference trees, the computationally efficient toolset GTDB-Tk allows automatic and objective taxonomic categorisation of archaeal and bacterial genomes (Chaumeil *et al.*, 2020).

3.3.4. Functional annotation

The curated metagenomic read datasets for the two lichen species were compared against local copies of the GenBank (Clark *et al.*, 2016), eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) (Huerta-Cepas *et al.*, 2016) and KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa and Goto, 2000) databases.

EggNOG is a phylogenetically and functionally annotated hierarchical orthologs library (Huerta-Cepas *et al.*, 2016). Annotations against eggNOG v.4.5.1 were performed with eggNOG-mapper v.1.0.3 (Cantalapiedra *et al.*, 2021), using accelerated profile HMM searches, following the recommended settings for large annotation jobs (Cantalapiedra *et al.*, 2021). Orthologous Groups (OGs) of proteins at various taxonomic levels are provided through the public eggNOG database, each with integrated and condensed functional annotations (Huerta-Cepas *et al.*, 2016; Cantalapiedra *et al.*, 2021). KEGG is a knowledge repository that connects genomic data with higher level functional information to enable the systematic analysis of gene activities (Kanehisa and Goto, 2000). The MEROPS repository which is a knowledge base for peptidases, proteases and proteolytic enzymes was also used to predict the enzymes involved in the degradation of polypeptides within the metagenomes of lichens (Rawlings et al., 2018). Searches against the KEGG and MEROPS databases were undertaken using DIAMOND (Buchfink *et al.*, 2015). The following settings were used: DIAMOND-fast: diamond blastx -t 48 -k 250 -min-score 40 and DIAMOND-sensitive: diamond blastx -t 48 -k 250 -sensitive -min-score 40.

To functionally annotate the predicted protein datasets (Prodigal output) for each metagenome, comparison against the Pfam (Finn *et al.*, 2016) database was performed. Pfam is a curated protein family database where each family is described by a profile hidden Markov model (HMM) (Finn *et al.*, 2014). Profile HMMs are probabilistic models constructed from an aligned set of curator-defined family-representative sequences that are used for statistical inference of homology. In the Pfam databases, all occurrences of the family are located by searching the profile HMM against a sizable sequence collection based on UniProt Knowledgebase (UniProtKB) (The UniProt Consortium, 2012; Finn *et al.*, 2014). The predicted proteins were also annotated against the dbCAN (Yin *et al.*, 2012) database. The dbCAN database has a collection of information on Carbohydrates-Active enZymes (CAZymes), which are enzymes involved in the metabolism of carbohydrates (Yin *et al.*, 2012). hmmsearch (Huerta-Cepas *et al.*, 2016) and hmmscan (HMMER v.3.2.1 package) were used to search against the Pfam and dbCAN databases, respectively, selecting hits with E-value < 0.001.

MinPath (Minimal set of Pathways) (Ye and Doak, 2009) was used to perform pathway prediction by comparison against the KEGG database (Kanehisa and Goto, 2000). MinPath eliminates several clearly false pathway annotations by identifying considerably fewer pathways for the genomes compiled in the KEGG database than the naive mapping approach (Ye and Doak, 2009). In addition, the metagenomes were run against the MetaCyc (Caspi *et*

al., 2018) database to further predict the possible pathways occurring within the symbiosis of the lichens. MetaCyc is a well-curated metabolic library that includes reactions, enzymes, metabolites, and metabolic pathways from all domains of life (Caspi *et al.*, 2018). Finally, METABOLIC (METabolic And BiogeOchemistry anaLyses In miCrobes) (Zhou *et al.*, 2022) was used to construct nutrient pathways that may be present within the metagenomes.

3.3. Results and Discussion

3.3.1. Metagenome assemblies of *P. tinctorum* and *F. flaventior*

The metagenomes representing the microbiomes of two lichen species were sequenced using the Illumina NovaSeq 6000 platform. Quality control of the reads yielded final datasets comprising 17,983,155 and 23,903,356 paired-end reads, for *P. tinctorum* and *F. flaventior*, respectively, with an average read length of 216 bp (range 200-232 bp). The read sets were initially assembled using MegaHit (Li *et al.*, 2015; Li *et al*., 2016) and metaSPAdes (Nurk *et al.*, 2017).

MegaHit produced 537,691 and 554,747 contigs, amounting to ~309 and 329 Megabases, for *P. tinctorum* and *F. flaventior*, respectively (Table 3.1). Conversely, metaSPAdes produced 23,198 and 31,729 contigs, amounting to ~ 61 and 67 Megabases for *P. tinctorum* and *F. flaventior*, respectively (Table 3.1). The results from the MegaHit assembler were used for downstream analysis in the present study. This decision is in line with previous research that concluded that MegaHit produced the highest number of contigs and the greater amount of assembled metagenome contigs compared to metaSPAdes (Llewellyn *et al.*, 2023). According to a study that compared the performance of eight widely used and freely available metagenome assembly tools, it depends on the aim and research question which assembler would give optimal results (Vollmers *et al.*, 2017). This study concluded that MegaHit and metaSPAdes are generally the best metagenome assemblers when identifying members of a complex habitat. However, if the research prioritises micro diversity, then MegaHit would be preferable. In the present study, MegaHit provided more assembled contigs which incorporates more sequences for downstream analysis.

Table 3.1: Final metagenome assembly report of *Parmotrema tinctorum* (65.61 % reads assembled) and *Flavopunctelia flaventior* (66.76 % reads assembled) using MegaHit (Li *et al.*, 2015; Li *et al*., 2016) and metaSPAdes (Nurk *et al.*, 2017).

	Parmotrema tinctorum		*Flavopunctelia flaventior*	
Statistics without reference	metaSPAdes	MegaHit	MegaHit	metaSPAdes
Number of contigs	23,198	537,691	554,747	31,729
Largest contig (bp)	48,608	32,802	23,579	27,035
Total length (bp)	61,413,412	309,140,312	329,175,876	67,266,763
N50 value (bp)	3,033	561	590	2,172
GC (%)	55.91	56	55	53.87
Mismatches	0	0	0	0

3.3.2. Metagenome contig classification

Metagenome contigs were classified as belonging to the domains prokaryotes and eukaryotes, using Whokaryote (Pronk and Medema, 2022). Tiara (Karlicki *et al.*, 2022) predictions were used as an additional feature in Whokaryote for improved contig classification (Pronk and Medema, 2022). A total of 49 and 44% of assembled contigs of the *P. tinctorum* and *F. flaventior* metagenomes were of eukaryotic origin. The remaining contigs were classified as belonging to prokaryotes.

Table 3.2: The classification (prokaryote or eukaryote) of contigs from the *Parmotrema tinctorum* and *Flavopunctelia flaventior* metagenomes.

Contigs	*Parmotrema tinctorum*	*Flavopunctelia flaventior*
Eukaryotic	261,124	245,672
Prokaryotic	276,567	309,075

3.3.3. Metagenome binning analysis of *P. tinctorum* and *F. flaventior*

The assembled contigs were binned using three distinct tools, namely CONCOCT, MaxBin2, and MetaBAT2 (Kang *et al.*, 2015; Kang *et al.*, 2019; Wu *et al.*, 2016; Sieber *et al.*, 2018) and refined using DAS tool (Sieber *et al.*, 2018). This yielded bins corresponding to seven and six MAGs (Table 3.3) for *P. tinctorum* and *F. flaventior*, respectively.

CheckM (Parks *et al.*, 2015; Schulz *et al.*, 2020) was used to check contamination and the completeness of the MAGs. Of the seven MAGs derived from the *P. tinctorum* metagenome, three showed ≥ 50% completeness, while all six MAGs from *F. flaventior* were deemed ≥50% complete (Table 3.3). Genomes with completeness ≥50% are considered relatively complete and have a broad range of applications (Parks *et al.*, 2015) such as possible metabolic analysis of microorganisms within an ecosystem, resulting in a discovery of enzymes that can be used for the purposes of biotechnology (Grimm *et al.*, 2021). Similarly, contamination of ≤ 20% was considered acceptable, as anything higher than 20% increases limitations for good quality downstream analysis (Parks *et al.*, 2015). For the *P. tinctorum* metagenome, five out of the seven MAGs demonstrated contamination levels ≤ 20% while four MAGs from *F. flaventior* had contamination levels ≤ 20% (Table 3.3). Overall, only one MAG (MAG4) from *P. tinctorum* and four MAGs (MAG8,9,10 and 12) from *F. flaventior* met both completeness and contamination criteria.

Table 3.3: Metagenome binning quality assessment results for *Parmotrema tinctorum* and *Flavopunctelia flaventior*, using CheckM (Parks *et al.*, 2015).

	MAGs	Number of genomes	MAG Completeness (%)	MAG Contamination (%)	Domain
Parmotrema tinctorum	MAG 1	387	72,81	50,88	Bacteria
	MAG 2	5449	69,91	40,37	Bacteria
	MAG 3	2258	32,05	9,62	Bacteria
	MAG 4	88	64,32	4,08	Bacteria
	MAG 5	26	31,50	4,89	Bacteria
	MAG 6	26	21,23	1,75	Bacteria
	MAG 7	5449	16,16	1,44	Bacteria
Flavopunctelia flaventior	MAG 8	2258	59.36	15.09	Bacteria
	MAG 9	2258	57.34	18.94	Bacteria
	MAG 10	2258	54.80	17.29	Bacteria
	MAG 11	63	53.35	20.20	Bacteria
	MAG 12	26	51.47	18.01	Bacteria
	MAG 13	2231	50.53	24.35	Bacteria

3.3.3.1. Refinement and taxonomic classification of lichen-derived metagenomes.

To further improve the assemblies and contig orientation of the lichen-derived MAGs, the five good quality MAGs (MAG4, 8, 9, 10 and 12) were scaffolded using a reference genome (identified by TYGS) on the MeDuSa web server (Bosi *et al.*, 2015). Moderate improvements in both N50 values and lower numbers of contigs were achieved for all MAGs (Table 3.4).

Table 3.4: Refinement of metagenome assembled genomes (MAGs) from the metagenomes of the two lichen species: *Parmotrema tinctorum* (**pink**) and *Flavopunctelia flaventior* (**green**), using MeDuSa (Bosi *et al.*, 2015).

MAGs		**NCBI Blast/TYGS**	**Input draft**			**Output scaffold**		
	Phylum	Genus	# Contigs	Length (bp)	N50 (bp)	# Contigs	Length (bp)	N50 (bp)
MAG 4	Verrucomicrobiota		992	3,144,091	3,416	984	3,144,391	3,482
MAG 8	Pseudomonadota	*Caulobacter*	1,317	2,425,866	1,895	1,234	2,429,766	1,939
MAG 9	Pseudomonadota	*Rhodopseudomonas*	2,866	4,737,538	1,723	2.853	4,738,038	1,726
MAG 10	Acidobacteriota	*Granulicella*	1,672	3,461,183	2,016	1,620	3,463,483	2,042
MAG 12	Acidobacteriota	*Terriglobus*	2,274	3,877,024	1,759	2,247	3,878,424	1,766

MAG4, from the *P. tinctorum* metagenome, was assigned to the phylum Verrucomicrobiota, class *Verrucomicrobiae* and order *Chthoniobacterales*, but could not be assigned further at the family or genus level (Table 3.4). Members of this order are Gram-negative rods that are capable of breaking down complex carbohydrates such as xylan and cellulose to support the cycling of carbon (Herlemann *et al.*, 2013). In a study on transcriptomic analysis of the

microbiome of the tree lungwort lichen, *Chthoniobacterales* was shown to play a role in the synthesis of different vitamins, the breakdown of phenolic chemicals, and resistance to oxidative stress and antibiotics (fluoroquinolones) (Cernava *et al.*, 2017). Additionally, another study on the microbiome composition of *Lobaria pulmonaria* highlighted the possible roles of *Chthoniobacterales* in facilitating the resistance against toxic compounds and oxidative stress (Grimm *et al.*, 2021).

