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The plurality of photobionts within single lichen thalli

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Abstract

Photobiont plurality refers to the presence of multiple photobionts within a single lichen thallus. This phenomenon was described at the end of the last century but has received deeper attention in the last decade. The available literature on this topic is limited and studies address only a few groups of lichens. Here, the most recent and important findings on photobiont plurality reported to date are revisited. The most commonly reported photobionts coexisting in a lichen thallus are algae of the genus *Trebouxia*, though other algae and cyanobacteria more or less phylogenetically related may also coexist together. A thallus with multiple photobionts may be formed at the beginning by the association of hyphae with several photobionts at once or by the acquisition of additional photobionts during life. The most important factors affecting the cooccurrence of multiple photobionts in a single thallus are likely the specificity and selectivity of mycobionts and local environmental conditions. Photobiont plurality has been investigated so far in about fifty species of lichen-forming fungi among the approximately 20,000 mycobiont species described. Coexisting photobionts differ in responses to several environmental, mostly stress conditions, suggesting that the presence of multiple photobionts in a thallus may provide an advantage for colonizing new habitats and may be essential for survival in localities with harsh or frequently changing conditions.

Keywords Coexistence · Specificity · Selectivity · Symbiosis · Thallus · Phycobiont

1 Introduction

Lichens represent one of the longest-known examples of symbiotic relationships. As early as 1869, Simon Schwendener described this unique close association between a fungus and algae (green or blue-green; Schwendener 1869). Later, these two main partners of lichens became well-known as the mycobiont (Scott 1957) and photobiont (Hawksworth and Honegger 1994). Although the fundamental concepts of how precisely lichens are defined remained relatively unchanged for over 140 years (Lücking et al. 2021; Sanders 2024), the perspective on this symbiotic relationship has been evolving over the past decade. According to the most updated definition, lichens are stable, self-supporting associations involving multiple symbiotic partners (Lücking et al. 2021). One of the findings that contributed to this idea was the discovery of the coexistence of two or more photobionts within a single lichen thallus, known as photobiont plurality (e.g. Casano et al. 2011; Muggia et al. 2014; Dal Grande et al. 2018; Vančurová et al. 2020). Besides that, it was the discoveries of bacteria, yeasts, and other groups of microorganisms in lichens (e.g. Uphof 1925; Wilkinson et al. 2015; Spribille et al. 2016; Muggia and Grube 2018; Petrzik et al. 2019).

While recent discoveries have expanded our understanding of lichens as complex symbiotic systems involving multiple partners (Hawksworth and Grube 2020), the term ‘lichen’ is still widely used to describe the traditional symbiotic relationship between a fungal partner, the mycobiont (Scott 1957) and photosynthetic microalgae or cyanobacteria, the photobiont (Ahmadjian 1993; Hawksworth and Honegger 1994; Sanders 2024). Despite the presence of diverse microorganisms within the thalli, the mycobiont and photobiont are essential, serving as the foundational elements that drive the formation and sustain the unique ecological identity of lichens (Honegger 1993; Sanders 2024). The

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presented text, therefore, does not consider epiphytic algae or cyanobacteria as photobionts or components of lichens in general. Views on the nature of this close relationship between both partners have varied among scientists. In the first description of the dual nature of lichens, Schwendener quite explicitly interpreted the fungus as a parasite upon the algae (Schwendener 1872) and so did more than a hundred years later Ahmadjian and Jacobs (1981). On the other hand, De Bary (1879) considered this fungal-algal symbiotic relationship mutualistic. Some authors view the relationship as a form of domestication, where the mycobiont (host) merely cultivates its algae for nutrition sources (Lücking et al. 2009; Leavitt et al. 2015; Dal Grande et al. 2018; Rolshausen et al. 2020; Sanders 2023 and references therein).

In recent years, several studies describing higher diversity of photobionts (e.g. Casano et al. 2011; Park et al. 2015; Dal Grande et al. 2018) and other symbiotic partners within a single thallus have emerged. Here, we summarize the current knowledge and various hypotheses regarding photobiont plurality within lichen thalli, i.e. the occurrence of more than one photobiont lineage within a single lichen thallus (Leavitt et al. 2015; Dal Grande et al. 2018; Vančurová et al. 2020). We focus mainly on the mechanisms of its formation, the taxa involved (both photobionts and mycobionts), the factors influencing it, and the overall significance of photobiont plurality for lichen symbioses. We also compile a review of the methods used to study photobiont plurality, as they play a crucial role in obtaining comprehensive insights that can be comparable and complementary among studies.

2 The lichen photobionts and their associations with the mycobionts

The term ‘photobiont’ refers to the photosynthetic symbiont of a lichen, whose role is the production of organic molecules – polyols in the case of algae, glucose in the case of cyanobacteria (e.g. Smith and Douglas 1987; Kranner et al. 2022; Spribille et al. 2022). It thus typically refers to most often unicellular (Sanders and Masumoto 2021) or occasionally filamentous (e.g. *Phycopeltis*; Sanders and Lücking 2002) green algae or cyanobacteria. The historical term ‘phycobiont’ has also been used in the scientific literature (Scott 1957; Ahmadjian 1993); however, that currently refers specifically to the green microalgae. Also, to distinguish between cyanobacterial and green algal photobionts, the terms cyanobiont and chlorobiont are used, respectively (Kosugi et al. 2014).

The significance of photobionts is broad. The acquired organic substances not only serve as an energy source for the mycobiont but perhaps also act as osmoregulators during desiccation and rehydration of the thallus (Bewley and Krochko 1982; Spribille et al. 2022) or as stimulants for the thallus growth (Wang et al. 2009). Both processes are also influenced by the production of phytohormones (Pichler et al. 2023). Photobionts further produce cyclic peptides, which serve as attractants for the mycobiont before they become symbiotic partners (Meeßen et al. 2013; Pichler et al. 2023).

Several groups of algae and cyanobacteria form associations with fungi to create lichens. Most commonly, these are green algae from the classes Trebouxiophyceae and Ulvophyceae. Over 50 genera of photobionts (including green algae and cyanobionts) are known to be involved in lichen formation, most of which can be involved in symbiosis with several different species of mycobionts (Muggia et al. 2018; Sanders and Masumoto 2021; Jung et al. 2021; Veselá et al. 2024). Therefore, significantly more mycobionts participate in lichen symbiosis than photobionts (e.g. Lücking et al. 2009; Dal Forno et al. 2021).

Scientific literature often deals with the terms ‘specificity’ and ‘selectivity’; however, there is considerable variability in how these terms are defined and used across different studies (e.g., Beck et al. 2002). For the purposes of this review, we understand ‘specificity’ as the number of photobionts with which a mycobiont can engage in this mutual relationship, i.e., the higher the specificity, the lower the number of photobiont species with which a mycobiont lichenizes (Leavitt et al. 2015). Similarly, we consider ‘selectivity’ as the frequency of a mycobiont’s interactions with a particular photobiont (Leavitt et al. 2015). Based on the degree of selectivity, mycobionts can be classified as specialists, generalists, and those whose selectivity falls between these two categories (Yahr et al. 2004). Generally, species with both low selectivity and specificity are considered generalists, as they can colonise a greater variety of habitats, having a higher chance of finding a suitable photobiont in a given area (Yahr et al. 2004; Blaha et al. 2006). Species on the opposite end of the spectrum are considered specialists whose photobionts and mycobionts are better adapted to each other (Yahr et al. 2004).

A photobiont significantly influences the ecological relations of the entire lichen (Rolshausen et al. 2020). Its requirements for the type of associated mycobiont and the environmental conditions in which they live can also be broad or, on the contrary, very specific. For example, representatives of the genera *Trebouxia* and *Asterochloris* associate with hundreds of different fungal species, while genera like *Neocystis* or *Leptosira* form symbioses with only a few mycobiont species (Sanders and Masumoto 2021). This often means that it is the photobiont that determines the width of the ecological niche of a given lichen species (Peksa and Škaloud 2011). Peksa and Škaloud (2011) demonstrated that the occurrence of individual *Asterochloris* lineages in studied lichens of the genera *Lepraria* and *Stereocaulon* is primarily determined by ecology rather than the taxonomic affiliation of the mycobionts. In contrast, other studies suggest that the presence of a photobiont in an ecosystem depends more on the genotype of the mycobiont than on the prevailing conditions at a site due to its selectivity (Piercey-Normore and DePriest 2001; Leavitt et al. 2015).

Another significant factor influencing the diversity of photobionts is the mode of reproduction of a lichen and, thus, the way of their spreading. Sexually reproducing lichens possibly share different photobionts than species that reproduce vegetatively (Peksa et al. 2022). These findings support the existence of so-called 'lichen guilds'. Lichens of the same guild grow in the same area and often contain the same photobionts in their thalli, sharing a so-called 'photobiont pool' (Rikkinen et al. 2002; Fedrowitz et al. 2011; Duran-Nebreda and Valverde 2023). This phenomenon is known as photobiont sharing (Lücking et al. 2009). According to Ohmura et al. (2019), photobiont sharing occurs more frequently in sexually reproducing lichens. Onuț-Brännström et al. (2018) further argue that it can also transpire between phylogenetically distant mycobiont lineages occurring in similar ecological conditions.

Another mechanism of photobiont sharing can be the so-called 'photobiont stealing', where one lichen actively appropriates the photobiont of another lichen (Lücking et al. 2009). This probably occurs mainly in juvenile parasitic species (Friedl 1987; Blaha et al. 2006). The occurrence of a specific photobiont in a lichen is thus affected by several different factors, which may vary in the extent of their influence among distinct groups of lichens (Peksa and Škaloud 2011; Leavitt et al. 2015).

The degree of specificity of a mycobiont may, to some extent, be related to the type of the lichen's reproduction. In general, it can be assumed that lichens reproducing sexually via spores should have a lower specificity, as different photobionts may be available at a new site compared to their original locality. In contrast, both main components of vegetatively reproducing lichens spread together, addressing the common problem of the unavailability of a compatible partner at a new site; thus, a tighter association of the mycobiont and the photobiont can be expected (Leavitt et al. 2015). This hypothesis has been confirmed in only a few chlorolichens (Cao et al. 2015; Steinová et al. 2019) and cyanolichens (Fedrowitz et al. 2011), whereas in other cases, this trend has not been demonstrated (e.g. Leavitt et al. 2015).

In general, there are several ways in which the mycobiont can acquire a compatible photobiont. The first possibility is the association of germinating hyphae with free-living photobionts or with photobionts from other lichens (Friedl 1987). The lichenized photobionts then gradually proliferate within the thallus (Blaha et al. 2006; Mansournia et al. 2012; Sanders 2014). To acquire additional photobionts into the thallus during its growth, the lichen may form a so-called prothallus (a layer of mycobiont hyphae without photobiont cells), providing space for potential association with another photobiont (Sanders and Lücking 2002).