MAG8 and 9 derived from the *F. flaventior* metagenome were assigned to the Gram-negative phylum Pseudomonadota and the genera *Caulobacter and Rhodopseudomonas*, respectively (Table 3.4). *Caulobacter* spp. are typified by their capacity to generate stalked cells and an atypical cell division cycle (Brun *et al.*, 1994). The genus *Caulobacter* has been detected in *Umbilicaria americana*, *Umbilicaria phaea*, *Parmelia sulcata*, and *Rhizoplaca chrysoleuca* but its role within the lichen symbioses remains unclear (Bates *et al.*, 2011). The genus *Rhodopseudomonas* comprises purple non-sulphur photoheterotrophic and chemoheterotrophic bacteria that utilise nitrogenase enzymes to fix nitrogen in the atmosphere by converting nitrogen gas into ammonia and hydrogen gas (Humphrey *et al.*, 2022). Given their metabolic versatility, members of this genus are of biotechnological interest for the production of various value-added products (Oh *et al.*, 2004; Carlozzi, 2012; Brown *et al.*, 2022). Further, they have been observed as part of the bacteriome composition of the lichen *L. pulmonaria* under the order *Rhizobiales* (Grube and Berg, 2009; Erlacher *et al.*, 2015). Members of the order *Rhizobiales* have been implicated in functions such as producing vitamins (involved in gene regulation and photosynthesis) and auxins (promoting biomass growth), fixing nitrogen and offering protection against osmotic and oxidative stress within the lung lichen symbioses (Erlacher *et al.*, 2015).

MAG10 and 12 belong to the Gram-negative phylum Acidobacteriota and the genera *Granulicella* and *Terriglobus*, respectively (Table 34). Previous research using a 16S rRNA amplicon sequencing-based approach identified these as the most abundant bacterial genera in *P. tinctorum* and *F. flaventior* (Naude, 2023). *Terriglobus* are moderately acidophilic, non-nitrate reducing bacteria that generate an extracellular matrix that help cells float freely in liquid media (Eichorst *et al.*, 2007; Campbell, 2014). They are capable of breaking down starch and pectin but not cellulose and chitin (Campbell, 2014). Additionally, *Terriglobus* has been isolated from *U. americana*, *U. phaea*, *P. sulcata*, and *R. chrysoleuca* and proposed to be involved in the cycling of carbon and nitrogen (Bates *et al.*, 2011; Eichorst *et al.*, 2018). *Granulicella* are chemoheterotrophic strict aerobic organisms (Campbell, 2014). This genus

has been observed in *Cladonia* lichens and are capable of breaking down pectin and laminarin and producing polysaccharides that are important for the formation of extracellular polymeric substances (EPS) (Pankratov and Dedysh, 2010).

A previous study on three lichens: *Peltigera*, *Cladonia*, and *Lobaria* identified MAGs assigned to the Pseudomonadota (20 MAGs), Bacteroidota (3 MAGs), Acidobacteriota (3 MAGs), and Verrucomicrobiota (1 MAG) (Wicaksono *et al.*, 2020). The phyla Pseudomonadota, Acidobacteriota and Verrucomicrobiota generally predominate the MAGs of lichen species (Grimm *et al.*, 2021).

3.3.4. Overview of the functions facilitated by the *F. flaventior* and *P. tinctorum* microbiome

The lichen microbiome may play a wide range of roles in the lichen symbiotic system (Grube and Berg, 2009; Grimm *et al.*, 2021). These include vital processes such as nutrient uptake and/or assimilation of nutrients like nitrogen, phosphate, iron, amino acids, sulphur, xylose, dipeptides, and sugars (Huneck and Yoshimura, 1996; Liba *et al.*, 2006; Grube and Berg, 2009; Cernava *et al.*, 2017). To characterise the functionality of the *P. tinctorum* and *F. flaventior* microbiome, proteins were functionally annotated using a range of functional annotation tools.

A total of 475,873 and 387,741 proteins were predicted from the *P. tinctorum* and *F. flaventior* metagenomes, respectively. These were placed into 22 Cluster of Orthologous Groups (COGs) categories under the broader supra-functional categories: metabolism, information storage and processing, cellular processes and signalling, unknown function (Figure 3.1). In both cases, proteins involved in metabolism predominate (*P. tinctorum*: 38%; *F. flaventior*: 44%) (Figure 3.1). Within this supra-functional category, the largest proportion of proteins are involved in energy production and conversion (C: 6% for *P. tinctorum* and 7% for *F. flaventior*) and amino acid (E: 8% for *P. tinctorum* and 7% for *F. flaventior*) and carbohydrate (G: 7% for both *P. tinctorum* and *F. flaventior*) transport and metabolism (Figure 3.2). In a study that assessed the functions of the lung lichen associated microbiome, 7.9, 14.5, and 83.6% of bacterial, fungal and green algae proteins, respectively, were involved in energy production and conversion (Schneider *et al.*, 2011). Carbohydrate metabolising proteins were largely attributed to the fungal component of the microbiome, accounting for 15% of the identified proteins, followed by green algae at 10.3% (Schneider *et al.*, 2011). Additionally, proteins responsible for amino acid transport and metabolism in the tree lungwort lichen were found in bacteria and

fungi, supporting evidence that bacteria within the lichen microbiome produce amino acids (Liba *et al.*, 2006; Schneider *et al.*, 2011).

For the information storage and processing supra-functional category, the *P. tinctorum* (29%) metagenome identified more proteins than *F. flaventior* (16%). Within this supra-functional category more proteins were involved in translation, ribosomal structure and biogenesis (J: 11% for *P. tinctorum* and 5% for *F. flaventior*), transcription (K: 12% for *P. tinctorum* and 4% for *F. flaventior*) and replication, recombination and repair (L: 6% for *P. tinctorum* and 7% for *F. flaventior*) (Figure 3.2). Transcription and translation are important processes that enable microorganisms to produce proteins essential for their adaptation and response to stress in their specific environment (Balleza *et al.*, 2009; Wang *et al.*, 2023). Similarly, proteins involved in replication, recombination and repair are important for microorganisms to survive exposure to different environmental conditions such ultraviolet (UV) radiation (Singh *et al.*, 2010; Gagunashvili and Andrésson, 2018). In a study on the genomes of members of the genera *Nostoc*, commonly associated with lichens, more proteins identified were involved in the COG categories J (5.83%) and L (4.9%) (Gagunashvili and Andrésson, 2018) in comparison to other categories. It was suggested that continuous exposure to UV radiation warranted for cyanobacteria to have mechanisms in place to repair DNA damage induced by radiation (Singh *et al.*, 2010).

The cellular processing and signalling supra-functional category had the smallest proportion of proteins attributed to it (*P. tinctorum*: 13%; *F. flaventior*: 16%) (Figure 3.1). More proteins were involved in cell wall, membrane, envelope biogenesis (M: 4% for *P. tinctorum* and 5% for *F. flaventior*), post-translational modification, protein turnover, chaperons (O: 3% for *P. tinctorum* and 4% for *F. flaventior*) and signal transduction mechanisms (T: 4% for *P. tinctorum* and 4% for *F. flaventior*) (Figure 3.2). The proteins involved in the M category are responsible for the synthesis of structures that provide the members of the lichen microbiome with structural support and protection against the external environment (Silhavy *et al.*, 2010; Dörr *et al.*, 2019). The O category associated proteins are responsible for assessing the quality of folded proteins and ensuring the removal of damaged proteins which can be cytotoxic to microorganisms (Cloutier and Coulombe, 2013; Wang *et al.*, 2013; Macek *et al.*, 2019). The proteins involved in the T category enables microorganisms to receive and respond to stimuli such as nutrient availability, sunlight and osmotic stress in the environment (Simon, 1995; Galperin, 2004).

For the function unknown supra-functional category, 20 and 24% of total proteins from the *P. tinctorum* and *F. flaventior* metagenomes, respectively, were identified (Figure 3.1 and 3.2). These are proteins whose functions have not yet been characterised (Galperin *et al.*, 2015).

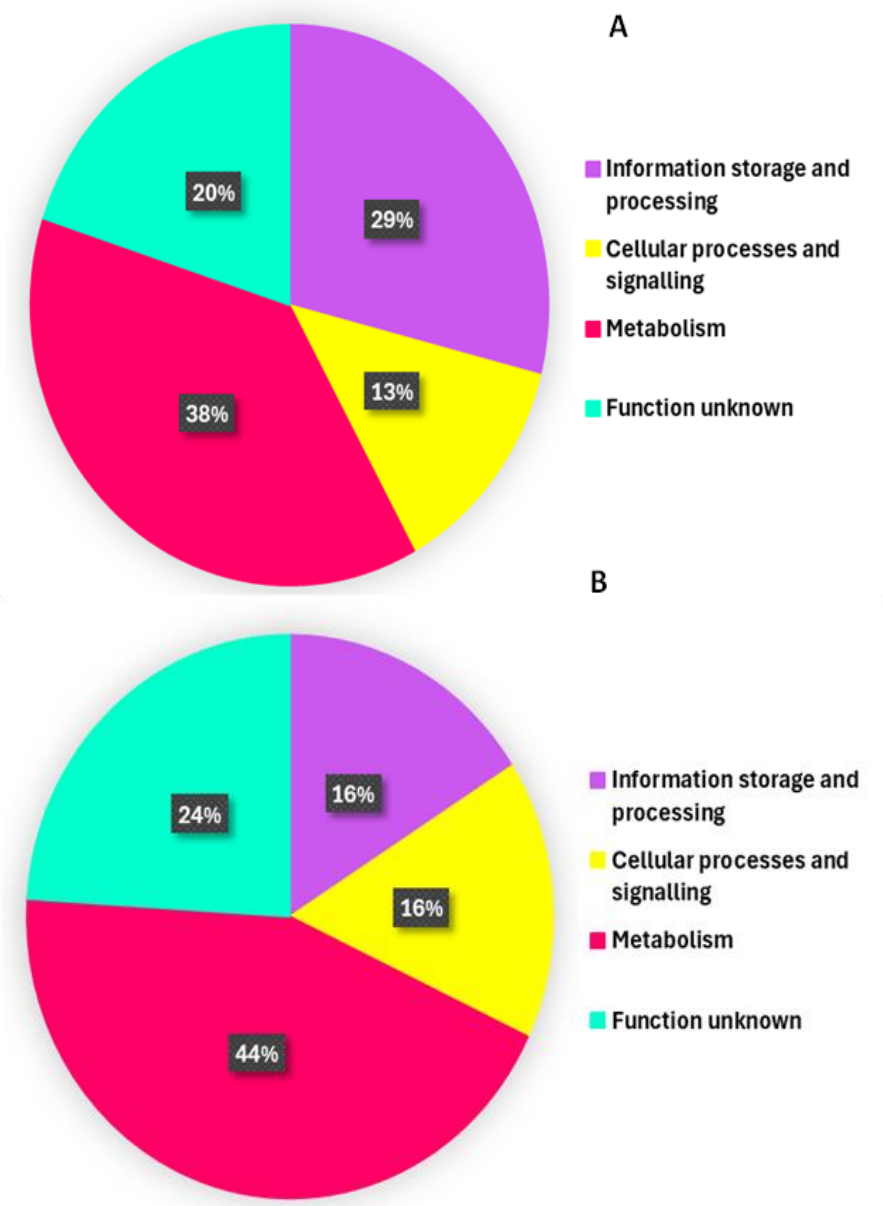

Figure 3.1: Classification of proteins into Orthologous Groups, comparing *Parmotrema tinctorum* (**A**) and *Flavopunctelia flaventior* (**B**) metagenomes.

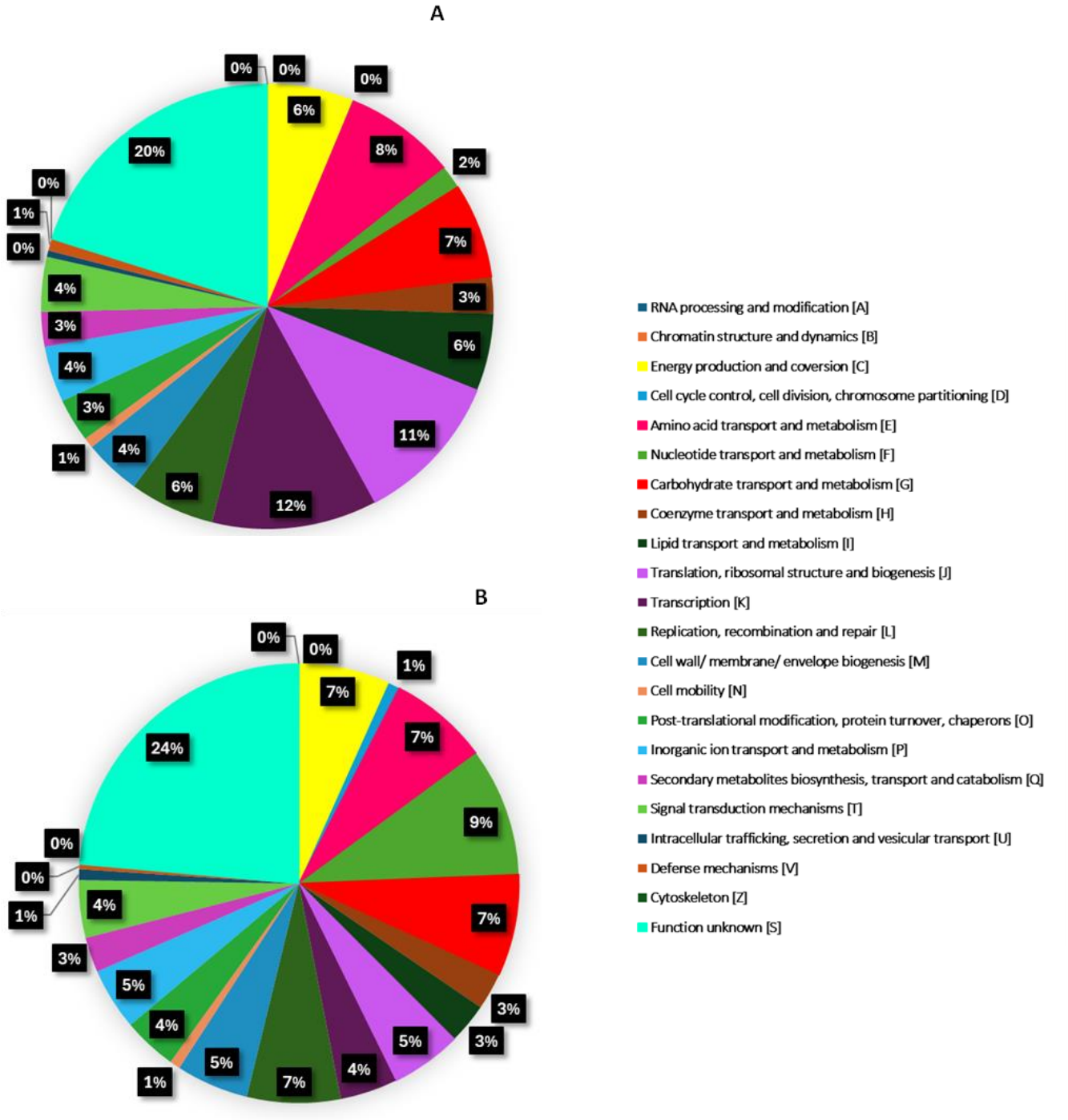

Figure 3.2: The number of proteins grouped in each of the 22 Cluster of Orthologous Group (COGs) (Galperin *et al.*, 2015) categories for the *Parmotrema tinctorum* (**A**) and *Flavopunctelia flaventior* (**B**) metagenomes.

3.3.5. The lichen microbiome and carbon cycling

The carbon cycle represents the flow of carbon in many different forms and is a crucial aspect of the biogeochemical dynamics on Earth (Pulselli and Marchi, 2015). It consists of five primary processes namely, combustion, respiration, sedimentation, exchange and photosynthesis. The majority of these processes use carbon dioxide as a carbon source (Humayun and Anwar, 2021). The carbon cycle of the two lichen species in this study were reconstructed using METABOLIC (Zhou *et al.*, 2022).

The occurrence of genes coding for proteins involved in reactions such as organic carbon, ethanol, and acetate oxidation (Figure 3.3) indicates the prevalence of aerobic microorganisms that are involved in the *P. tinctorum* and *F. flaventior* carbon cycle (Grimm *et al.*, 2021). Carbon oxidation has been observed for all the prokaryotic and fungal phyla identified previously (Chapter 2). Members of the bacterial phyla Pseudomonadota (genus *Methylobacterium*) (Du *et al.*, 2024), Firmicutes (genus *Clostridium*) (Kutscha and Pflügl, 2020) and archaeal phyla Candidatus Lokiarchaeota (Orsi *et al.*, 2020) and Euryarchaeota (Leigh and Whitman, 2013; Castro-Fernandez *et al.*, 2017) are capable of both ethanol and acetate oxidation and these taxa were likewise identified in the *P. tinctorum* and *F. flaventior* metagenomes.

A global schematic of the carbon cycle (Figure 3.3) in the two lichens demonstrates the near-identical nature of their carbon cycles. However, the combustion reaction of ethanol to carbon dioxide and water which is present only in *P. tinctorum* (Figure 3.3A). Ethanol is a common product of carbohydrate fermentation by microorganisms such as yeasts (e.g. *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii*), filamentous fungi (e.g. *Aspergillus* spp. and *Trichoderma viride*) and bacteria (e.g. *Clostridium thermocellum*, *Escherichia coli* and *Zymomonas mobilis*) (Lin and Tanaka, 2006). Within symbiotic relationships, either acetic acid bacteria can absorb ethanol and use it as an energy source, or ethanol can function as an antiseptic against bacteria and other microorganisms (Lin and Tanaka, 2006).

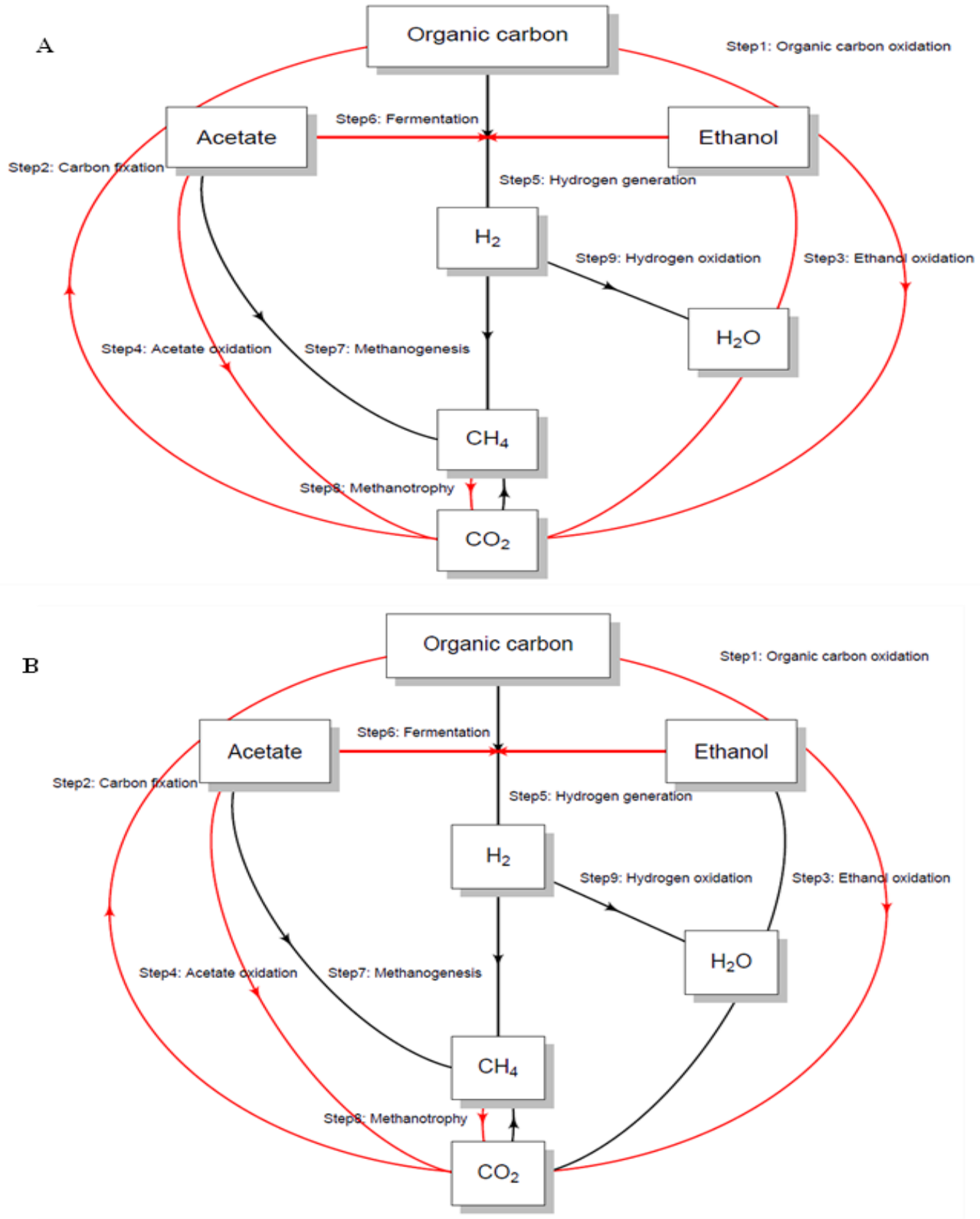

Figure 3.3: Carbon cycle taking place within the *Parmotrema tinctorum* (A) and *Flavopunctelia flaventior* (B) lichen symbiosis. **Red** arrows suggests that the pathways are present while the **Black** arrows symbolise the absence of the pathway using METABOLIC (Zhou *et al.*, 2022).

KEGG annotation of the metagenome-encoded proteins (Kanehisa and Goto, 2000) identified 2,169 pathways per metagenome for both the *P. tinctorum* and *F. flaventior*. Of these pathways, 220 were associated with carbohydrate metabolism. Central metabolic pathways (76 hits) such as glycolysis, gluconeogenesis, pentose phosphate pathway, and citrate cycle were present in both lichen species. Carbon fixation (109 hits) was also identified as being common between the two lichen species by METABOLIC (Zhou *et al.*, 2022) and KEGG (Kanehisa and Goto, 2000) (Figure 3.3). The reductive pentose phosphate cycle (Calvin cycle) was present in both the *P. tinctorum* and *F. flaventior* metagenomes (Table A2.1). In this pathway, photoautotrophic organisms such as cyanobacteria convert atmospheric carbon dioxide into organic molecules in the presence of sunlight (Pribil and Leister, 2017). However, there were key differences observed in the carbon fixation pathways of the *P. tinctorum* and *F. flaventior* metagenomes.