The term 'photobiont switching' refers to the phenomenon where the mycobiont relinquishes one photobiont and acquires another (Sanders and Masumoto 2021), thereby potentially expanding its ecological niche and increasing the size of its population (Ertz et al. 2018). This often occurs based on specific environmental gradients (e.g. elevation). For example, different photobiont species may be associated with the same mycobiont in lowlands compared to areas a few hundred meters higher. Between these two areas lies a transition zone, where the ecological niches of both photobiont species (green microalgae or cyanobacteria) overlap, leading to a photobiont exchange within the lichen (Rolshausen et al. 2020, 2022). In some lichens, the genetic relatedness of the switched photobionts may not even be important. For instance, Ertz et al. (2018) demonstrated a photobiont switch between a *Trebouxia* and a Trentepohlioid photobiont from the family Ulvophyceae in the lichen *Lecanographa amylacea*. The exchange of phylogenetically distant photobionts often occurs in sterile lichens (Blaha et al. 2006; Ohmura et al. 2019). Photobiont switching takes place not only due to environmental changes but also among different generations (Sanders and Masumoto 2021), contributing to the longevity of mycobionts (Wornik and Grube 2010). It often occurs in the same lichens where photobiont plurality has been described (e.g. Muggia et al. 2014; Dal Grande et al. 2018; Ohmura et al. 2019; Moya et al. 2024).

3 Photobiont plurality

The term 'photobiont plurality' refers to the occurrence of more than one photobiont within a single lichen thallus (Leavitt et al. 2015; Dal Grande et al. 2018; Vančurová et al. 2020). This phenomenon has been more thoroughly described with the development of molecular methods and has been studied in more detail in the last 15 years (e.g. Del Campo et al. 2010, 2013; Casano et al. 2011; Moya et al. 2017). A general assumption for the occurrence of multiple photobionts is the low specificity of the mycobiont because such mycobiont species can generally associate with a larger number of photosynthetic partners (Leavitt et al. 2015).

The photobiont that is most abundant in the lichen is referred to by authors as the 'predominant' (Vančurová et al. 2018, 2020; Onuț-Brännström et al. 2018) or 'primary' photobiont (Voytsekhovich and Beck 2016; Molins et al. 2018b; Chiva et al. 2023), or when its abundance exceeds 90%, it may be sometimes described as 'dominant' (Dal Grande et al. 2018; Smith et al. 2020). In addition, other species of photobionts may also be present in the thallus, which may not even have any evident significance for the lichen and may only serve as a source of photobionts for other lichens in the locality (Vančurová et al. 2020). These photobionts are referred to as 'additional', 'accessory' (Voytsekhovich et al. 2011; Voytsekhovich and Beck 2016) or 'secondary' (Schmull et al. 2011; Paul et al. 2018). However, the latter term is misleading in this context since it should mainly be used to describe cyanobionts in tripartite lichens. Therefore, it is advisable to avoid the terms 'primary' and 'secondary' photobiont in the context of photobiont plurality (Voytsekhovich and Beck 2016) and instead use the terms 'predominant' or 'main' photobiont to refer to the most abundant one and 'additional' or 'accessory' when mentioning the minority photobionts. Tripartite lichens represent about 3–4% of all lichen species (Henskens et al. 2012; Cornejo and Scheidegger 2013). The plurality of secondary cyanobacterial photobionts in cephalodia has been reported in a few studies (e.g. Myllys et al. 2007; Prieto et al. 2023); however, they will not be reviewed in detail here.

The possibility of the coexistence of multiple algae within a thallus was mentioned as early as in 1967 (Ahmadjian 1967), where both described photobionts of the lichen *Staurothele catalepta* (currently known as *Verrucaria aethiobola*) were cultivated. In the last century, at least four other studies mentioning this phenomenon appeared (Ott 1987a; Friedl 1987; Bhattacharya et al. 1996; Aoki et al. 1998). In the first decade of the 21st century, several studies that detected photobiont plurality in analysed thalli as a secondary outcome of their focus were published. The authors of these papers attempted to comment at least partially on the coexistence of photobionts within a thallus (Helms et al. 2001; Romeike et al. 2002; Piercey-Normore 2006; Ohmura et al. 2006; Guzow-Krzemińska 2006; Bačkor et al. 2010). It was not until 2010 that Del Campo et al. published the first study specifically focusing on the topic of photobiont plurality in the thallus. In this work, the authors addressed the diversity of photobionts within the thallus of the species *Ramalina farinacea*, using both the Sanger sequencing method (Sanger et al. 1977) and transmission electron microscopy (TEM) of the ultrastructure of photobiont pyrenoids. The coexistence of two different algae of the genus *Trebouxia*, *T. jamesii* and *T. lynnae*, designated at that time as TR1 and TR9 (Del Campo et al. 2010; Moya et al. 2017, 2024; Barreno et al. 2022), was subsequently confirmed in multiple studies by molecular and morphological analyses. The work of Del Campo et al. (2010) was just the first step in the research on this phenomenon. In the following years, photobiont plurality was revealed also in other lichen species. Initially, however, most studies were conducted on thalli of the species *R. farinacea*, which was taken as a reference model to enhance the methods used and compare the physiology of the two recorded algal species. In the work of Moya et al. (2017), the authors used the 454-pyrosequencing method to reveal the overall diversity of photobionts in the thallus of this lichen. They discovered more than 30 photobiont taxa, which is the largest recorded diversity of photobionts within a single lichen thallus to date. However, the authors note that some of the sequences may have been obtained due to contamination by epiphytic algae (Moya et al. 2017).

Photobiont plurality has been discovered in several different lichen genera to date (Fig. 1). The majority belongs to the phylum Ascomycota, class Lecanoromycetes. Most studies have focused on lichen species of the genus *Ramalina* (Del Campo et al. 2010; Campo et al. 2013; Casano et al. 2011, 2015; Del Hoyo et al. 2011; Álvarez et al. 2012, 2015; Catalá et al. 2016; Voytsekhovich and Beck 2016; Moya

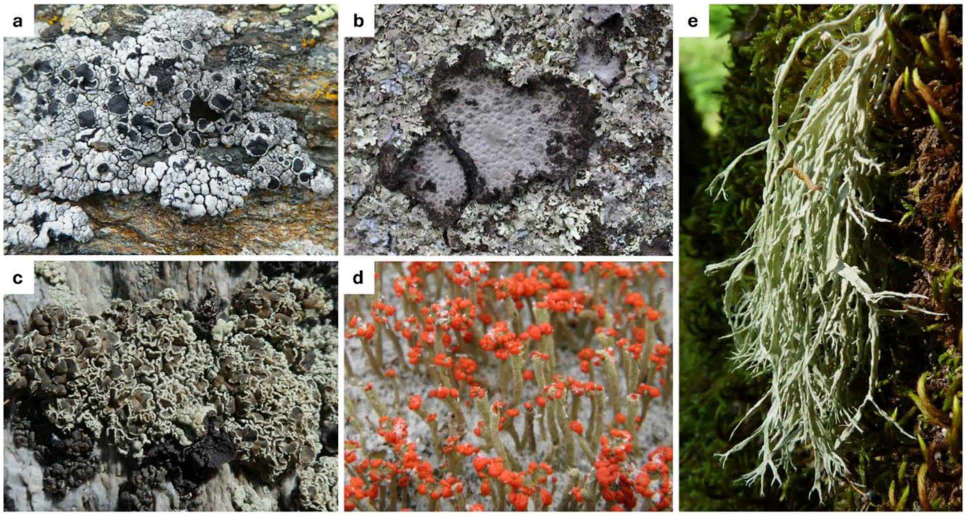

Fig. 1 Examples of lichens where photobiont plurality was reported. **a** *Tephromela atra*, **b** *Umbilicaria pustulata*, **c** *Rhizoplaca melanophthalma*, **d** *Cladonia macilenta*, **e** *Ramalina farinacea*. (Photos: J. Malíček – b; L. Muggia – a, c, e; J. Steinová – d)

et al. 2017, 2024; Molins et al. 2021; Blázquez et al. 2022; Chiva et al. 2023), mostly the species *R. farinacea*. The second and third most studied groups are lichens belonging to the families Cladoniaceae – *Cladonia borealis*, *C. cariosa*, *C. gracilis*, *C. humilis*, *C. macilenta*, *C. pyxidata*, *C. rei*, *C. squamosa*, *C. subturgida*, *C. subulata* (Bačkor et al. 2010; Park et al. 2015; Noh et al. 2020; Osyczka et al. 2021; Pino-Bodas et al. 2023) and Umbilicariaceae – *Umbilicaria antarctica*, *U. decussata*, *U. hispanica*, *U. phaea* and *U. pustulata* (Romeike et al. 2002; Park et al. 2015; Dal Grande et al. 2018; Paul et al. 2018; Rolshausen et al. 2020).

Furthermore, photobiont plurality has been recorded in the lichen families Lecanoraceae (Guzow-Krzemińska 2006; Muggia et al. 2013b; De Carolis et al. 2022), Megasporaceae (Voytsekhovich and Beck 2016; Molins et al. 2018a, b), Parmeliaceae (Piercey-Normore 2006; Ohmura et al. 2006, 2019; Mansournia et al. 2012; Molins et al. 2013; Onuț-Brännström et al. 2018; Meyer et al. 2023; Chiva et al. 2023), Physciaceae (Helms et al. 2001; Dal Grande et al. 2014), Tephromelataceae (Muggia et al. 2014; De Carolis et al. 2022) and several others.

Within the phylum Ascomycota, several photobionts from a single thallus have also been described among the classes Lichinomycetes (Voytsekhovich and Beck 2016; Chrismas et al. 2021) and Eurotiomycetes (Voytsekhovich and Beck 2016). Regarding lichens of the phylum Basidiomycota, photobiont plurality was recorded in most genera of the family Hygrophoraceae, in particular, in the species *Lichenomphalia meridionalis* (Gasulla et al. 2020), and representatives of the genera *Acantholichen*, *Cora*, *Corella* and *Dictyonema* (Dal Forno et al. 2021). For more detailed information, refer to Table 1.

Photobiont plurality of the genus *Trebouxia* has also been described in the parasitic species *Diploschistes muscorum* (Friedl 1987; Wedin et al. 2016; Osyczka et al. 2021). The hyphae of the mycobiont first ‘steal’ *Asterochloris* algae from a *Cladonia* lichen to form the thallus and subsequently exchange it for a *Trebouxia* photobiont, which seems to be preferred for long-term symbiosis (Friedl 1987; Wedin et al. 2016).

4 Methods applied to study photobiont plurality

At first, efforts were made to demonstrate the phenomenon of photobiont plurality using several methods simultaneously (Table 1). These methods primarily consisted of molecular techniques and microscopic analysis. The most common photobionts entering this symbiosis are representatives of the genus *Trebouxia* (Nash 2008), with some species of this genus differing in the morphology of pyrenoids or even entire chloroplasts (e.g. Del Campo et al. 2010, 2013; Casano et al. 2011; Bordenave et al. 2022). For this reason, both transmission electron microscopy (TEM; Del Campo et al. 2010; Campo et al. 2013; Casano et al. 2011; Molins et al. 2013, 2018a, b) and light microscopy (LM; Friedl 1987; Casano et al. 2011; Voytsekhovich et al. 2011; Muggia et al. 2014; Catalá et al. 2016; Voytsekhovich and Beck 2016) played a significant role in confirming the plurality of photobionts within the thallus (Fig. 2). In the initial study focusing purely on the phenomenon of plurality, microscopic techniques were even more crucial, since by using Sanger sequencing of the ITS region of nuclear rDNA only one of the two observed photobionts was detected (Del Campo et al. 2010).