The 4-hydroxyburyrate/3-hydroxypropionate (HB/HP), 3-hydroxypropionate (3HP) bi-cycle, and C4-dicarboxylic acid (DC/HB) cycles were only present in the *P. tinctorum* metagenome (Table A2.1). The 3HP bi-cycle is an aerobic process used by photosynthetic green non sulphur bacteria to fix carbon dioxide and bicarbonate into pyruvate and acetyl-CoA (Tabita, 2009). The 3HP bi-cycle has been attributed to members of the phyla Cloroflexota (*Chloroflexus aurantiacus*) (Holo, 1989; Gayathri *et al.*, 2021), Pseudomonadota and Actinomycetota (Garritano *et al.*, 2022). The DC/HB cycle involves the carboxylation of acetyl-CoA to succinyl-CoA catalysed, by PEP carboxylase (Garritano *et al.*, 2022). More succinyl-CoA is subsequently produced by an incomplete reverse tricarboxylic acid cycle and one molecule of acetyl-CoA is a final product (Garritano *et al.*, 2022). This cycle appears to be restricted to anaerobic archaea of the phyla Asgardarchaeota (class Lokiarchaeia), and Thermoproteota (genera *Sulfolobus* and *Ignicoccus*) (Garritano *et al.*, 2022).The genus *Ignicoccus* was among the unique archaeal genera within the *P. tinctorum* metagenome (Chapter 2) and may contribute to the production of acetyl and succinyl-CoA within the symbiosis.

In the HB/HP cycle the carboxylation of acetyl-CoA to succinyl-CoA occurs first (Garritano *et al.*, 2022). Following this, succinyl-CoA ends up being converted to more acetyl-CoA molecules, with 4-hydroxybutyrate as a by-product (Berg, 2011). The HB/HP cycle has been found in the members of the phylum Pseudomonadota (genus *Luminiphilus*), some bacteria within the family *Bradyrhizobiaceae* (Garritano *et al.*, 2022) and in the archaeal species *Metallosphaera sedula* (Berg *et al.*, 2007) and *Nitrosopumilus maritimus* (Berg, 2011).

3.3.6. Enzymes involved in the cycling of carbon

An extensive range of polysaccharides are enzymatically recycled in the carbon cycle, producing simple sugars that serve as the primary energy source for microorganisms (Gavande *et al.*, 2023). The carbohydrate active enzymes (CAZymes) are crucial for recycling organic compounds because they break down and synthesise polysaccharides, including biosynthesis and hydrolysis of glycosidic bonds, beta-elimination, and removal of ester bonds (Cantarel *et al.*, 2009). The key enzyme classes include the glycosyltransferase (GT – enzymes that use activated donors to create glycosidic bonds and transfer sugars to particular acceptors such as proteins, lipids, or other glycans) (Yip and Withers, 2006), polysaccharide lyases (PL – degrade polysaccharides containing uronic acids) (Cantarel *et al.*, 2009), glycosyl hydrolases (GH – break down glycosidic bonds) (Henrissat, 1991) and carboxylesterases (CE – break down ester bonds) (Cantarel *et al.*, 2009). The primary natural producers of CAZymes are bacteria and fungi (Ameri *et al.*, 2022).

A total of 83 CAZyme families per metagenome were identified using dbCAN2 (Yin *et al.*, 2012). GHs were the most prevalent enzyme class, with 78 families identified (Figure 3.4). GHs have also been identified in *Alectoria sarmentosa* sampled from Idaho County and were the most prevalent (Tagirdzhanova, Saary, J. P. Tingley, *et al.*, 2021). The remaining five CAZymes were identified as PLs. No GTs were identified by dbCAN2, however, some were detected by Prodigal (Hyatt *et al.*, 2010) when run against the KEGG pathway (Kanehisa and Goto, 2000) database as enzymes involved in metabolism (Table A2.2).

The CAZYme family GH013 was the most prevalent, with 35 and 26 hits in the *P. tinctorum* and *F. flaventior* metagenomes, respectively (Figure 3.4). Members of this family breakdown α-glucoside linkages in starch, pullulan and sucrose (Sharma *et al.*, 2022). These enzymes are key drivers for the regeneration of cell wall constituents and the modification of specific compounds, including antibiotics (Faure, 2002). Fungi, archaea and bacteria can harbour the GH013 enzymes (Tashkandi and Baz, 2023).

A total of ten GH families were unique to *P. tinctorum* and nineteen were unique to *F. flaventior* (Figure 3.5). Among the unique *P. tinctorum* CAZymes, GH065 (maltose phosphorylase) and GH025 (lysozyme) occurred in the highest number of copies (Figure 3.5). GH025 family enzymes, cleave the β-1,4-glycosidic bond between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) in bacterial peptidoglycan (Moroz *et al.*, 2021), while GH065 family enzymes break down α-glucosidic bonds in oligosaccharides (Nakamura *et al.*, 2021).

In contrast, enzymes belonging to the GH029 (α-L-fucosidase) and GH092 (mannosyl-oligosaccharide α-1,2-mannosidase) families were the most prevalent GH among those unique to the *F. flaventior* metagenome (Figure 3.5). GH029 family enzymes catalyse the hydrolysis of fucose (Grootaert *et al.*, 2020), while the breakdown of N-glycans having α-mannose chains is catalysed by GH092 family enzymes (Alonso-Gil *et al.*, 2022).

Glycosyltransferases (predicted by KEGG) were predominated by members of GT family 1, with 316 and 250 hits in *P. tinctorum* and *F. flaventior* metagenomes, respectively (Table A2.2). GT1 can be found in archaea, bacteria, fungi and even viruses, with bacteria accounting for the majority (Zhang *et al.*, 2020). This family of enzymes can act on substrates such as terpernoids, steroids, polyphenols and flavonoids (Teze *et al.*, 2022).

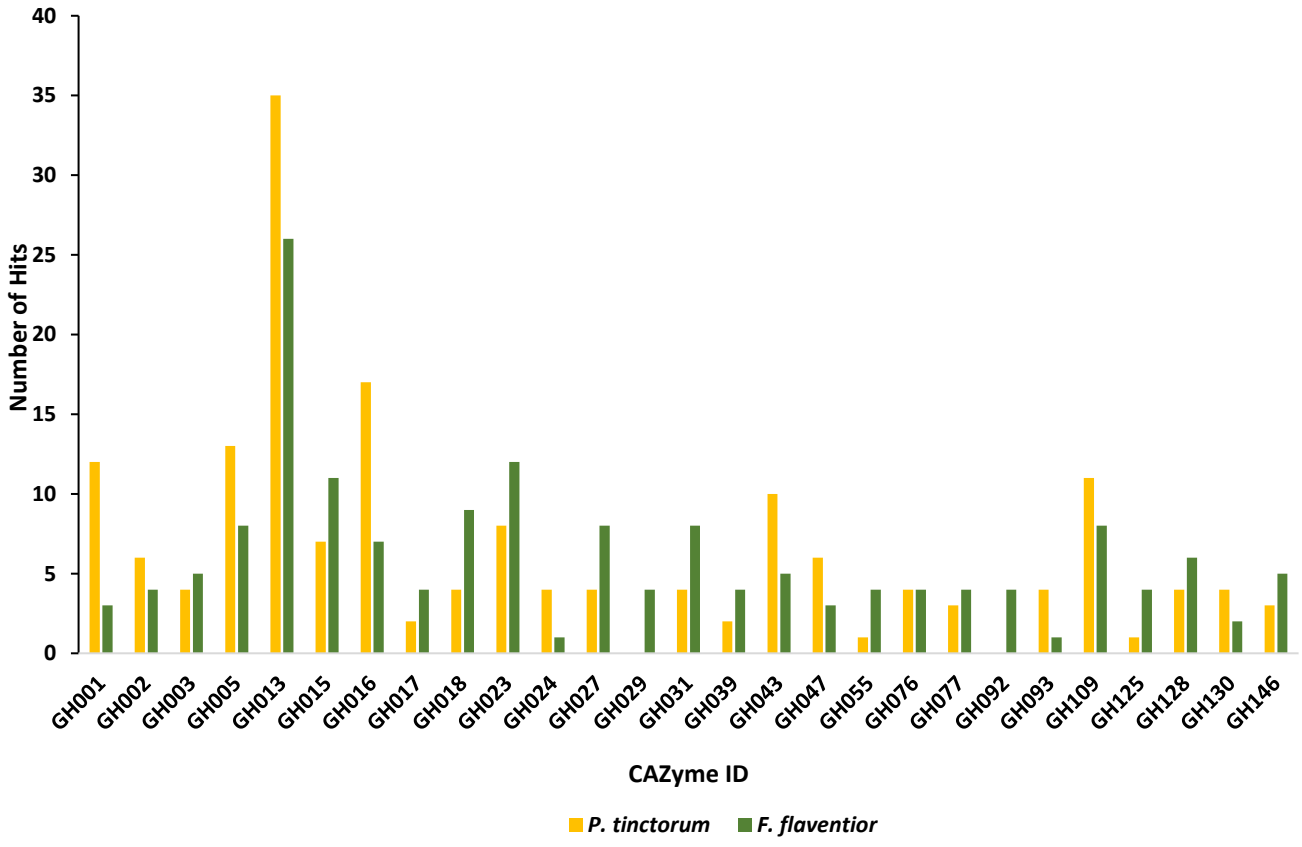

Figure 3.4: The most dominant CAZymes across the *Parmotrema tinctorum* (**yellow**) and *Flavopunctelia flaventior* (**green**) metagenomes, using dbCAN2 (Yin *et al.*, 2012).

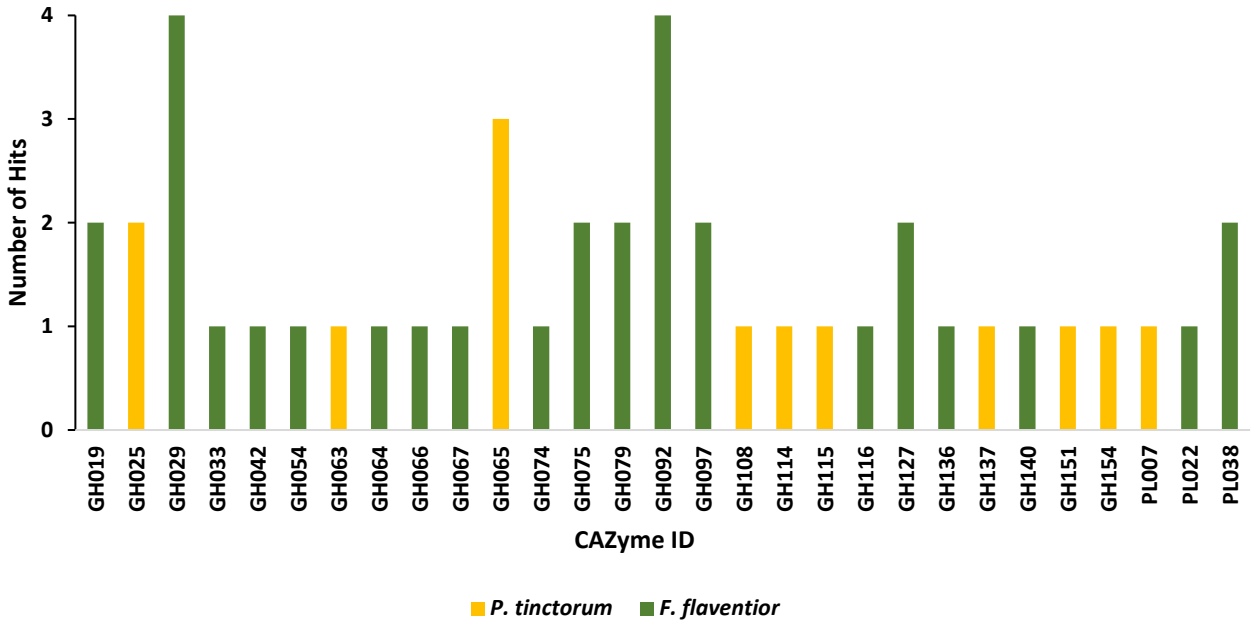

Figure 3.5: The CAZymes that are unique to the *Parmotrema tinctorum* (**yellow**) and *Flavopunctelia flaventior* (**green**) metagenomes, using dbCAN2 (Yin *et al.*, 2012).

3.3.7. The lichen microbiome and nitrogen cycling.

The nitrogen cycle is fundamental to all life processes and involves everything from the food we consume to the air we breathe (Thiemens and Shaheen, 2014). It also plays a crucial role in the synthesis and break down of nucleic acids and amino acids. Generally, there is competition for nitrate and nitrite in the environment between two microbial anaerobic respiration processes: dissimilatory nitrate reduction to ammonia (DNRA) and denitrification (Van den Berg *et al.*, 2016). Nitrogen is released into the atmosphere as dinitrogen gas when nitrate is reduced through denitrification, whereas nitrogen is retained in the habitat as ammonium through DNRA (Pandey *et al.*, 2020).

The reconstructed nitrogen cycles of both *F. flaventior,* and *P. tinctorum* microbiomes suggest that they favour the retention of ammonia within the symbiosis, through the nitrite ammonification pathway (Figure 3.6). The anaerobic process known as nitrite ammonification involves reducing nitrate to nitrite and then reducing nitrite to ammonium using six electrons (Cabello *et al.*, 2009). Microorganisms largely drive nitrate ammonification by catalysing the reduction of nitrite to ammonium through the pentaheme cytochrome c nitrite reductase (NrfA) (Saghaï *et al.*, 2023). Bacterial genera capable of nitrate ammonification include *Campylobacter* (De Vries *et al.*, 1980), *Desulfovibrio* (Almeida *et al.*, 2003), *Escherichia*

(Bamford *et al.*, 2002), *Thialkalivibrio* (Tikhonova *et al.*, 2012) and *Geobacter* (Campeciño *et al.*, 2020).

The nitrogen cycles of the two lichen species were similar except for the reduction reaction of nitrate to nitrite, which was only present in the *P. tinctorum* metagenome (Figure 3.6). Reduced nitrate can be used as a nitrogen source to promote growth of microorganisms within a mutualistic relationship through nitrate assimilation (Moreno-Vivián *et al.*, 1999). Furthermore, it can also be used to produce energy when nitrate is used as a final electron acceptor through nitrate respiration (Zhu *et al.*, 2020).

Nitrogen fixation, the conversion of atmospheric nitrogen gas to ammonia catalysed by nitrogenase, is absent in both lichen microbiomes (Figure 3.6). There is however, some evidence in literature suggesting the presence of nitrogen fixation in lichens (Grube *et al.*, 2009; Bates *et al.*, 2011; Jiang *et al.*, 2017; Almendras *et al.*, 2018). Studies done on four lichen species (*P. sulcata, R. chrysoleuca, U. americana* and *U. phaea*) revealed the presence of nitrogen fixing taxa in the bacterial genera *Azospirillum* (Bates *et al.*, 2011; Almendras *et al.*, 2018), *Bacillus*, *Bradyrhizobium, Clostridium* (Grube *et al.*, 2009; Almendras *et al.*, 2018), *Frankia* (Grube *et al.*, 2009; Bates *et al.*, 2011; Almendras *et al.*, 2018) and *Pseudomonadales* (Grube *et al.*, 2009; Jiang *et al.*, 2017).

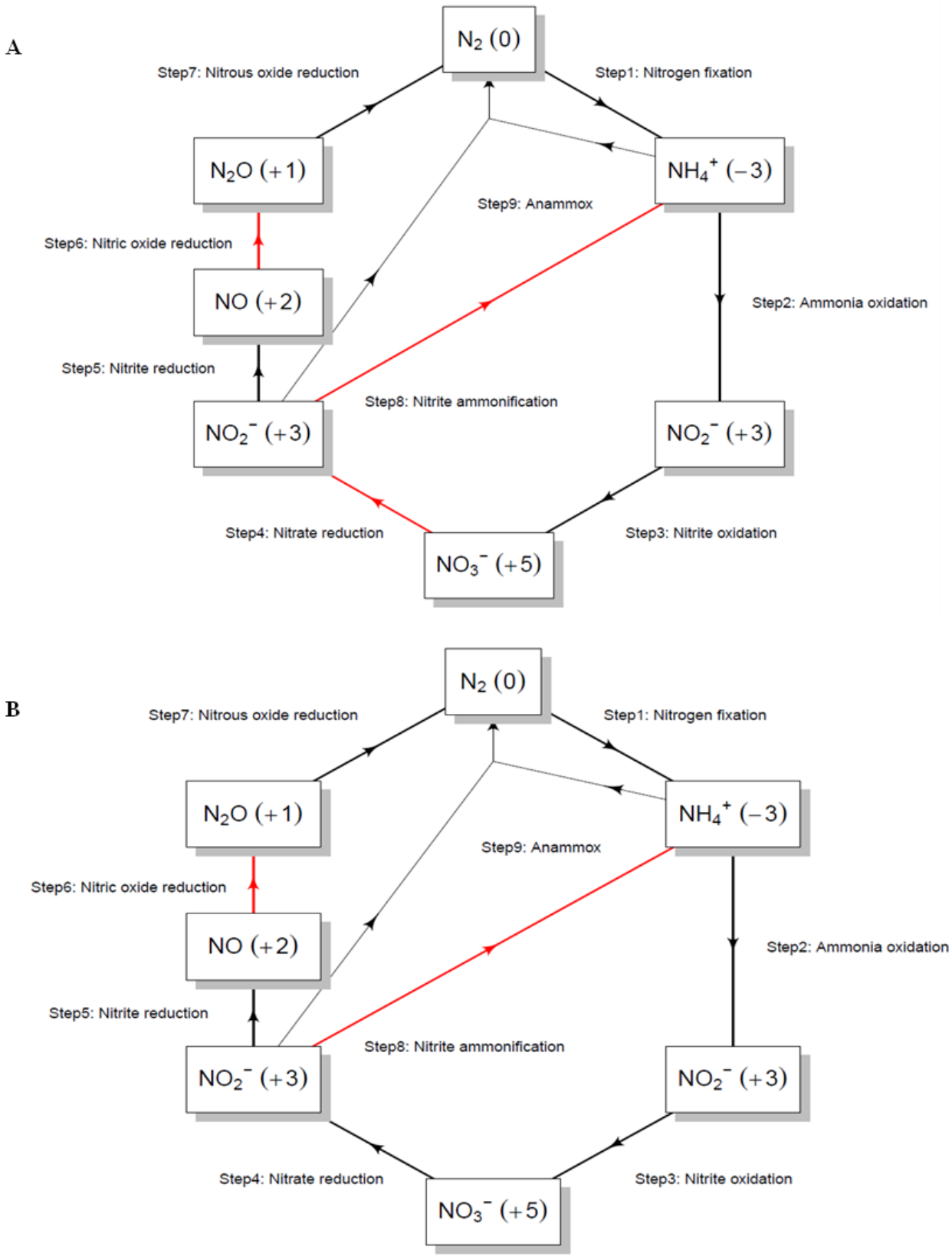

Figure 3.6: The reconstructed nitrogen cycle for *Parmotrema tinctorum* (A) and *Flavopunctelia flaventior* (B) lichen symbiosis. **Red** arrows suggests that the pathways are present while the **Black** and **Grey** arrows symbolise the absence of the pathway.

3.3.8. Enzymes involved in the cycling of nitrogen

In soil and aquatic ecosystems, proteinaceous molecules are common sources of organic nitrogen (Nguyen *et al.*, 2019). Microorganisms degrade these proteinaceous molecules using peptidase enzymes that cleave peptides and proteins (Rawlings *et al.*, 2018). Peptidases are thought to have originated early during the evolution process and are present in all microorganisms (Nguyen *et al.*, 2019). Several peptidase super-families, including aspartic, cysteine, metallo-, serine, and threonine peptidases, have emerged as a result of subsequent diversification and are named based on the type of amino acid present at the centre of the active site of the enzymes (Mooshammer *et al.*, 2014).

The lichen metagenomic protein datasets were compared against the MEROPS database (Rawlings *et al.*, 2018). A total of 117 peptidase across eight super-families were identified in both the *P. tinctorum* and *F. flaventior* metagenomes (Figure 3.7). The cysteine (19%), metallo- (38%) and serine (26%) super-families together accounted for ~ 83% of the total identified peptidases. The majority of aspartic peptidases are produced by fungi, metallopeptidases are frequently discovered in bacteria, and serine and cysteine peptidases appear to be prevalent among all microorganisms (Page and Cera, 2008). Aspartic and threonine peptidases are less common in archaea and bacteria, respectively (Nguyen *et al.*, 2019). Prolyl aminopeptidases (S33 family) (*P. tinctorum* :89 hits and *F. flaventior* :85 hits) were the dominant peptidases in both lichen species (Figure 3.7). Members of this family are exopeptidases that selectively eliminate proline from the N-terminus of peptides or proteins and have a range of different physiological functions across all domains of life (Dong *et al.*, 2022).

A total of twenty-six and nine peptidase families were unique to the *P. tinctorum* and *F. flaventior* metagenomes, respectively. In both cases, these were dominated by lichen-specific cysteine proteases (31 and 22%) and metalloproteases (31 and 67%) in *P. tinctorum* and *F. flaventior*, respectively (Figure 3.8).

In a large-scale study that examined the distribution of secreted peptidases among prokaryotic lineages the Acidobacteriota, Actinomycetota, Bacteroidota, Planctomycetota, and Pseudomonadota were shown to be key producers of such enzymes (Nguyen *et al.*, 2019). In particular, members of the Acidobacteriota had the largest mean number of secreted peptidases per genome (Nguyen *et al.*, 2019). Peptidase enzymes have been isolated from lichens such as *Cladonia rangiferina*, *L. pulmonaria*, *Parmelia sulcata* and *P. arnoldii* (Rodriguez *et al.*, 2012).

Within the lichen microbiomes of *P. tinctorum* and *F. flaventior*, members of the Acidobacteriota (genera *Granulicella* and *Terriglobus*) may be responsible for secreting the majority of the peptidases.

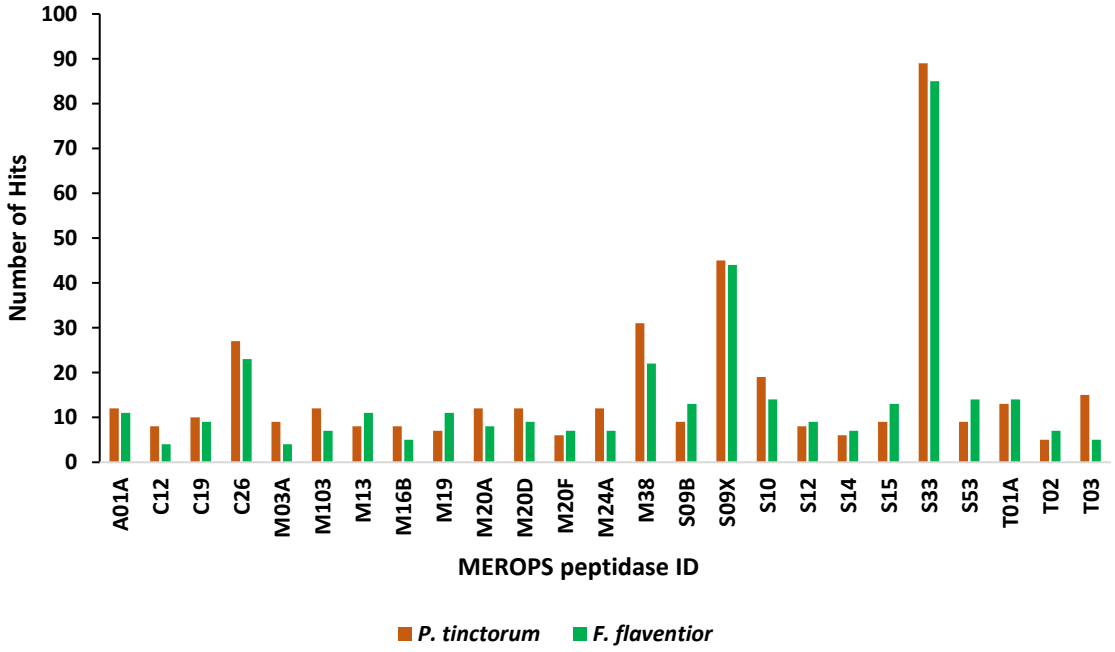

Figure 3.7: The most dominant peptidase across the *Parmotrema tinctorum* (**brown**) and *Flavopunctelia flaventior* (**green**) metagenomes, using MEROPS (Rawlings *et al.*, 2018).