From the early stages of photobiont plurality research, molecular methods have gained significance (e.g. Park et al. 2015; Chrismas et al. 2021). Originally, authors applied Sanger sequencing as it was the most common sequencing method at the time (Del Campo et al. 2010; Campo et al. 2013; Casano et al. 2011). However, this method can only accurately detect the predominant photobiont, whose DNA is most abundantly represented after amplification. Sanger sequencing can yield high-quality results only if the photobiont with minor abundance constitutes a maximum of 30% of the thallus photobionts. If the proportion of the additional photobiont in the thallus is higher, readable sequencing data cannot be obtained (Paul et al. 2018; Moya et al. 2021). Thus, sequencing has often failed in thalli that were confirmed, e.g. morphologically, to contain several photobionts (Voytsekhovich and Beck 2016). In 2015, the first study was published in which information on photobiont plurality was obtained by using next-generation sequencing (NGS, HTS), specifically DNA metabarcoding (Park et al. 2015). Subsequent studies routinely employed this technique (e.g. Onuț-Brännström et al. 2018; Paul et al. 2018; Vančurová et al. 2020; Molins et al. 2021), and the results were often no longer supplemented with microscopic observations. Among the most used NGS platforms are Illumina (Fig. 2; Vančurová et al. 2020; Smith et al. 2020; Chrismas et al. 2021, 2024; Molins et al. 2021; Moya et al. 2021, 2024; Blázquez et al. 2022; Meyer et al. 2023; Pino-Bodas et al. 2023), Ion Torrent (Onuț-Brännström et al. 2018), and the now less commonly used 454 pyrosequencing method (e.g. Park et al. 2015; Moya et al. 2017; Molins et al. 2018a, b; Noh et al. 2020; Dal Forno et al. 2021). In some studies, metabarcoding was used to sequence particularly those samples for which authors were not able to obtain reliable data using Sanger sequencing (Muggia et al. 2014; Vančurová et al. 2020), and they understood this situation as a possibility of multiple algal sequences being present (Ohmura et al. 2006). Photobiont plurality in these samples was subsequently confirmed by metabarcoding. This method is also suitable for detecting the coexistence of photobiont species that cannot be distinguished microscopically (Moya et al. 2017; Dal Forno et al. 2021).

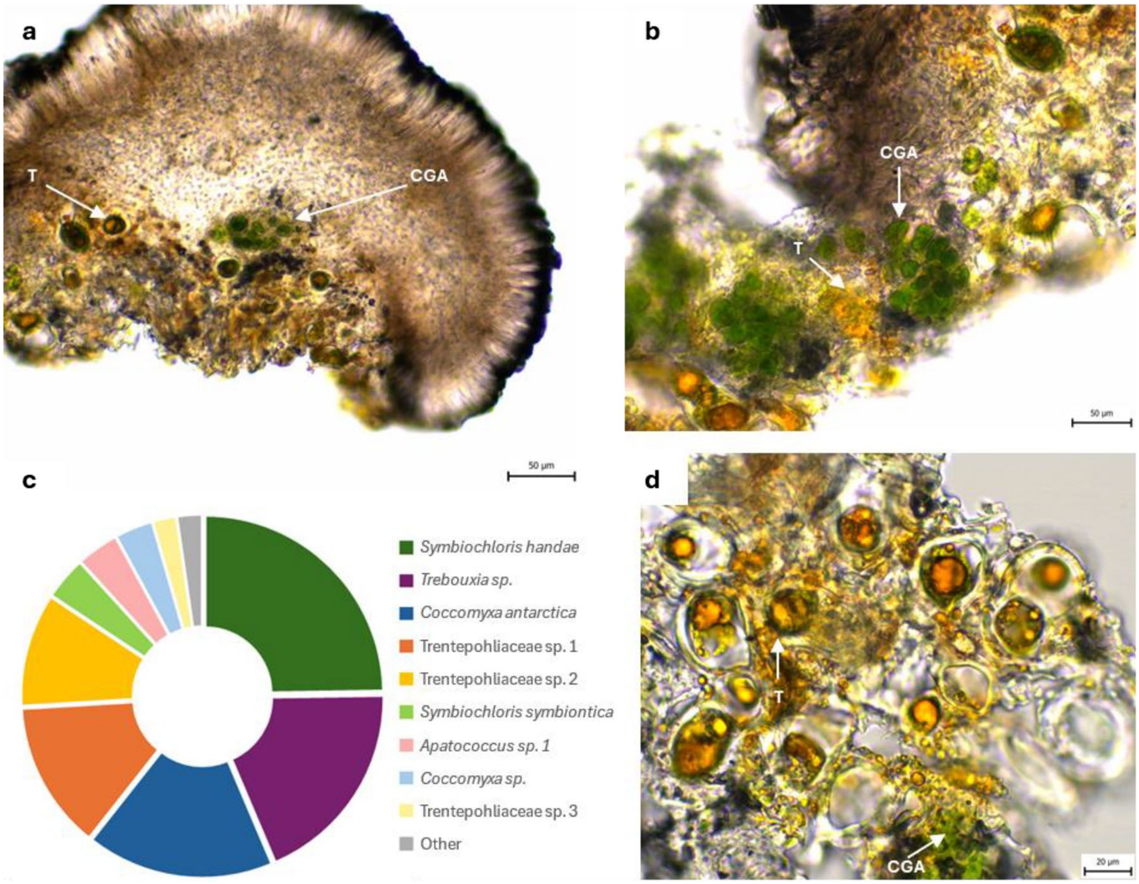

Fig. 2 Photobiont plurality proven in a thallus of *Biatora globulosa*. **a**, **b**, **d** LM pictures – Trentepohliaceae species (T, orange), coccoid green algae (CGA, green). **c** Proportion of photobionts detected in the thallus by Illumina metabarcoding. For detailed methodology of LM and Illumina metabarcoding applied, see Supplementary material S1

The most sequenced region was the internal transcribed spacer (ITS) in nuclear rDNA (e.g. Piercey-Normore 2006; Guzow-Krzemińska 2006; Del Campo et al. 2013; Dal Grande et al. 2018; Gasulla et al. 2020; De Carolis et al. 2022). Otherwise, sequencing of chloroplast DNA, i.e. of the *rbcL* marker, was performed (e.g. Del Campo et al. 2010; Casano et al. 2011; Molins et al. 2013; Ruprecht et al. 2014; Catalá et al. 2016; Ohmura et al. 2019). The chloroplast genome is more conservative and may be more suitable for distinguishing between individual algal species in some algal groups such as *Trebouxia* species (Del Campo et al. 2010; Casano et al. 2011).

Besides the conventional sequencing methods, fingerprinting methods have also been applied to detect photobiont plurality (Table 1). Fingerprinting techniques allow to distinguish between photobionts of different genotypes/haplotypes of the same species (Piercey-Normore 2006; Muggia et al. 2013b; Nyati et al. 2013; Ohmura et al. 2019). By comparing the obtained sequences, photobiont plurality was revealed, for example, in the thalli of the lichen *Evernia mesomorpha* using the restriction fragment length polymorphism (RFLP) based on the different lengths of fragments from different genotypes (Piercey-Normore 2006). The denaturing gradient gel electrophoresis (DGGE) method separates equally long DNA fragments after PCR amplification based on the differences in their sequences after denaturation (Schäfer and Muyzer 2001). Ohmura et al. (2019) applied this method to study photobiont plurality in the species *Parmotrema tinctorum* on the chloroplast gene *rbcL*. Single-strand conformation polymorphism (SSCP) separates different fragments of genetic information based on differences in the conformation of single-strand DNA (Dong and Zhu 2005). This method also confirmed the coexistence of algae in the thalli of lichens *Protoparmeliopsis muralis* and *Tephromela atra* (Muggia et al. 2013b, 2014). Lastly, the authors of the study by Nyati et al. (2013) used the randomly amplified polymorphic DNA (RAPD), a method based on the random PCR amplification of DNA segments (Nyati et al. 2013).

A crucial condition for the successful determination of the photobiont diversity in a thallus is the selection of primers, which are essential for amplifying the DNA of the present photobiont(s). In some cases, this has led to the need to design specific primers targeting specific groups of photobionts (Del Campo et al. 2010; Casano et al. 2011; Moya et al. 2017), as existing primers might not be most suitable to reflect the true diversity in the thallus. Universal primers typically amplify the predominant photobiont, while specifically designed primers are applied for detecting minor photobionts (Piercey-Normore 2006; Del Campo et al. 2013; Álvarez et al. 2015; Molins et al. 2020).

The phenomenon of photobiont plurality was probably overlooked in the past for several reasons. Part of this was due to misinterpretation of the ambiguous signal obtained by Sanger sequencing. Such signals were considered methodological errors. Thus, sequences with these double peaks on the electropherogram were typically excluded from the results (Leavitt et al. 2015). However, current knowledge suggests that the problem with sequencing photobionts using the Sanger method, on the contrary, often indicates the presence of multiple photobionts in the thallus (Vančurová et al. 2020). On the other hand, it should be noted that due to the high success rate of sequencing photobionts directly from the thallus using this method, most lichens probably contain only one predominant photobiont (Paul et al. 2018; Blázquez et al. 2022). Therefore, it seems that the final identification of the number of photobionts in the thallus significantly depends on the method used, the accuracy and precision with which it was performed, and even on the part of the thallus authors used for the analysis (Molins et al. 2018a).

As mentioned above, different species or lineages of photobionts often cannot be distinguished from one another using microscopy techniques alone (Kroken and Taylor 2000; Helms et al. 2001; Del Campo et al. 2013; Catalá et al. 2016), and it is a common practice in recent years to follow an integrative approach for species determination. However, in some cases within the genera *Trebouxia* and *Asterochloris*, different algae may be morphologically determined based on the visualisation of the entire three-dimensional chloroplast morphology using confocal microscopy (CLSM; Škaloud and Peksa 2008; Bordenave et al. 2022), pyrenoid ultrastructure using TEM (e.g. Casano et al. 2011; Del Campo et al. 2013; Bordenave et al. 2022), cultivation and subsequent light microscopy of all stages of their life cycle (Kroken and Taylor 2000), visualisation of cell wall thickness using scanning electron microscopy (SEM; Casano et al. 2011, 2015; Muggia et al. 2011; Álvarez et al. 2012), or cell size itself (Casano et al. 2011).

It is important to keep in mind that many discrepancies in the study of photobionts (e.g. failure to capture sequences of all photobionts or detection of algae/cyanobacteria that are not photobionts of the thallus) may be due to methodological errors. In addition to the previously mentioned limits of Sanger sequencing, results may be influenced by errors in the steps prior to the sequencing process itself. For example, during PCR amplification of DNA extracted directly from the thallus, DNA of epiphytic algae which are not part of the holobiont (Guzow-Krzemińska 2006; Thüs et al. 2011; Moya et al. 2017; Škaloud et al. 2018), or DNA of photobionts from other lichens occurring in the same area as the studied species may be amplified causing a bias in the results (Helms et al. 2001). To some extent, these contaminations can be prevented by washing the thallus (Mansournia et al. 2012; Moya et al. 2017; Dal Grande et al. 2018; Chrismas et al. 2021); however, even with thorough washing, some epiphytic algae may remain trapped on the thallus in cavities or cracks (Thüs et al. 2011). In the study by Chrismas et al. (2021) on *Lichina pygmaea*, the extrathalline community extracted from the wash water filters was also analysed to distinguish contaminations from photobionts.