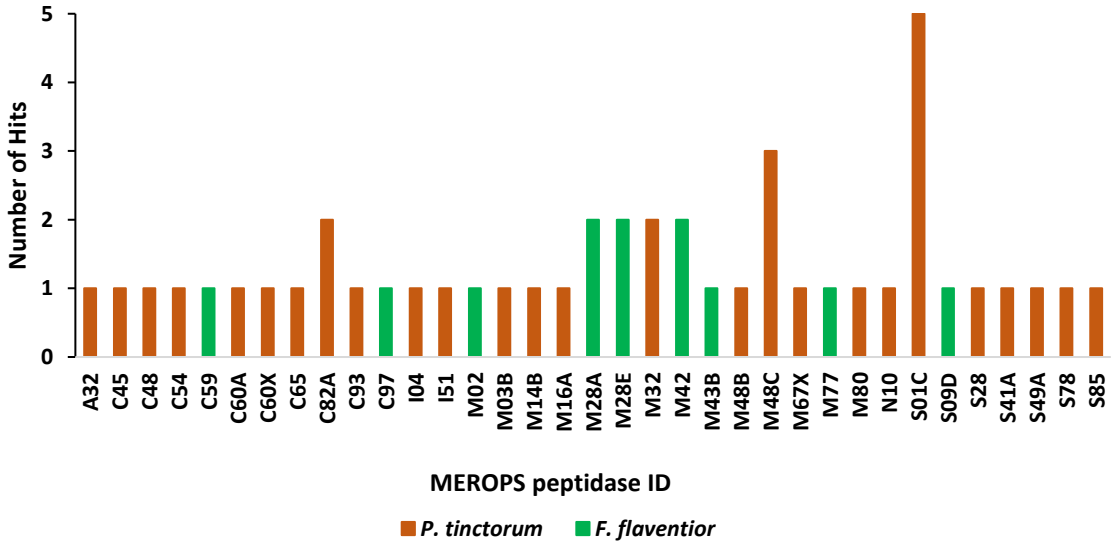

Figure 3.8: Unique peptidases across the *Parmotrema tinctorum* (**brown**) and *Flavopunctelia flaventior* (**green**) metagenomes, using MEROPS (Rawlings *et al.*, 2018).

3.4. Conclusions

The analysis of metagenome sequencing, assembly, and functional annotations of *F. flaventior* and *P. tinctorum* lichens has provided insight on the possible roles played by the lichen microbiome. It also highlighted the importance of the lichen microbiome in the self-sustaining ecosystem. The data analysis has also shown the metabolic capacities and putative functions of microorganisms such as carbon and amino acid transport and metabolism linked with *F. flaventior* and *P. tinctorum*. While protein distribution among the supra-functional categories were similar between the two lichen metagenomes, more proteins were identified as being involved in the information storage and processing supra-functional category for *P. tinctorum*. Since proteins involved in this supra-functional category are responsible for the adaptation of microorganisms to their environment, it suggests that the *P. tinctorum* lichen microbiome may be less sensitive to changes in environmental conditions compared to *F. flaventior*. Although the nitrogen and carbon cycles of *F. flaventior* and *P. tinctorum* were similar, there were also notable differences. The *P. tinctorum* lichen metagenome incorporates pathways involved in ethanol oxidation and nitrate reduction steps which were absent in the *F. flaventior* metagenome. There were also differences in the carbon fixation pathways, where members of the *P. tinctorum* lichen microbiome also fixed carbon through the HB/HP, 3HP bi-cycle, and DC/HB cycles. The differences observed between the nutrient cycles of the two lichen microbiomes may be attributed to the accessory microbiome of the *P. tinctorum* lichen. Novel insights into the function, acquisition and impact of the lichen microbiome on the lichen holobiont could be obtained by correlations among the fungal/algal genotypes, climatic variations and lichen microbiome compositions. Undoubtedly, further research is needed to fully comprehend the intricate interactions that lichens have with the microorganisms that inhabit them.

3.5. References

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Summary

Lichens are conventionally defined as mutualistic relationships between a mycobiont, a photobiont and/or phycobiont. However, recent research has proven the lichen symbiosis to be far more complex, integrating yeasts, archaea, viruses, bacteria, and filamentous fungi in their microbiome. Thus, the lichen microbiome must be investigated, to shed more light on the lichen archaeome, mycobiome, bacteriome and virome composition. The roles that the lichen microbiome plays within the lichen association also needs to be explored. In addition, the roles of environmental factors such as pH and temperature in shaping the composition of the lichen microbiome is another possible study avenue. Given that South African lichen species are understudied, there is an opportunity to explore the lichen microbiomes of lichen species found across the country.

In this study, the metagenomes of two lichen species *Flavopunctelia flaventior* and *Parmotrema tinctorum*, sampled from the same location in Bryanston, Gauteng, South Africa, were sequenced, assembled and annotated. This was done to categorise the composition of the lichen microbiome and identify the core and accessory microbiomes of the two lichens. In addition, we wanted to functionally annotate the metagenomes to identify what each component of the lichen microbiome may contribute towards sustaining the symbiosis.

The bacteriome constituted a large proportion of the lichen microbiome and the phyla Actinomycetota and Pseudomonadota and genera *Bradyrhizobium*, *Methylobacterium*, *Nacordioides*, *Pseudomonas*, *Sphingomonas* and *Streptomyces* were prevalent. The mycobiome was the second largest component of the lichen microbiome, with the phylum Ascomycota and genera *Aspergillus* and *Fusarium* being the most prevalent. Low numbers of reads mapping to both archaea and viruses were also identified in both metagenomes. The virome comprised mainly of giant viruses and bacteriophages, which are responsible for regulating the population of microorganisms such as bacteria and archaea within the symbioses.

The lichen microbiome composition of *F. flaventior*, and *P. tinctorum* were very similar and this may be because they were sampled from the same location and were subjected to the same climatic conditions. The two lichens sharing the same family (*Parmeliaceae*) taxa may also contribute to the consistency and similarities observed between the two microbiomes.

Members of the lichen microbiome are involved in functions such as the cycling of carbon and nitrogen and produce a broad range of carbohydrate hydrolysing enzymes and proteases. While both the *F. flaventior* and *P. tinctorum* lichen microbiome were shown to perform carbon fixation via the Calvin cycle, it was noted that the *P. tinctorum* microbiome had members fixing

carbon via the HB/HP, 3HP bi-cycle, and DC/HB cycles. Additionally, ethanol oxidation and nitrate reduction steps were only observed in the *P. tinctorum* metagenome. The differences observed between the two lichen species may be ascribed to the accessory microbiome.

The metagenomes sequenced and analysed in this study provide primary insights into the microbial diversity associated with lichens beyond the direct mycobiont and photobiont as well as their functional potential. Future work could look at employing meta-proteomics to identify proteins produced by members of the lichen microbiome and meta-transcriptomics to study the genes they express. This may further provide an understanding of the biological pathways involved in sustaining the lichen symbiosis. Studying the secondary metabolites produced by members of the lichen microbiome may also contribute to the discovery of novel antimicrobial compounds.

Appendix

Appendix 1

Table A1: The concentration and quality of metagenomic DNA (mDNA) of *Parmotrema tinctorum* (**pink**) and *Flavopunctelia flaventior* (**green**) assessed using the Nanodrop 1000 spectrophotometer (ThermoFisher, United States of America).

	Concentration (ng/µl)	260:280 value
Sample 1	93.7	1.91
Sample 2	91	1.9
Sample 3	213.4	1.8
Sample 4	239	1.91
Sample 5	83.8	1.92
Sample 6	174	1.92

Appendix 2

Table A2.1: KEGG (Kanehisa and Goto, 2000) module step hits that were unique to *Parmotrema tinctorum* and *Flavopunctelia flaventior* metagenomes.