Direct isolation of photobionts from the algal layer and cultivation may also reduce or even avoid contamination by epithalline algae. Once the individual photobionts are grown in cultures, they are identified morphologically and by sequencing. However, contaminations may occur even in this case, when accidentally cultivating non-lichenized algae if their association with the mycobiont hyphae is not microscopically verified before cultivation (Thüs et al. 2011). Another bias could be caused by using overly specific primers that do not comprehensively capture the diversity of photobionts in the thallus (Jadrná 2017). For instance, this happened when primers specific to the algal genera *Trebouxia* and *Asterochloris* were used for Sanger sequencing when investigating the photobiont diversity of the species *Psora decipiens* (Ruprecht et al. 2014). This mycobiont generally associates with the phycobiont genus *Myrmecia* (Williams et al. 2017; Jadrná 2017), but due to the wrong primer choice it was not captured in the results.

5 The origins of photobiont plurality

There are several hypotheses regarding the origin of photobiont plurality in lichen thalli. In the case of sexually reproducing lichens, a so-called horizontal transmission of photobionts usually occurs (see, for instance, Dal Grande et al. 2018). The germinating fungal spore may associate with several photobionts present in the surrounding environment simultaneously, which is followed by the selection of the most suitable photosynthesising partner(s) (Bhattacharya et al. 1996; Werth and Sork 2010; Dal Grande et al. 2014, 2018; Muggia et al. 2014). Although horizontal transmission is usually linked to sexually reproducing species, it may also play an important role in asexual lichens. In this case, the mycobiont and the predominant photobiont spread together (so-called vertical transmission). Other photobionts may enter the newly forming thallus from outside during the redifferentiation of the vegetative propagules or thallus fragments (horizontal transmission; Ohmura et al. 2019). During this process, not only hyphae from these germinating propagules may incorporate new photobionts, but also the original photobiont may be replaced by a more suitable photobiont(s) from the surrounding environment (Ohmura et al. 2006; Moya et al. 2024), leading to photobiont switching (Piercey-Normore 2006). Multiple different photobionts may also seldom coexist already in the isidia (Mansournia et al. 2012; Molins et al. 2013). The spreading of several photobionts together via the same vegetative propagule belongs to the category of vertical transmission (Werth and Sork 2010).

Photobiont plurality may also arise from the incorporation of additional photobionts into the lichen thallus during its life (horizontal transmission; Piercey-Normore 2006; Ohmura et al. 2006; Mansournia et al. 2012; Dal Grande et al. 2014; Onuț-Brännström et al. 2018). This explanation is supported, for example, by cases where the podetia (erect three-dimensional structures of the thalli of the genus *Cladonia*) of two *Cladonia* species contained multiple photobionts only in their upper part. In the basal part, which is the oldest, the occurrence of only one photobiont was recorded, indicating that new photobionts must have entered the thallus while it was growing (Bačkor et al. 2010). Furthermore, it is presumed that additional photobionts may enter the thallus during the thallus regeneration process, for example, after herbivory (Dal Grande et al. 2014, 2018). Another explanation is that the photobiont plurality may arise from the fusion of two originally distinct thalli (Friedl 1987; Ohmura et al. 2006; Nash 2008). This theory is based on a witnessed fusion of hyphae (Ott 1987b) and soredia (Schuster et al. 1985). However, neither of these explanations has been experimentally confirmed, and they are based only on indirect observations. To precisely determine the source of photobionts and understand the mechanism of photobiont plurality formation, it would be necessary to analyse free-living photobionts from thalli surrounding the thallus of interest (Guzow-Krzemińska 2006).

Generally, it is assumed that if photobionts are not commonly free-living, they were most likely acquired from other lichens growing nearby and later incorporated into the thallus (Ahmadjian 1988). Alternatively, free-living algae and cyanobacteria from the surrounding environment enter the thallus (Mansournia et al. 2012; Voytsekhovich and Beck 2016; Chrismas et al. 2021).

The publication of Mansournia et al. (2012) suggests that genetic variability of photobionts within the thallus can also arise when algae reproduce sexually inside the thallus. However, in the vast majority of cases, as far as it is known, photobionts in the thallus undergo only asexual reproduction, and cases of sexual reproduction occur very rarely and have been observed in axenic cultures only (Mansournia et al. 2012; Škaloud et al. 2015; Gasquez et al. 2024). According to some authors, the presence of distinct haplotypes in the thallus may also be further attributed to mutations in the photobiont genome (Nash 2008; Dal Grande et al. 2014; Moya et al. 2024). However, this explanation is not commonly used in most of the available literature.

6 Coexisting photobionts within a lichen thallus

The co-occurrence of multiple photobionts was mostly observed within the genera *Trebouxia* and *Asterochloris*. These genera are prevalent among phycobionts, prompting authors to revisit their examination (Casano et al. 2011; Ruprecht et al. 2014; Dal Grande et al. 2018). *Trebouxia jamesii* appears to be the most frequently documented species among the recorded coexisting photobionts. The classification of the genus *Trebouxia* into four clades: 'A' (*arboricola*/*gigantea*), 'C' (*corticola*/*galapagensis*/*usneae*), 'I' (*impressa*/*gelatinosa*), and 'S' (*simplex*/'*letharii*'/*jamesii*) was revised most recently by Muggia et al. (2020). Later published studies have utilized this classification for phylogenetic analysis, such as Molins et al. (2021) and De Carolis et al. (2022). Most species of the genus *Trebouxia* coexisting with other photobionts in lichens are found within the clade 'A', followed by 'S' and 'I' (Voytsekhovich and Beck 2016; Dal Grande et al. 2018; Molins et al. 2021; De Carolis et al. 2022).

Other photobionts that have been reported to coexist with another photobiont include, for example, *Coccomyxa*, *Diplosphaera* (e.g. Molins et al. 2021), *Symbiochloris* (Wedin et al. 2016), and *Pseudochlorella* (e.g. Park et al. 2015) (Trebouxiophyceae), as well as *Blidingia*, *Paulbroadia* (Ulvophyceae) (Chrismas et al. 2021; Veselá et al. 2024). Within prokaryotic organisms, this primarily involved cyanobacteria from the genera *Pleurocapsa*, *Rivularia* (Chrismas et al. 2021), and *Rhizonema* (Dal Forno et al. 2021). To a lesser extent, the genera *Acaryochloris*, *Phormidesmis*, and *Synechocystis* were also involved (Chrismas et al. 2021). For further details, see Table 1.

Phylogenetic analyses of photobionts within lichen thalli do not reveal any clear trend regarding the coexistence of photobionts based on their relatedness. In some cases, closely related species were found within the thallus (Friedl 1987; Helms et al. 2001; Guzow-Krzemińska 2006; Del Campo et al. 2010; Campo et al. 2013; Casano et al. 2011, 2015; Del Hoyo et al. 2011; Mansournia et al. 2012; Álvarez et al. 2012, 2015; Muggia et al. 2013b; Catalá et al. 2016; Voytsekhovich and Beck 2016; Dal Grande et al. 2018; Molins et al. 2018a, 2020; Onuț-Brännström et al. 2018; Rolshausen et al. 2020; Blázquez et al. 2022; De Carolis et al. 2022; Meyer et al. 2023; Chiva et al. 2023; Pino-Bodas et al. 2023; Moya et al. 2024) and even the coexistence of different haplotypes/genotypes of algae of the same species has also been considered photobiont plurality (Romeike et al. 2002; Piercey-Normore 2006; Ohmura et al. 2006, 2019; Mansournia et al. 2012; Muggia et al. 2013b; Dal Grande et al. 2014; Catalá et al. 2016; Jadrná 2017; Gasulla et al. 2020; Dal Forno et al. 2021; Osyczka et al. 2021; Vančurová et al. 2021). On the other hand, algae from different genera or even further taxonomically distant algae were observed coexisting together within a thallus (Fig. 2; Ahmadjian 1967; Muggia et al. 2011, 2014; Schmull et al. 2011; Molins et al. 2013, 2018b, 2021; Ruprecht et al. 2014; Park et al. 2015; Wedin et al. 2016; Voytsekhovich and Beck 2016; Moya et al. 2017, 2021; Vančurová et al. 2018, 2020; Noh et al. 2020; Chrismas et al. 2021; Chiva et al. 2023). Once, even the cooccurrence of several eukaryotic and prokaryotic photobionts was demonstrated (Chrismas et al. 2021).

The ratios of coexisting photobionts within individual thalli in various studies exhibit a similar trend. The majority of thalli indeed contain a single predominant photobiont, which has a higher abundance compared to others (Muggia et al. 2014; Dal Grande et al. 2018; Smith et al. 2020; Molins et al. 2021; Moya et al. 2021; Blázquez et al. 2022; Pino-Bodas et al. 2023). Dal Grande et al. (2018), in a study focusing on lichens of the genus *Umbilicaria* (formerly known as *Lasallia*), observed that for the species *U. pustulata* the abundance of the predominant photobiont accounted for more than 90% in 43.4% of the cases (among all samples including those with a single photobiont). They observed a similar pattern for *U. hispanica* in 11.7% of the cases. Likewise, Blázquez et al. (2022) pointed out in the study of several species of the *Ramalina decipiens* group that 94% of the examined thalli exhibiting photobiont plurality had indeed one dominant photobiont. On the other hand, *Biatora globulosa*, presented in this work, was associated with several photobionts of high abundance in its thallus with none being clearly dominant (Fig. 2).

As mentioned above, the term 'photobiont pool' refers to the set of all algal species present in a given environment (Yahr et al. 2004; Guzow-Krzemińska 2006; Molins et al. 2021). It likely serves as a source of additional photobionts (Voytsekhovich and Beck 2016), although, Vančurová et al. (2020) discovered that the algal communities within the studied *Stereocaulon* thalli and the soil from the sampling locality were not overlapping very much. However, it can be assumed that the availability of photobionts has a significant impact on the selectivity of the mycobiont. A species that appears to be highly selective may actually have a low degree of selectivity, but compatible photobionts may not be available in the given location (Dal Forno et al. 2021). Closely related species are usually found on various substrates of similar type, e.g. eutrophic substrates (Guzow-Krzemińska 2006). From this perspective, photobionts are first selected by the environment and then by the mycobiont (Molins et al. 2021).