Module	Module.Category	*P. tinctorum*	*F. flaventior*
Glycolysis (Embden-Meyerhof pathway), glucose => pyruvate	Central carbohydrate metabolism	Present	Absent
Glucuronate pathway (uronate pathway)	Other carbohydrate metabolism	Present	Absent
Proline biosynthesis, glutamate => proline	Arginine and proline metabolism	Present	Absent
Proline biosynthesis, glutamate => proline	Arginine and proline metabolism	Present	Absent
Methionine biosynthesis, aspartate => homoserine => methionine	Cysteine and methionine metabolism	Present	Absent
Threonine biosynthesis, aspartate => homoserine => threonine	Serine and threonine metabolism	Present	Absent
Valine/isoleucine biosynthesis, pyruvate => valine / 2-oxobutanoate => isoleucine	Branched-chain amino acid metabolism	Present	Absent
Tryptophan biosynthesis, chorismate => tryptophan	Aromatic amino acid metabolism	Absent	Present
Histidine biosynthesis, PRPP => histidine	Histidine metabolism	Absent	Present
Histidine biosynthesis, PRPP => histidine	Histidine metabolism	Present	Absent
Lysine biosynthesis, mediated by LysW, 2-aminoadipate => lysine	Lysine metabolism	Present	Absent
Methionine salvage pathway	Cysteine and methionine metabolism	Absent	Present
Methionine salvage pathway	Cysteine and methionine metabolism	Present	Absent
Methionine salvage pathway	Cysteine and methionine metabolism	Absent	Present
Methionine salvage pathway	Cysteine and methionine metabolism	Present	Absent
Tryptophan metabolism, tryptophan => kynurenine => 2-aminomuconate	Aromatic amino acid metabolism	Absent	Present
Monolignol biosynthesis, phenylalanine/tyrosine => monolignol	Biosynthesis of other secondary metabolites	Absent	Present
Tyrosine biosynthesis, chorismate => arogenate => tyrosine	Aromatic amino acid metabolism	Absent	Present
Tyrosine degradation, tyrosine => homogentisate	Aromatic amino acid metabolism	Absent	Present
Histidine degradation, histidine => N-formiminoglutamate => glutamate	Histidine metabolism	Present	Absent
Pyrimidine degradation, uracil => beta-alanine, thymine => 3-aminoisobutanoate	Pyrimidine metabolism	Present	Absent
Pyrimidine degradation, uracil => beta-alanine, thymine => 3-aminoisobutanoate	Pyrimidine metabolism	Present	Absent
Creatine pathway	Arginine and proline metabolism	Absent	Present
De novo pyrimidine biosynthesis, glutamine (+ PRPP) => UMP	Pyrimidine metabolism	Absent	Present
N-glycan precursor biosynthesis	Glycan biosynthesis	Present	Absent
N-glycan precursor biosynthesis	Glycan biosynthesis	Absent	Present
CMP-KDO biosynthesis	Lipopolysaccharide metabolism	Absent	Present
N-glycan biosynthesis, high-mannose type	Glycan biosynthesis	Present	Absent
N-glycan biosynthesis, complex type	Glycan biosynthesis	Present	Absent
Heparan sulfate degradation	Glycosaminoglycan metabolism	Absent	Present
Fatty acid biosynthesis, elongation	Fatty acid biosynthesis and degradation	Present	Absent
beta-Oxidation, acyl-CoA synthesis	Fatty acid biosynthesis and degradation	Present	Absent
beta-Oxidation	Fatty acid biosynthesis and degradation	Present	Absent
Ketone body biosynthesis, acetyl-CoA => acetoacetate/3-hydroxybutyrate/acetone	Lipid metabolism	Present	Absent
Ketone body biosynthesis, acetyl-CoA => acetoacetate/3-hydroxybutyrate/acetone	Lipid metabolism	Present	Absent
Triacylglycerol biosynthesis	Lipid metabolism	Absent	Present
Phosphatidylcholine (PC) biosynthesis, choline => PC	Lipid metabolism	Present	Absent
Ceramide biosynthesis	Lipid metabolism	Absent	Present
Ceramide biosynthesis	Lipid metabolism	Absent	Present
C5 isoprenoid biosynthesis, mevalonate pathway	Terpenoid backbone biosynthesis	Present	Absent
C5 isoprenoid biosynthesis, mevalonate pathway	Terpenoid backbone biosynthesis	Absent	Present
C5 isoprenoid biosynthesis, mevalonate pathway	Terpenoid backbone biosynthesis	Present	Absent
C5 isoprenoid biosynthesis, non-mevalonate pathway	Terpenoid backbone biosynthesis	Absent	Present
C5 isoprenoid biosynthesis, non-mevalonate pathway	Terpenoid backbone biosynthesis	Present	Absent
beta-Carotene biosynthesis, GGAP => beta-carotene	Other terpenoid biosynthesis	Absent	Present
Acylglycerol degradation	Lipid metabolism	Absent	Present
Sphingosine biosynthesis	Lipid metabolism	Absent	Present
Sphingosine biosynthesis	Lipid metabolism	Absent	Present
Sphingosine degradation	Lipid metabolism	Absent	Present
Cholesterol biosynthesis, squalene 2,3-epoxide => cholesterol	Sterol biosynthesis	Absent	Present
Cholesterol biosynthesis, squalene 2,3-epoxide => cholesterol	Sterol biosynthesis	Absent	Present
Ascorbate biosynthesis, plants, fructose-6P => ascorbate	Other carbohydrate metabolism	Present	Absent
NAD biosynthesis, aspartate => quinolinate => NAD	Cofactor and vitamin metabolism	Absent	Present
NAD biosynthesis, aspartate => quinolinate => NAD	Cofactor and vitamin metabolism	Present	Absent
NAD biosynthesis, aspartate => quinolinate => NAD	Cofactor and vitamin metabolism	Present	Absent
Menaquinone biosynthesis, chorismate (+ polyprenyl-PP) => menaquinol	Cofactor and vitamin metabolism	Present	Absent
Menaquinone biosynthesis, chorismate (+ polyprenyl-PP) => menaquinol	Cofactor and vitamin metabolism	Present	Absent
Menaquinone biosynthesis, chorismate (+ polyprenyl-PP) => menaquinol	Cofactor and vitamin metabolism	Present	Absent
Menaquinone biosynthesis, chorismate (+ polyprenyl-PP) => menaquinol	Cofactor and vitamin metabolism	Present	Absent
Coenzyme A biosynthesis, pantothenate => CoA	Cofactor and vitamin metabolism	Absent	Present
Cobalamin biosynthesis, cobyrinate a,c-diamide => cobalamin	Cofactor and vitamin metabolism	Absent	Present
Cobalamin biosynthesis, cobyrinate a,c-diamide => cobalamin	Cofactor and vitamin metabolism	Present	Absent
Biotin biosynthesis, pimeloyl-ACP/CoA => biotin	Cofactor and vitamin metabolism	Absent	Present
Biotin biosynthesis, pimeloyl-ACP/CoA => biotin	Cofactor and vitamin metabolism	Absent	Present
Biotin biosynthesis, pimeloyl-ACP/CoA => biotin	Cofactor and vitamin metabolism	Absent	Present
Riboflavin biosynthesis, plants and bacteria, GTP => riboflavin/FMN/FAD	Cofactor and vitamin metabolism	Present	Absent
Thiamine biosynthesis, prokaryotes, AIR (+ DXP/tyrosine) => TMP/TPP	Cofactor and vitamin metabolism	Absent	Present
GABA biosynthesis, prokaryotes, putrescine => GABA	Polyamine biosynthesis	Present	Absent
Flavanone biosynthesis, phenylalanine => naringenin	Biosynthesis of other secondary metabolites	Absent	Present

Reductive pentose phosphate cycle (Calvin cycle)	Carbon fixation	Absent	Present
Reductive pentose phosphate cycle, ribulose-5P => glyceraldehyde-3P	Carbon fixation	Absent	Present
CAM (Crassulacean acid metabolism), light	Carbon fixation	Present	Absent
C4-dicarboxylic acid cycle, NAD - malic enzyme type	Carbon fixation	Present	Absent
C4-dicarboxylic acid cycle, NADP - malic enzyme type	Carbon fixation	Present	Absent
Methane oxidation, methanotroph, methane => formaldehyde	Methane metabolism	Absent	Present
Formaldehyde assimilation, ribulose monophosphate pathway	Methane metabolism	Absent	Present
Formaldehyde assimilation, ribulose monophosphate pathway	Methane metabolism	Absent	Present
Formaldehyde assimilation, serine pathway	Methane metabolism	Present	Absent
Formaldehyde assimilation, serine pathway	Methane metabolism	Present	Absent
Formaldehyde assimilation, serine pathway	Methane metabolism	Absent	Present
Coenzyme M biosynthesis	Methane metabolism	Present	Absent
C10-C20 isoprenoid biosynthesis, bacteria	Terpenoid backbone biosynthesis	Present	Absent
C10-C20 isoprenoid biosynthesis, archaea	Terpenoid backbone biosynthesis	Present	Absent
C10-C20 isoprenoid biosynthesis, archaea	Terpenoid backbone biosynthesis	Present	Absent
C10-C20 isoprenoid biosynthesis, plants	Terpenoid backbone biosynthesis	Present	Absent
C10-C20 isoprenoid biosynthesis, plants	Terpenoid backbone biosynthesis	Present	Absent
C10-C20 isoprenoid biosynthesis, non-plant eukaryotes	Terpenoid backbone biosynthesis	Present	Absent
Ethylmalonyl pathway	Other carbohydrate metabolism	Present	Absent
Ethylmalonyl pathway	Other carbohydrate metabolism	Present	Absent
Ethylmalonyl pathway	Other carbohydrate metabolism	Present	Absent
Ethylmalonyl pathway	Other carbohydrate metabolism	Present	Absent
Ethylmalonyl pathway	Other carbohydrate metabolism	Present	Absent
Ethylmalonyl pathway	Other carbohydrate metabolism	Present	Absent
Dicarboxylate-hydroxybutyrate cycle	Carbon fixation	Present	Absent
Hydroxypropionate-hydroxybutylate cycle	Carbon fixation	Present	Absent
Hydroxypropionate-hydroxybutylate cycle	Carbon fixation	Present	Absent
Hydroxypropionate-hydroxybutylate cycle	Carbon fixation	Present	Absent
3-Hydroxypropionate bi-cycle	Carbon fixation	Present	Absent
3-Hydroxypropionate bi-cycle	Carbon fixation	Present	Absent
3-Hydroxypropionate bi-cycle	Carbon fixation	Present	Absent
F420 biosynthesis, archaea	Methane metabolism	Absent	Present
Fatty acid elongation in endoplasmic reticulum	Fatty acid biosynthesis and degradation	Present	Absent
Toluene degradation, anaerobic, toluene => benzoyl-CoA	Aromatics degradation	Present	Absent
Assimilatory nitrate reduction, nitrate => ammonia	Nitrogen metabolism	Absent	Present
Photorespiration	Other carbohydrate metabolism	Present	Absent
Homoprotocatechuate degradation, homoprotocatechuate => 2-oxohept-3-enedioate	Aromatic amino acid metabolism	Present	Absent
Homoprotocatechuate degradation, homoprotocatechuate => 2-oxohept-3-enedioate	Aromatic amino acid metabolism	Present	Absent
Benzoate degradation, cyclohexanecarboxylic acid =>pimeloyl-CoA	Aromatics degradation	Present	Absent
EHEC/EPEC pathogenicity signature, T3SS and effectors	Pathogenicity	Present	Absent
Carbazole degradation, carbazole => 2-oxopent-4-enoate + anthranilate	Aromatics degradation	Present	Absent
Trans-cinnamate degradation, trans-cinnamate => acetyl-CoA	Aromatic amino acid metabolism	Present	Absent
Trans-cinnamate degradation, trans-cinnamate => acetyl-CoA	Aromatic amino acid metabolism	Present	Absent
Purine degradation, xanthine => urea	Purine metabolism	Present	Absent
Purine degradation, xanthine => urea	Purine metabolism	Present	Absent
Purine degradation, xanthine => urea	Purine metabolism	Present	Absent
Ascorbate degradation, ascorbate => D-xylulose-5P	Other carbohydrate metabolism	Present	Absent
Benzoate degradation, benzoate => catechol / methylbenzoate => methylcatechol	Aromatics degradation	Present	Absent
Benzoate degradation, benzoate => catechol / methylbenzoate => methylcatechol	Aromatics degradation	Present	Absent
D-galactonate degradation, De Ley-Doudoroff pathway, D-galactonate => glycerate-3P	Other carbohydrate metabolism	Absent	Present
Nucleotide sugar biosynthesis, galactose => UDP-galactose	Other carbohydrate metabolism	Present	Absent
Betaine biosynthesis, choline => betaine	Serine and threonine metabolism	Present	Absent
Methanogenesis, methylamine/dimethylamine/trimethylamine => methane	Methane metabolism	Present	Absent
Helicobacter pylori pathogenicity signature, cagA pathogenicity island	Pathogenicity	Absent	Present
Trehalose biosynthesis, D-glucose 1P => trehalose	Other carbohydrate metabolism	Present	Absent
Methanogenesis, CO2 => methane	Methane metabolism	Present	Absent
Catechol ortho-cleavage, catechol => 3-oxoadipate	Aromatics degradation	Present	Absent
Catechol meta-cleavage, catechol => acetyl-CoA / 4-methylcatechol => propanoyl-CoA	Aromatics degradation	Present	Absent
Isoleucine biosynthesis, threonine => 2-oxobutanoate => isoleucine	Branched-chain amino acid metabolism	Present	Absent
Biotin biosynthesis, BioI pathway, long-chain-acyl-ACP => pimeloyl-ACP => biotin	Cofactor and vitamin metabolism	Absent	Present
Biotin biosynthesis, BioI pathway, long-chain-acyl-ACP => pimeloyl-ACP => biotin	Cofactor and vitamin metabolism	Absent	Present
Pertussis pathogenicity signature, T1SS	Pathogenicity	Present	Absent
Pertussis pathogenicity signature, T1SS	Pathogenicity	Present	Absent
Biotin biosynthesis, BioW pathway, pimelate => pimeloyl-CoA => biotin	Cofactor and vitamin metabolism	Absent	Present
Biotin biosynthesis, BioW pathway, pimelate => pimeloyl-CoA => biotin	Cofactor and vitamin metabolism	Absent	Present
Biotin biosynthesis, BioW pathway, pimelate => pimeloyl-CoA => biotin	Cofactor and vitamin metabolism	Absent	Present
Pentose phosphate pathway, archaea, fructose 6P => ribose 5P	Central carbohydrate metabolism	Absent	Present
Pentose phosphate pathway, archaea, fructose 6P => ribose 5P	Central carbohydrate metabolism	Absent	Present
Nicotinate degradation, nicotinate => fumarate	Cofactor and vitamin metabolism	Absent	Present
Nicotinate degradation, nicotinate => fumarate	Cofactor and vitamin metabolism	Present	Absent