The nature of the relationship between algae within the thallus can vary. Most studies focus solely on the relationship between the mycobiont and the photobiont without considering how different algae may influence each other. For example, some authors consider the species *Trebouxia decolorans* to be a parasite partially nourished by another alga (photobiont) living within the same lichen thallus (e.g. Rikkinen 1995). It is also possible that only those algae selected by the mycobiont, and with no competition between them, may coexist within the thallus. (Piercey-Normore 2006). This hypothesis can be explained using the example of transition zones in elevation gradients. Rolshausen et al. (2020) defined this transition zone based on temperature and precipitation in the warmest part of the year. Other studies have delimited it as a zone within the range of mean altitudes. In areas of low or high altitudes, lichens with only one photobiont adapted to local conditions are usually found. In thalli growing in the transition zone, multiple photobionts are often present simultaneously (Dal Grande et al. 2018; Gasulla et al. 2020; De Carolis et al. 2022). In these zones, two photobiont pools (from lower and higher altitudes) merge, leading to competition among photobionts within the thalli. At lower or higher altitudes, algae adapted to those specific conditions have a competitive advantage. At the end of the transition zone, a less adapted photobiont species unable to compete with the better-adapted photobiont will usually be outcompeted, resulting in thalli with a single photobiont (Rolshausen et al. 2020). Therefore, the transition zone *de facto* represents an ecotone located in the boundary area between two or more adjacent communities and is typically characterised by a higher diversity of organisms (Holland and Risser 1991).

The interaction of coexisting photobionts was also indicated by the results published by Bhattacharya et al. (1996). The authors suggest that horizontal transfer of group I introns within nuclear-encoded ribosomal DNA probably occurred in the past between two different species of the genus *Trebouxia* in close physical contact within the thallus.

7 Factors affecting photobiont plurality in lichens

Several factors influence the diversity and abundance of individual photobiont species within a lichen thallus. The literature commonly mentions the genotype of the mycobiont, geographic location, abiotic conditions in the area, microenvironment within the thallus and reproduction mode (e.g. Muggia et al. 2014; Molins et al. 2021). The level of influence of each of these factors varies among lichens.

Mycobiont - The occurrence of multiple photobionts within the thallus is related to the degree of specificity and selectivity of the given mycobiont. The hypothesis, assuming low specificity of the mycobiont, has been supported several times, particularly in the family Physciaceae (Helms et al. 2001) and in species such as *Evernia mesomorpha* (Piercey-Normore 2006), *Protoparmeliopsis muralis* (Guzow-Krzemińska 2006), and *Psora decipiens* (Ruprecht et al. 2014).

In the case of the species *Ramalina farinacea*, in which photobiont plurality has been studied most extensively, a high level of mycobiont selectivity was recorded, as the same two lineages of the genus *Trebouxia* were consistently present in the vast majority of thalli across different sampling sites and studies (Del Campo et al. 2010; Campo et al. 2013; Casano et al. 2011, 2015; Del Hoyo et al. 2011; Álvarez et al. 2012, 2015; Moya et al. 2017; Molins et al. 2021). The degree of selectivity probably varies with thallus age. Young thalli may contain many different photobionts that are available in the surrounding environment. As the thallus develops, the best-adapted photobiont for the given location is selected and favoured as the predominant one (Molins et al. 2021). A rather high level of selectivity is also indicated by a study focused on the lichen *Lichina pygmaea* (Chrismas et al. 2021). Chrismas et al. (2021) demonstrated that the mycobiont also carefully selects its photosynthetic partners, as there was a significantly higher diversity of algae and cyanobacteria outside of the thallus compared to the diversity within it. Lichens from the Physciaceae family, which also exhibited photobiont plurality, showed a higher level of selectivity as well (Helms et al. 2001). On the other hand, Bačkor et al. (2010) reported that in the genus *Cladonia*, two different photobionts per thallus were detected in four out of seventeen studied species. These four species were identified as having low selectivity (Bačkor et al. 2010). Low selectivity was further documented in *Ramalina fraxinea* (Catalá et al. 2016), *Stereocaulon vesuvianum* sensu lato (s.l.; Vančurová et al. 2018), lichens of the genus *Umbilicaria* growing in Antarctica (Romeike et al. 2002), and in studies mainly focusing on crustose lichens (Voytsekhovich et al. 2011; Voytsekhovich and Beck 2016). Crustose lichens are generally assumed to exhibit lower selectivity (Helms et al. 2001) than foliose or fruticose ones. It is also possible that selectivity varies not only with the age of the thallus but also depending on the geographic region. According to Ohmura et al. (2006), in areas with less favourable conditions, the mycobiont must choose a suitable photobiont more thoroughly, resulting in higher selectivity.

In the thalli of some lichens, the presence of a particular species as the predominant alga often depends on the specific phylogenetic lineage of the mycobiont (Dal Grande et al. 2018; Rolshausen et al. 2020; Vančurová et al. 2020). In contrast, species of algae found in these thalli in minority overlap across different mycobiont lineages growing in the same locality (Rolshausen et al. 2020; Vančurová et al. 2020). However, this trend cannot be generalised to other genera of mycobionts, as, for example, the lichen *Circinaria hispida* contained four different species of the genus *Trebouxia* as the predominant photobionts in its thalli (Molins et al. 2018b). In the same study, the authors examined three additional species of the genus *Circinaria*, of which two species collected from the same locality shared the same photobionts, but no locality effect was demonstrated when two different species were compared. Similarly, differences between photobionts within a single lichen species were only recorded in some cases. Thus, there seems to be no universally applicable model describing which factors are most crucial for the selection of photobionts into lichen thalli. Most likely, it differs among individual lichen species (Molins et al. 2018b).

In some lichens, the degree of selectivity can vary depending on the specific haplotype of the mycobiont and thus can be variable even within a species (Muggia et al. 2014; Molins et al. 2021). At the same time, mycobiont haplotypes vary geographically, meaning that even an apparent correlation between locality and the presence or abundance of photobiont in the thallus may, in fact, be determined by the identity of the mycobiont (Molins et al. 2021).

The dependence of the photobiont on the mycobiont has been studied, for example, using transplantation experiments, where it was observed that the original algal composition of the thallus was preserved even after transplantation (Williams et al. 2017). Regarding plurality, differences have been noted particularly among the predominant algae of different lichen species collected in the same localities. Predominant photobionts were usually unique to only one of the examined lichen species. On the other hand, within the same lichen species, predominant photobionts varied between different sites. Thus, the influence of the mycobiont appears to be crucial for the occurrence of photobionts in the thalli of different lichen species, while environmental factors primarily influence the selection of photobionts by these same mycobionts in different localities (Park et al. 2015; Dal Grande et al. 2018; Molins et al. 2018b).

Geography - Photobiont plurality has so far been discovered in lichens with two distinct distribution patterns. Either, in lichens that are cosmopolitan, such as *Ramalina farinacea* (Casano et al. 2011, 2015; Moya et al. 2017), *Stereocaulon alpinum*, *S. vesuvianum* (Vančurová et al. 2018), *Tephromela atra* (Muggia et al. 2014; De Carolis et al. 2022), *Psora decipiens* (Ruprecht et al. 2014; Jadrná 2017), *Protoparmeliopsis muralis* (Guzow-Krzemińska 2006; Muggia et al. 2013b), *Rhizoplaca melanophthalma* (De Carolis et al. 2022), or in lichens growing in areas with extreme conditions, such as *Amandinea coniops*, *Cladonia borealis*

(Park et al. 2015), or *Lichina pygmaea* (Chrismas et al. 2021). However, for the species *Protoparmeliopsis muralis* and *Ramalina farinacea* no clear correlation was found between the geographic region of sampling and the algal species present in the thallus (Guzow-Krzemińska 2006; Casano et al. 2011; Del Hoyo et al. 2011; Muggia et al. 2013b; Catalá et al. 2016).

Lichens collected at different localities are likely to differ not in the species composition of photobionts, but rather in the abundance of individual lineages within the thallus (Del Campo et al. 2010; Molins et al. 2021). Molins et al. (2021) conducted several consecutive studies on lichens collected from two distinct geographic locations – the Iberian Peninsula and the Canary Islands (Molins et al. 2013, 2018a, b, 2020, 2021). In their 2021 study, they compared the photobiont plurality in the species *Ramalina farinacea* at both locations using Illumina metabarcoding. While the thalli from the Canary Islands were dominated by an OTU designated *Trebouxia lynnae* (Barreno et al. 2022; formerly identified as *Trebouxia* TR9), in thalli from the Iberian Peninsula *Trebouxia jamesii* was dominant (Del Campo et al. 2010; Campo et al. 2013; Molins et al. 2021; Moya et al. 2024). No difference in the abundance of the predominant algae was observed among the individual islands of the Canary Islands. Similarly, Moya et al. (2024) studied the distribution of the same two main photobiont species of *R. farinacea* – *T. jamesii* and *T. lynnae* – and indeed found that their predominance and occurrence in the thalli correlates with their geographical distribution as the temperature and precipitation values differ.

Environment - The occurrence of photobionts in the environment is determined by local conditions and substrate rather than by geographic area (Romeike et al. 2002; Guzow-Krzemińska 2006; Del Hoyo et al. 2011; Catalá et al. 2016; Gasulla et al. 2020). It is assumed that different species of photobionts vary in sensitivity to substrate changes, meaning that less specialised algae occur in various habitats in different lichens, e.g. *Trebouxia jamesii* (Voytsekhovich and Beck 2016; Moya et al. 2017). Conversely, species with narrow ecological amplitudes will associate with selected mycobionts only in places with optimal conditions (Voytsekhovich et al. 2011; Park et al. 2015; Voytsekhovich and Beck 2016).

Vegetatively reproducing *Umbilicaria pustulata* and sexually reproducing *U. hispanica* are two closely related species with very distinct ecological requirements (Dal Grande et al. 2018). Comparing them has enabled the assessment of the influence of ecological conditions. The results indicate that these species have ecological optima in different ecological niches, and they engage in photobiont sharing in areas of overlap. Proportionally, photobiont plurality was recorded much more frequently in *U. pustulata* (64.1% of all samples) than in *U. hispanica* (28.4%), suggesting that despite their close genetic relation, they differ in their degree of selectivity and optimal ecological conditions of the environment. In contrast, the overall diversity of associated algae was higher in *U. hispanica*, which is attributed to its mode of reproduction (Dal Grande et al. 2018). Reproductive mode probably influences the specificity rather than the selectivity of the mycobiont.

When considering environmental influences, anthropogenic impacts must also be considered. Within a single thallus of the lichen *Parmotrema tinctorum*, Ohmura et al. (2006) found a smaller number of haplotypes of the photobiont *Trebouxia corticola* in specimens collected in urban areas compared to those sampled outside. The authors attribute this finding either again to different selectivity of the mycobiont, or to the bottleneck effect or founder effect, which assume a reduction in algal diversity due to poor air quality (Ohmura et al. 2006). In contrast, other publications describe a higher frequency of photobiont plurality in lichens growing in eutrophic or polluted areas, which is explained as a survival strategy in inhospitable or even toxic conditions (Guzow-Krzemińska 2006; Bačkor et al. 2010; Muggia et al. 2013b; Osyczka et al. 2021).

Thallus parts and reproduction mode of the mycobiont - Another yet insufficiently answered question is the extent to which the frequency of photobiont plurality varies depending on the part of the analysed thallus. Existing results do not provide a clear answer. For example, Molins et al. (2021) did not find significant differences in photobiont plurality abundance between the apical, middle, and basal parts of the thallus branches of *Ramalina farinacea*. On the other hand, Moya et al. (2017) and Noh et al. (2020) observed significant differences between different parts of the thallus of *R. farinacea* and *Cladonia squamosa*, respectively. In both studies, a higher diversity of photobionts was found in its basal part, which is attributed to higher, and above all more stable, humidity and temperature conditions, which create a more favourable environment for the microalgae. Conversely, the apical part is more exposed to sunlight, higher desiccation, and mechanical stresses (e.g. breakage), which are conditions where fewer species of algae can survive (Moya et al. 2017; Noh et al. 2020). This process, where photobionts are exchanged within the thallus as the conditions change, may be seen as a succession within the lichen thallus ecosystem. In this context, photobiont species at the margins of the thallus may be considered pioneer communities (Moya et al. 2017). This trend was observed in fruticose lichens, where temperature and humidity conditions can differ at small spatial scales in the apical and basal parts of the thallus, similarly as in distinct regions of macroecosystems on a larger scale (Moya et al. 2017). Another explanation arises from the influence of the age of the thallus. *R. farinacea* exhibits higher photobiont diversity in smaller and younger thalli than in older, larger thalli collected from the same trees/branches (Molins et al. 2021).

The importance of the particular part of the thallus being analysed has also been demonstrated in the case of *Parmotrema tinctorum*, where different parts of the thallus contained a single photobiont, which in some cases was different from the photobionts in other parts of the same thallus (Mansournia et al. 2012). High photobiont diversity in the thallus of the species *Ramalina farinacea* might also have been discovered due to the systematic investigation of several parts of each thallus (Moya et al. 2017), unlike in previous studies of the same lichen (e.g. Del Campo et al. 2010, 2013; Casano et al. 2011).

Muggia et al. (2014) presented yet another alternative explanation for why there might be higher photobiont diversity in the older parts of the thallus, using the example of the lichen *Tephromela atra*. This crustose lichen reproduces sexually, which means that it relies on germinating spores to find a suitable photosynthetic partner to form a thallus. In such a case, it may associate with several photobiont species, from which it selects the most suitable one during its development, which then becomes the most abundant in the thallus. The remaining photobionts are retained in the thallus only as remnants of thallus formation and as a reserve in case environmental conditions change (Muggia et al. 2014).

8 Significance of photobiont plurality for the lichen symbiosis

A fundamental question regarding photobiont plurality is ‘Why do some lichens have multiple photobionts?’ Several hypotheses are available in the scientific literature. The strategy of lichens to accumulate multiple photosynthetic partners within their thalli was hypothesized to be advantageous in overcoming adverse environmental conditions (Romeike et al. 2002; Casano et al. 2011, 2015; Molins et al. 2013; Moya et al. 2017). For instance, this may be the case for the species *Evernia mesomorpha*, which survives in polluted areas (Gunn et al. 1995). Photobionts of the lichen *Ramalina farinacea* (*Trebouxia jamesii* and *T. lynnae*) have been tested and compared for their different responses to stressful conditions (Del Hoyo et al. 2011; Álvarez et al. 2012; Casano et al. 2015). Furthermore, whether these photobionts were tested for resistance to stress caused by heavy metals (Álvarez et al. 2012, 2015; Casano et al. 2015), oxidative stress (Del Hoyo et al. 2011; Álvarez et al. 2015), or the effects of temperature and irradiance (Casano et al. 2011; Del Campo et al. 2013), the conclusion was always that each of the two species responded differently to stress, thereby offering the lichen symbiosis two different ways to cope with unfavourable conditions.

In the case of heavy metals, the *T. jamesii* used intracellular mechanisms as a defence against stress, while the *T. lynnae* algae relied primarily on a more extensive extracellular apparatus and an approximately three times thicker cell wall (Álvarez et al. 2012; Casano et al. 2015). The thicker cell wall seems to compensate for its relatively easier lysis (compared to the cell wall of *T. jamesii*). During oxidative stress, *T. lynnae* better preserves essential components of the photosynthetic apparatus, but *T. jamesii* has a faster response to the stress (Del Hoyo et al. 2011). In terms of the thermostability of RNA secondary structure, *T. lynnae* is again more resistant (Del Campo et al. 2013). Overall, *T. lynnae* is a partner with generally higher tolerance to stress factors; thus, it is found as the predominant photobiont in warm areas exposed to sunlight. Alternatively, *T. jamesii* is more abundant in thalli exposed to lower temperatures and lower irradiance (Del Hoyo et al. 2011). The overall response to a given stress factor is mostly determined by the predominant photobiont (Álvarez et al. 2015); however, it is assumed that algal species coexisting in one thallus have physiological properties that complement each other at least partially, thereby enhancing the fitness of the whole lichen (Casano et al. 2011, 2015; Del Hoyo et al. 2011; Del Campo et al. 2013). However, it is important to note that all of these studies were conducted only on a single lichen species, so it is still not possible to draw any generally applicable conclusions from their results.

The advantage of a thallus with multiple photobionts may also lie in its ability to survive in frequently changing conditions. For example, the coastal lichen *Lichina pygmaea*, in addition to algae from the Ulvophyceae family, has both marine cyanobacteria for survival after seawater coverage due to tidal cycles and freshwater cyanobacteria for periods when the thallus may be exposed to rain. This allows it to maintain photosynthetic activity both in water and in dry conditions (Chrismas et al. 2021). Chrismas et al. further studied this phenomenon and, in 2023, published results that clearly prove this statement. The two most abundant *L. pygmaea* photobionts, *Rivularia* and *Pleurocapsa*, indeed alternate in dominance in photosynthesis according to the tidal conditions. *Rivularia* dominates in high tide periods, *Pleurocapsa* in dry low tide. Furthermore, they complement each other in terms of different sugar and sugar alcohol production (Chrismas et al. 2024).

Vančurová et al. (2020) found photobiont plurality in lichens of the genus *Stereocaulon* in unstable environments along mountain rivers. This is also related to the lichens’ attempt to expand their ecological niche. It seems that photobiont plurality occurs in ecologically less demanding species, which, due to the coexistence of photobionts, can expand their tolerance to environmental changes (Ruprecht et al. 2014; Muggia et al. 2014; Molins et al. 2020; Prieto et al. 2023) and/or colonise new habitats (Guzow-Krzemińska 2006; Vančurová et al. 2018). It is assumed that this ability may bring advantages to lichens in adapting to global climate change in the future (Rolshausen et al.

2020). Meyer et al. (2023) investigated the algal community shifts in the lichen *Evernia mesomorpha* and discovered that with increasing temperature, abundances of individual *Trebouxia* species present in the thallus change. *Trebouxia* of the clade S dominated the thallus at lower temperatures, but thalli growing under warmer temperatures exhibited a bigger proportion of *Trebouxia* of the clade I. The authors, however, also discuss whether this observed phenomenon was partly due to seasonal change in the algal communities since sampling was performed twice, six months apart. *Protoparmeliopsis muralis*, due to its low selectivity and ability to harbour multiple photobionts in the thallus, is one of the most successful colonisers of anthropogenic, eutrophic and disturbed substrates (Guzow-Krzemińska 2006). A clear correlation was found in a study focusing on polluted environments between the degree of pollution and the number of thalli exhibiting photobiont plurality. Therefore, it can be assumed that in polluted environments, lichens with multiple photobionts survive better (Osyczka et al. 2021).

The effort to maximise photosynthetic efficiency may also play a role (Piercey-Normore 2006; Casano et al. 2011; Molins et al. 2020; Chrismas et al. 2024). For example, the photobionts of the lichen *Buellia zoharyi* differ in the intensity of photosynthesis in environments with different temperatures (Molins et al. 2020). Epiphytic lichens growing in tree canopies may have an ecological advantage when containing multiple photobionts, since they may manage more efficiently with varying light intensities passing through the canopy (Piercey-Normore 2006; Casano et al. 2011).

On the contrary, Blázquez et al. (2022) argue about the real significance of the additional photobionts for the thallus, since in their study of *Ramalina decipiens* species-group they found that a vast majority of the thalli with multiple photobionts had one algae that was clearly more abundant than the others.

9 Conclusions and outlooks

Photobiont plurality has been observed in lichens of different families, including both ascomycetes and basidiomycetes, diverse growth forms, and originating from a wide range of substrates and habitats. Here we have reviewed the occurrence of multiple photobionts within single thalli in 62 studies, four fungal classes, 15 orders, 26 families, 46 genera, and at least 100 species (Fig. 3). Both cyanobacteria and green algae, including representatives from the Trebouxiophyceae and Ulvophyceae classes, are found to coexist within the thallus. Across the cited studies, six genera of cyanobacteria and 20 genera of algae were described as participating in the phenomenon of photobiont plurality within the lichen thallus (Table 1). Photobionts found within the same thallus can be closely related (at the level of haplotypes within the same lineage) or phylogenetically distant (at the level of genera or even families). It is generally believed that the greatest influence on the occurrence of photobiont plurality in the thallus is the available photobiont pool in the environment, itself influenced by the prevailing biotic and abiotic conditions, as well as the degree of specificity and selectivity of the mycobiont (e.g. Park et al. 2015; Dal Grande et al. 2018; Molins et al. 2018b, 2021; De Carolis et al. 2022; Meyer et al. 2023).

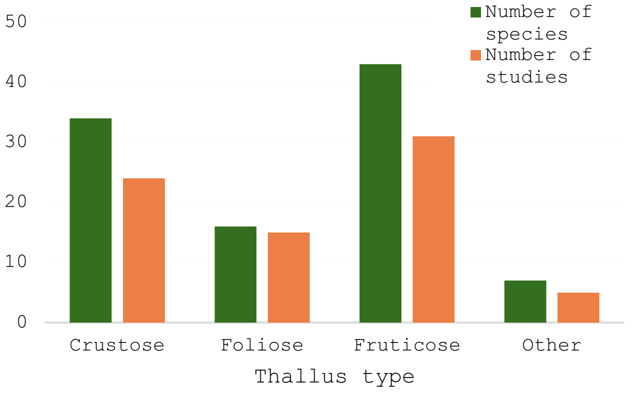

Fig. 3 Summary of Table 1 representing the number of lichen species (green) which proved to have multiple photobionts and number of studies (orange) which described this phenomenon, according to the thallus growth form

Photobiont plurality is likely a relatively common phenomenon in lichen thalli; however, the number of lichen species in which the phenomenon was studied so far is limited, which makes interpretation of the data and its generalisation still difficult. To deepen our understanding of this phenomenon, further studies on a larger number of lichen species with different characteristics (thallus types, modes of reproduction, localities, environmental conditions, etc.) are needed. Most of the available literature focuses on the plurality of chlorobionts, partly due to the choice of primers for amplifying only eukaryotic DNA. Most studies have focused only on one or a small number of lichen species (e.g. Noh et al. 2020; Chrismas et al. 2021), while only a few broadly focused studies are available (e.g. Voytsekhovich et al. 2011; Voytsekhovich and Beck 2016).

Foliose lichens have been the least explored in terms of photobiont plurality within the thallus. Multiple photobionts within the thallus have been most frequently observed in crustose and fruticose lichens. Fruticose lichens have also been studied most extensively in terms of photobiont physiology and differences in the analysed parts of the thallus.