beta-Lactam resistance, Bla system	Drug resistance	Present	Absent
Galactose degradation, Leloir pathway, galactose => alpha-D-glucose-1P	Other carbohydrate metabolism	Present	Absent
Multidrug resistance, efflux pump MexEF-OprN	Drug resistance	Absent	Present
Multidrug resistance, efflux pump MexEF-OprN	Drug resistance	Present	Absent
Multidrug resistance, efflux pump MexJK-OprM	Drug resistance	Present	Absent
Multidrug resistance, efflux pump MexXY-OprM	Drug resistance	Present	Absent
Vancomycin resistance, D-Ala-D-Lac type	Drug resistance	Absent	Present
Vancomycin resistance, D-Ala-D-Lac type	Drug resistance	Present	Absent
Vancomycin resistance, D-Ala-D-Lac type	Drug resistance	Present	Absent
Xanthomonas spp. pathogenicity signature, T3SS and effectors	Plant pathogenicity	Present	Absent
Nodulation	Symbiosis	Present	Absent
Multidrug resistance, efflux pump AcrEF-TolC	Drug resistance	Present	Absent
Multidrug resistance, efflux pump MdtEF-TolC	Drug resistance	Present	Absent
Multidrug resistance, efflux pump BpeEF-OprC	Drug resistance	Present	Absent
Multidrug resistance, efflux pump BpeEF-OprC	Drug resistance	Present	Absent
Methylaspartate cycle	Other carbohydrate metabolism	Absent	Present
Propanoyl-CoA metabolism, propanoyl-CoA => succinyl-CoA	Other carbohydrate metabolism	Present	Absent
Propanoyl-CoA metabolism, propanoyl-CoA => succinyl-CoA	Other carbohydrate metabolism	Present	Absent
Propanoyl-CoA metabolism, propanoyl-CoA => succinyl-CoA	Other carbohydrate metabolism	Present	Absent
Imipenem resistance, repression of porin OprD	Drug resistance	Absent	Present
Imipenem resistance, repression of porin OprD	Drug resistance	Absent	Present
Ornithine biosynthesis, mediated by LysW, glutamate => ornithine	Arginine and proline metabolism	Present	Absent
Multidrug resistance, efflux pump MexPQ-OpmE	Drug resistance	Absent	Present
Tylosin biosynthesis, methylmalonyl-CoA + malonyl-CoA => tylactone => tylosin	Macrolide biosynthesis	Absent	Present
Avermectin biosynthesis, 2-methylbutanoyl-CoA/isobutyryl-CoA => 6,8a-Seco-6,8a-deoxy-5-oxoavermectin 1a/1b aglycone => avermectin A1a/B1a/A1b/B1b	Macrolide biosynthesis	Present	Absent
Avermectin biosynthesis, 2-methylbutanoyl-CoA/isobutyryl-CoA => 6,8a-Seco-6,8a-deoxy-5-oxoavermectin 1a/1b aglycone => avermectin A1a/B1a/A1b/B1b	Macrolide biosynthesis	Absent	Present
Dihydrokalafungin biosynthesis, octaketide => dihydrokalafungin	Type II polyketide biosynthesis	Present	Absent
Tetracenomycin C/8-demethyltetracenomycin C biosynthesis, tetracenomycin F2 => tetracenomycin C/8-demethyltetracenomycin C	Type II polyketide biosynthesis	Present	Absent
Elloramycin biosynthesis, 8-demethyltetracenomycin C => elloramycin A	Type II polyketide biosynthesis	Absent	Present
Rebeccamycin biosynthesis, tryptophan => rebeccamycin	Biosynthesis of other secondary metabolites	Present	Absent
Pyrrolnitrin biosynthesis, tryptophan => pyrrolnitrin	Biosynthesis of other secondary metabolites	Present	Absent
dTDP-L-rhamnose biosynthesis	Polyketide sugar unit biosynthesis	Present	Absent
dTDP-L-rhamnose biosynthesis	Polyketide sugar unit biosynthesis	Absent	Present
dTDP-L-mycarose biosynthesis	Polyketide sugar unit biosynthesis	Present	Absent
dTDP-L-mycarose biosynthesis	Polyketide sugar unit biosynthesis	Present	Absent
dTDP-L-oleandrose biosynthesis	Polyketide sugar unit biosynthesis	Present	Absent
dTDP-L-oleandrose biosynthesis	Polyketide sugar unit biosynthesis	Present	Absent
dTDP-L-megosamine biosynthesis	Polyketide sugar unit biosynthesis	Present	Absent
dTDP-L-olivose biosynthesis	Polyketide sugar unit biosynthesis	Present	Absent
dTDP-D-forosamine biosynthesis	Polyketide sugar unit biosynthesis	Present	Absent
dTDP-D-angolosamine biosynthesis	Polyketide sugar unit biosynthesis	Present	Absent
Complete nitrification, comammox, ammonia => nitrite => nitrate	Nitrogen metabolism	Present	Absent
C-1027 beta-amino acid moiety biosynthesis, tyrosine => 3-chloro-4,5-dihydroxy-beta-phenylalanyl-PCP	Enediyne biosynthesis	Present	Absent
Kedarcidin 2-aza-3-chloro-beta-tyrosine moiety biosynthesis, azatyrosine => 2-aza-3-chloro-beta-tyrosyl-PCP	Enediyne biosynthesis	Present	Absent
Calicheamicin biosynthesis, calicheamicinone => calicheamicin	Enediyne biosynthesis	Absent	Present
Calicheamicin biosynthesis, calicheamicinone => calicheamicin	Enediyne biosynthesis	Absent	Present
Calicheamicin biosynthesis, calicheamicinone => calicheamicin	Enediyne biosynthesis	Absent	Present
Pyocyanine biosynthesis, chorismate => pyocyanine	Biosynthesis of other secondary metabolites	Present	Absent
Tetrahydrofolate biosynthesis, mediated by ribA and trpF, GTP => THF	Cofactor and vitamin metabolism	Present	Absent
Arginine biosynthesis, glutamate => acetylcitrulline => arginine	Arginine and proline metabolism	Present	Absent
Heme biosynthesis, archaea, siroheme => heme	Cofactor and vitamin metabolism	Absent	Present
C5 isoprenoid biosynthesis, mevalonate pathway, archaea	Terpenoid backbone biosynthesis	Present	Absent
C5 isoprenoid biosynthesis, mevalonate pathway, archaea	Terpenoid backbone biosynthesis	Present	Absent
C5 isoprenoid biosynthesis, mevalonate pathway, archaea	Terpenoid backbone biosynthesis	Absent	Present
KDO2-lipid A modification pathway	Lipopolysaccharide metabolism	Present	Absent
Fatty acid biosynthesis in mitochondria, animals		Absent	Present
Fatty acid biosynthesis in mitochondria, fungi		Absent	Present
Molybdenum cofactor biosynthesis, GTP => molybdenum cofactor		Absent	Present
Roseoflavin biosynthesis, FMN => roseoflavin		Present	Absent
UDP-N-acetyl-D-glucosamine biosynthesis, eukaryotes, glucose => UDP-GlcNAc		Absent	Present
Thiamine biosynthesis, prokaryotes, AIR (+ DXP/glycine) => TMP/TPP		Absent	Present
Thiamine biosynthesis, archaea, AIR (+ NAD+) => TMP/TPP		Absent	Present
Thiamine salvage pathway, HMP/HET => TMP		Present	Absent
Ethynylserine biosynthesis, lysine => ethynylserine		Present	Absent
Ethynylserine biosynthesis, lysine => ethynylserine		Present	Absent
UDP-N-acetyl-D-glucosamine biosynthesis, prokaryotes, glucose => UDP-GlcNAc		Absent	Present
Phenylalanine biosynthesis, chorismate => arogenate => phenylalanine		Present	Absent
Riboflavin biosynthesis, fungi, GTP => riboflavin/FMN/FAD		Present	Absent
NAD biosynthesis, tryptophan => quinolinate => NAD		Absent	Present
NAD biosynthesis, tryptophan => quinolinate => NAD		Present	Absent
Phytosterol biosynthesis, squalene 2,3-epoxide => campesterol/sitosterol		Absent	Present
Phytosterol biosynthesis, squalene 2,3-epoxide => campesterol/sitosterol		Absent	Present
Cyclooctatin biosynthesis, dimethylallyl-PP + isopentenyl-PP => cyclooctatin		Present	Absent
Cobalamin biosynthesis, anaerobic, uroporphyrinogen III => sirohydrochlorin => cobyrinate a,c-diamide		Present	Absent
Cobalamin biosynthesis, anaerobic, uroporphyrinogen III => sirohydrochlorin => cobyrinate a,c-diamide		Absent	Present
Cobalamin biosynthesis, anaerobic, uroporphyrinogen III => sirohydrochlorin => cobyrinate a,c-diamide		Absent	Present
Cobalamin biosynthesis, anaerobic, uroporphyrinogen III => sirohydrochlorin => cobyrinate a,c-diamide		Present	Absent
Cobalamin biosynthesis, aerobic, uroporphyrinogen III => precorrin 2 => cobyrinate a,c-diamide		Present	Absent
Cobalamin biosynthesis, aerobic, uroporphyrinogen III => precorrin 2 => cobyrinate a,c-diamide		Absent	Present
Cobalamin biosynthesis, aerobic, uroporphyrinogen III => precorrin 2 => cobyrinate a,c-diamide		Present	Absent
Cobalamin biosynthesis, aerobic, uroporphyrinogen III => precorrin 2 => cobyrinate a,c-diamide		Absent	Present
Menaquinone biosynthesis, futalosine pathway		Present	Absent
Menaquinone biosynthesis, futalosine pathway		Present	Absent
Menaquinone biosynthesis, modified futalosine pathway		Present	Absent
Phylloquinone biosynthesis, chorismate (+ phytyl-PP) => phylloquinol		Present	Absent
Phylloquinone biosynthesis, chorismate (+ phytyl-PP) => phylloquinol		Present	Absent
Phylloquinone biosynthesis, chorismate (+ phytyl-PP) => phylloquinol		Present	Absent
Mycinamicin biosynthesis, malonyl-CoA + methylmalonyl-CoA => protomycinolide IV => mycinamicin II		Absent	Present
Pyrimidine degradation, uracil => 3-hydroxypropanoate		Absent	Present
D-Arginine racemization, D-arginine => L-arginine		Present	Absent
Hydroxyproline degradation, trans-4-hydroxy-L-proline => 2-oxoglutarate		Absent	Present
Biotin biosynthesis, BioU pathway, pimeloyl-ACP/CoA => biotin		Absent	Present
Biotin biosynthesis, BioU pathway, pimeloyl-ACP/CoA => biotin		Absent	Present
Guanine ribonucleotide degradation, GMP => Urate		Absent	Present

Table A2.2: Glycosyltransferases found within the *Parmotrema tinctorum* and *Flavopunctelia flaventior* metagenomes identified using Prodigal (Hyatt *et al.*, 2010).

KEGG +COG FUN	*Parmotrema tinctorum* (#Hits)	*Flavopunctelia flaventior* (#Hits)
acyl-Coa dehydrogenase	1	0
ADP-heptose:LPS heptosyltransferase	3	3
Alpha-N-acetylglucosamine transferase	6	5
capsule-associated protein CAP1	1	1
Cellobiose phosphorylase	101	90
Core-2/I-Branching enzyme	1	1
Cryptococcal mannosyltransferase 1	9	6
Glycogen synthase	2	2
Egl nine homolog	1	3
Exostosin	1	2
Flagellar hook-associated protein	1	1
Glycogen synthase	2	2
Glycosyl transferase Family 1	316	250
Glycosyl transferase Family 2	38	29
Glycosyl transferase Family 9	2	1
UDP-glucuronosyltransferase	15	10
Glycosyltransferases involved in cell wall biogenesis	150	136
Lipopolysaccharide biosynthesis protein	10	8
Lysophospholipase L1 and related esterases	8	10
MannoseP-dolichol utilization defect 1	5	5
Mannosyltransferase	9	5
Membrane glycosyltransferase	124	110
Methyltransferase	2	5
Sugar transferases involved in lipopolysaccharide synthesis	29	35
Teichoic acid biosynthesis protein A	4	8
UDP-N-acetylmuramyl pentapeptide phosphotransferase/UDP-N-acetylglucosamine-1-phosphate transferase	10	5