One of the greatest challenges in studying photobiont plurality lies in choosing an appropriate methodology or preferably a combination of methods. In most cases, authors have identified only two coexisting photobionts using microscopy or Sanger sequencing. However, these methods rarely

Table 1 Summary of literature and relevant data on the phenomenon of photobiont plurality. The table reports: the lichen species name (which corresponds with the name of the mycobiont) in which photobiont plurality has been detected, thallus growth form, genus or species name of the photobionts involved, the number of species/strains of photobionts found in a single thallus, the methodology used to detect photobiont plurality, the geographic origin, the referring study. The table is arranged alphabetically according to the lichens' scientific names. The first mention of a lichen genus is indicated in bold, and the following species belong to the indicated genus in bold above. Abbreviations: cru. = crustose thallus, fol. = foliose thallus, fru. = fruticose thallus, lep. = leprose thallus, vag. = vagrant thallus; 454 = pyrosequencing, DGGE = denaturing gradient gel electrophoresis, LM = light microscopy, RAPD = randomly amplified polymorphic DNA, RFLP = restriction fragment length polymorphism, Sanger = Sanger sequencing, SSCP = single-strand conformation polymorphism, TEM = transmission electron microscopy; NA = not specified in the study

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
NA	NA	*Trebouxia* spp.	NA	Sanger	NA	Bhattacharya et al. 1996	
***Acantholichen* sp.**	fol.	*Rhizonema* sp.	NA	454	South America	Dal Forno et al. 2021	*Rhizonema* sp. (different haplotypes)
Amandinea coniops	cru.	*Trebouxia impressa*, *T. potteri*, *T. flava*	2	454	Antarctica (King George Island)	Park et al. 2015	
Anaptychia ciliaris	fru.	*Trebouxia decolorans*	NA	Sanger (microsatelites)	Norway, Spain	Dal Grande et al. 2014	*T. decolorans* (different haplotypes)
Aspicilia cinerea	cru.	*Ttrebouxia. asymmetrica*, *T. solaris*	2	LM	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
Biatora globulosa	cru.	*Apatococcus* spp., *Coccomyxa antarctica*, *Coccomyxa*. sp., *Symbiochloris handae*, *S. symbiontica*, *Symbiochloris* spp., *Trebouxia* sp., *Trentepohliaceae* spp.	14	LM, Illumina	Czech Rep. (Beskydy)	This study (detected by Jiří Malíček and Lucie Vančurová)	
Bryoria fremontii	fru.	*Trebouxia* clade A, S	up to 4	Illumina	USA (Montana)	Smith et al. 2020	
B. tortuosa	fru.	*Trebouxia* clade S	up to 2	Illumina	USA (Montana)	Smith et al. 2020	
***Buellia* sp.**	cru.	*Elliptochloris bilobata*, *Trebouxia* cf. *impressa*	2	Cultivation, LM	Antarctica	Aoki et al. 1998	Extracted only from one sample
B. granulosa	cru.	*Trebouxia jamesii*, other unspecified sp.	NA	454	Antarctica (King George Island)	Park et al. 2015	
B. zoharyi	cru.	*Trebouxia asymmetrica*, *Trebouxia* sp. '*arnoldoi*', *T. cretacea*	2	Sanger, TEM, Cultivation, LM	Canary Islands (Fuerteventura, Lanzarote, Tenerife)	Molins et al. 2020	Differences in composition between the islands

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
B. zoharyi	cru.	*Asterochloris* spp., *Coccomyxa* sp., *Coenochloris* sp., *Diplosphaera* sp., *Elliptochloris* sp., *Myrmecia* sp., *Pseudostichococcus* sp., *Trebouxia* sp., *T. asymmetrica*, *T. cretacea*, *T. jamesii*, *Vulcanochloris* sp.	at least 3	Sanger, Illumina	Balearic Islands, Iberian Peninsula	Moya et al. 2021	
B. zoharyi	cru.	*Deuterostichococcus* sp. (6 strains), *Trebouxia* sp.	7	Cultivation, Sanger	Spain (Titulcia)	Chiva et al. 2023	
B. zoharyi	cru.	*Diplosphaera* sp., *Trebouxia* sp.	2	Cultivation, Sanger	Canary Islands (Tenerife)	Chiva et al. 2023	
B. zoharyi	cru.	*Diplosphaera chodatti*, *Trebouxia* sp.	2	Cultivation, Sanger	Canary Islands (Tenerife)	Chiva et al. 2023	
Candelariella medians	cru.	*Trebouxia crenulata* / *T. gigantea*, *T. decolorans*	2	LM, Sanger	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
C. vitellina	cru.	*Trebouxia* cf. *gigantea*, *T. solaris*	2	LM, Cultivation, Sanger	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
***Cetraria* sp.**	fru.	*Trebouxia* spp.	NA	Sanger, Ion Torrent	Sweden (Öland), Iceland	Onuț-Brännström et al. 2018	
Circinaria contorta	cru.	*Trebouxia asymmetrica* / *T.* cf. *gigantea*, *T. solaris*	2	LM	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
C. gyrosa	vag.	*Asterochloris* sp., *Elliptochloris* sp., *Trebouxia* spp.	NA	Sanger, 454, TEM	Spain	Molins et al. 2018b	
C. hispida	vag.	*Trebouxia* A12, *T. createa*	up to 4	Sanger, 454, Cultivation, TEM	Spain	Molins et al. 2018a	2 detected by 454, 4 from culture
C. hispida	vag.	*Asterochloris* sp., *Elliptochloris* sp., *Trebouxia* spp.,	NA	Sanger, 454, TEM	Spain	Molins et al. 2018b	
C. sp. '*oromediterranea*'	cru.	*Asterochloris* sp., *Elliptochloris* sp., *Trebouxia* spp.,	NA	Sanger, 454, TEM	Spain	Molins et al. 2018b	
C. sp. '*paramerae*'	vag.	*Asterochloris* sp., *Elliptochloris* sp., *Trebouxia* spp.	NA	Sanger, 454, TEM	Spain	Molins et al. 2018b	

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
Cladonia borealis	fru.	*Asterochloris erici* (related sp.), *Pseudochlorella*, *Trebouxia impressa*, *T. jamesii*, undefined lineage	5	454	Antarctica (King George Island)	Park et al. 2015	
C. cariosa	fru.	*Asterochloris*, *Trebouxia*	NA	Sanger	Poland (polluted areas)	Osyczka et al. 2021	*Asterochloris* (different haplotypes), *Trebouxia* (different haplotypes)
C. gracilis	fru.	*Asterochloris erici*, other unspecified sp.	NA	454	Antarctica (King George Island)	Park et al. 2015	
C. humilis	fru.	*Asterochloris* spp.	2	Sanger	Czech Rep. (former ore-sedimentation basin)	Bačkor et al. 2010	
C. macilenta	fru.	*Asterochloris* spp.	3	Sanger	Czech Rep. (former ore-sedimentation basin), Slovakia (Cu mine-spoil heaps)	Bačkor et al. 2010	
C. pyxidata	fru.	*Asterochloris* spp.	2	Sanger	Slovakia (Cu mine-spoil heaps)	Bačkor et al. 2010	
C. rei	fru.	*Asterochloris*, *Trebouxia*	NA	Sanger	Poland (polluted areas)	Osyczka et al. 2021	*Asterochloris* (different haplotypes), *Trebouxia* (different haplotypes)
C. rei	fru.	*Asterochloris* spp.	2	Sanger	Czech Rep. (former ore-sedimentation basin), Slovakia (Cu mine-spoil heaps)	Bačkor et al. 2010	
C. squamosa	fru.	*A. erici*, Trebouxiophyceae, Ulvophyceae	up to 20	454	Antarctica	Noh et al. 2020	
C. subturgida	fru.	*Myrmecia* sp. + *Asterochloris*, aff. *Hemichloris*, *Trebouxia*, *Vulcanochloris*	NA	Illumina	France (Provence-Alps-Côte D'Azur), Greece (Macedonia - Thrace), Greece (Peloponese), Italy (Sardinia), Spain (Toledo)	Pino-Bodas et al. 2023	*Myrmecia* sp. represented over 96% of all reads
C. subturgida	fru.	*Myrmecia* sp.	2	Illumina	Italy (Sardinia)	Pino-Bodas et al. 2023	*Myrmecia* sp. (2 different ASV)
C. subturgida	fru.	*Myrmecia* sp. + *Asterochloris*, aff. *Hemichloris*, *Trebouxia*, *Vulcanochloris*	NA	Illumina	Italy (Sardinia)	Pino-Bodas et al. 2023	2 *Myrmecia* ASV most abundant in the thallus
C. subulata	fru.	*Asterochloris* spp.	2	Sanger	Czech Rep. (former ore-sedimentation basin)	Bačkor et al. 2010	

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
***Coccocarpia* sp.**	fol.	*Rhizonema* sp.	NA	454	South America	Dal Forno et al. 2021	*Rhizonema* sp. (different haplotypes)
***Cora*sp.**	fru.	*Rhizonema* sp.	NA	454	South America	Dal Forno et al. 2021	*Rhizonema* sp. (different haplotypes)
***Corella* sp.**	fru.	*Rhizonema* sp.	NA	454	South America	Dal Forno et al. 2021	*Rhizonema* sp. (different haplotypes)
***Dictyonema* sp.**	fru.	*Rhizonema* sp.	NA	454	South America	Dal Forno et al. 2021	*Rhizonema* sp. (different haplotypes)
Diploschistes diacapsis	cru.	*Trebouxia* cf. *arboricola*, *T. vagua*	2	LM	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
D. muscorum	cru.	*Asterochloris irregularis*, *Trebouxia showmanii*	2	Cultivation, LM	France, Italy, Germany, Switzerland	Friedl 1987	*A. irregularis* identified in the study as *T. irregularis*
D. muscorum	cru.	*Asterochloris* sp., *Trebouxia* sp.	NA	Sanger	Poland (polluted areas)	Osyczka et al. 2021	
D. muscorum	cru.	*Asterochloris* sp., *Trebouxia* sp.	2	Sanger	Germany (Bavaria), Sweden (Öland)	Wedin et al. 2016	
D. muscorum	cru.	*Symbiochloris symbiontica*, *Trebouxia* sp.	2	Sanger	Germany (Bavaria), Sweden (Öland)	Wedin et al. 2016	*Symbiochloris symbiontica* marked as *Dictyochloropsis symbiontica*
Evernia mesomorpha	fru.	*T. jamesii*	NA	Sanger, RFLP	Canada (Manitoba)	Piercey-Normore 2006	*T. jamesii* (different genotypes)
E. mesomorpha	fru.	*Trebouxia* clade A, I, and S	NA	Illumina	USA (Minnesota)	Meyer et al. 2023	
Lecidea roseotincta	cru.	*Trebouxia jamesii*, *T.* ‘*roseotinctae*’	2	Sanger	NA	Schmull et al. 2011	
Lepraria membranacea	lep.	*Stichococcus bacillaris*, *Trebouxia* sp.	2	LM	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
***Leproloma* sp.**	lep.	*Elliptochloris bilobata*, *Trebouxia* cf. *impressa*	2	Cultivation, LM	Antarctica	Aoki et al. 1998	Isolated only from one specimen
Lichenomphalia meridionalis	cru.	*Coccomyxa subellipsoidea*	2	Sanger	Spain (mountains)	Gasulla et al. 2020	*Coccomyxa subellipsoidea* (different haplotypes)
Lichina pygmaea	fru.	*Blidingia*, *Paulbroadya*; *Rivularia*, *Pleurocapsa*, (*Acaryochloris*, *Phormidesmis*, *Synechocystis*)	NA	Illumina	South-west UK (intertidal zone)	Chrismas et al. 2021	Algae of the family Ulvophyceae; Cyanobacteria
L. pygmaea	fru.	*Pleurocapsa*, *Rivularia*	NA	Illumina	South-west UK (intertidal zone)	Chrismas et al. 2024	Algae of the family Ulvophyceae; Cyanobacteria

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
Micarea melanobola	cru.	*Elliptochloris subsphaerica*, *Pseudococcomyxa* sp.	2	LM	Ukraine (Transcarpathian)	Voytsekhovich et al. 2011	
M. misella	cru.	*Elliptochloris bilobata*, *Neocystis* sp., *Pseudococcomyxa* sp.	3	LM	Ukraine (Kyiv)	Voytsekhovich et al. 2011	
M. peliocarpa	cru.	*Elliptochloris reniformis*, *E. subsphaerica*	2	LM	Ukraine (Transcarpathian)	Voytsekhovich et al. 2011	
M. prasina	cru.	*Elliptochloris bilobata*, *E. subsphaerica*, *Interfilum* sp.	up to 3	LM	Ukraine (Donetsk, Luhansk)	Voytsekhovich et al. 2011	
Ochrolechia parella	cru.	*Trebouxia jamesii*, other unspecified sp.	NA	454	Antarctica (King George Island)	Park et al. 2015	
Parmotrema pseudotinctorum	fol.	*Asterochloris glomerata*, *Trebouxia* sp., *T. corticola*,	3	Sanger, TEM	Canary Islands	Molins et al. 2013	
P. pseudotinctorun	fol.	*Diplosphaera* sp., *Trebouxia* sp.	3	Cultivation, Sanger	Spain (San Sebastián)	Chiva et al. 2023	*Diplosphaera* sp. (2 strains)
P. tinctorum	fol.	*Trebouxia corticola*, *T. higginsiae*, *Trebouxia* sp. RG1	2, 5	Cultivation, Sanger (microsatelites)	Japan (Kujukurihama coast)	Mansournia et al. 2012	*T. corticola* (different genotypes)
P. tinctorum	fol.	*Trebouxia corticola*	NA	Cultivation, Sanger, LM	Japan (Shizuoka City)	Ohmura et al. 2006	*T. corticola* (different haplotypes)
P. tinctorum	fol.	*Trebouxia corticola*	NA	Sanger, DGGE	Japan (Shizuoka City)	Ohmura et al. 2019	*T. corticola* (different haplotypes)
Physcia biziana	fol.	*Trebouxia* clade I	2	Illumina	USA (Nevada)	Smith et al. 2020	
Physciella chloantha	fol.	*Trebouxia* clade A, I	3	Illumina	USA (Nevada)	Smith et al. 2020	
Placynthiella icmalea	cru.	*Elliptochloris subsphaerica*, *Interfilum massjukiae*, *Radiococcus signiensis*	up to 3	LM	Ukraine (Kherson, Transcarpathian)	Voytsekhovich et al. 2011	
P. uliginosa	cru.	*Asterochloris* sp., *Elliptochloris subsphaerica*, *Interfilum massjukiae*, *Leptosira* cf. *thrombii*, *Radiococcus signiensis*, *Trebouxia* sp.	2	LM	Ukrajina (Kherson, Luhansk)	Voytsekhovich et al. 2011	
Protoparmeliopsis muralis	cru.	*Trebouxia gigantea*, *T.* *'muralis I'*	2	Sanger	Poland (castle wall)	Guzow-Krzemińska 2006	
P. muralis	cru.	*Trebouxia* sp., *T. incrustata*, *T. 'muralis I'*	NA	Sanger, SSCP	Czech Rep., Italy, Germany, Austria, Sweden	Muggia et al. 2013b	

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
Psora decipiens	cru.	*Asterochloris* sp., *Trebouxia* sp.	NA	Sanger	Germany (Bavaria), Austria (Großglockner, Alps), Spain (Tabernas), Sweden (Öland)	Ruprecht et al. 2014	Used overly specific primers
P. decipiens	cru.	*Myrmecia israelisensis* (different genotypes)	NA	Cultivation, Sanger	Albania, Czech Rep., France, Croatia, Cyprus, Germany, Portugal, Russia, Slovakia, Turkey	Jadrná 2017	
Ramalina calicaris	fru.	*Asterochloris excentrica*, *Trebouxia jamesii*	2	Cultivation, LM, Sanger	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
R. capitata	fru.	*Trebouxia jamesii*, *T. solaris*	2	LM, Sanger	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
R. decipiens	fru.	*Trebouxia lynnae*, *Trebouxia* spp., *Trebouxia*	up to 9, mean 2.46	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. delicata	fru.	*Trebouxia lynnae*, *Trebouxia* spp., *Trebouxia*	up to 5, mean 2.33	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. erosa	fru.	*Trebouxia lynnae*, *Trebouxia* spp., *Trebouxia*	up to 2, mean 1.22	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. fariancea	fru.	*Asterochloris glomerata*, *Appatococcus* sp., *Coccomyxa* sp., *Diplosphaera* sp., *Elliptochloris* sp., *Stichococcus* sp., *Trebouxia* spp.	up to 6	Illumina	Spain (Canary Islands, Iberian Peninsula)	Molins et al. 2021	
R. farinacea	fru.	*Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9)	2	Cultivation, Sanger, LM, TEM	Spain (Canary Islands, Iberian Peninsula)	Del Campo et al. 2010	
R. farinacea	fru.	*Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9)	2	Sanger, TEM, LM	Spain (Canary Islands, Iberian Peninsula), USA (California)	Casano et al. 2011	
R. farinacea	fru.	*Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9)	2	Cultivation	Spain	Del Hoyo et al. 2011	
R. farinacea	fru.	*Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9)	2	Cultivation	Spain	Álvarez et al. 2012	

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
R. farinacea	fru.	*Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9)	2	Sanger	Spain (Canary Islands, Iberian Peninsula)	Del Campo et al. 2013	
R. farinacea	fru.	*Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9)	2	Cultivation, Sanger	Spain	Álvarez et al. 2015	
R. farinacea	fru.	*Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9)	2	Cultivation	Spain	Casano et al. 2015	
R. farinacea	fru.	*Asterochloris* spp., *Elliptochloris* sp., *Diplosphaera* sp., *Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9), *Vulcanochloris* sp.	31	Sanger, 454	Canary Islands (Tenerife)	Moya et al. 2017	2 types of washing
R. farinacea	fru.	*Diplosphaera* sp. (2 strains), *Trebouxia* sp., *Tritostichococcus coniocybes* (5 strains)	8	Cultivation, Sanger	Spain (El Toro)	Chiva et al. 2023	
R. farinacea	fru.	*T. jamesii*, *T. lynnae*	31	Sanger	Mediterranean Basin (Algeria, Iberian & Italian peninsulas, Balearic Islands)	Moya et al. 2024	
R. fastigiata	fru.	*Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9)	2	NA	Iberian Peninsula	Del Campo et al. 2013 (Catalá, unpublished)	
R. fortunatarum	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 2, mean 1.38	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde; *R. fortunatarum* marked as *R. fortunata*
R. fraxinea	fru.	*T. decolorans* (6 lineages), *T. jamesii*	up to 3	LM, TEM, Sanger	Iberian Peninsula	Catalá et al. 2016	
R. gomerana	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 7, mean 3	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. hamulosa	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 2, mean 1.30	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
R. maderensis	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 10, mean 2.23	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. nematodes	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 5, mean 2	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. papyracea	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 4, mean 3	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. pluviariae	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 7, mean 4.67	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. portosantana	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 5, mean 2	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. sabinosana	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 3, mean 2.25	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde; *R. sabinosana* marked as *R. sabinosae*
R. sampaiana	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 6, mean 1.92	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
R. sp. 2	fru.	*Trebouxia lynnae* (former TR9), *Trebouxia* spp., *Trebouxia*	up to 2, mean 1.5	Illumina	Canary Islands, Cape Verde, Madeira archipelago	Blázquez et al. 2022	Photobiont plurality mostly found in Canary Islands and Cape Verde
Rhizocarpon geographicum	cru.	*Trebouxia gelatinosa*/*Trebouxia* sp., *T.* cf. *gigantea*, *T. jamesii*	2	LM	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
Rhizoplaca melanophthalma	cru.	*Trebouxia* spp.	NA	Cultivation, Sanger, TEM	Globally (high altitudes)	De Carolis et al. 2022	
R. melanophthalma subsp. *crispa*	vag.	*Trebouxia* clade A	up to 2	Illumina	USA (Idaho)	Smith et al. 2020	
R. arbuscula	vag.	*Trebouxia* clade A	up to 2	Illumina	USA (Idaho)	Smith et al. 2020	
Rinodina atrocinerea	cru.	*Trebouxia* spp.	2	Sanger	Austria	Helms et al. 2001	
R. tunicata	cru.	*Trebouxia* spp.	2	Sanger	Greece (Crete)	Helms et al. 2001	
Rinodinella controversa	cru.	*Trebouxia* spp.	2	Sanger	Greece (Crete)	Helms et al. 2001	

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
Schaereria dolodes	cru.	*Chlorella ellipsoidea*, *Trebouxia jamesii*, unknown Trebouxyophyceae	3	Sanger	NA	Schmull et al. 2011	
Schizoxylon albescens	cru.	*Coccomyxa* sp., *Pseudococcomyxa* sp.	NA	Sanger, Cultivation, SEM	Norway (north), Sweden (north, middle)	Muggia et al. 2011	*Coccomyxa* sp. (different genotypes)
***Staurothele* sp.**	cru.	*Diplosphaera chodiatii*, *Trebouxia incrustata*	2	LM	Ukraine (Crimean Peninsula, Karadag massif)	Voytsekhovich and Beck 2016	
Stereocaulon alpinum	fru.	*Asterochloris irregularis*, *Asterochloris* spp., Trebouxiophyceae URa28	2, 3	Illumina	Switzerland (Bernina Range)	Vančurová et al. 2020	
S. alpinum	fru.	*Asterochloris* sp., *Sanguina* sp., Chlorophyceae sp.	NA	Illumina	Switzerland (Bernina Range)	Vančurová et al. 2020	Thallus where the Sanger sequencing method failed
S. alpinum	fru.	*Pseudochlorella* sp., *Sanguina* spp., *Trebouxia jamesii*, Trebouxiophyceae URa28	5	Illumina	Switzerland (Bernese Alps)	Vančurová et al. 2020	Thallus where the Sanger sequencing method failed
S. alpinum	fru.	*Asterochloris* spp., *Coelastrella* sp., *Elliptochloris* sp., *Chloroidium* sp., *Pseudochlorella* sp., Trebouxiophyceae URa28	NA	Illumina	Switzerland (Bernina Range)	Vančurová et al. 2020	
S. alpinum	fru.	*Asterochloris irregularis*, *Asterochloris* spp., Chlorophyceae sp., *Pseudochlorella* sp., *Sanguina* sp., Trebouxiophyceae URa28	NA	Illumina	Switzerland (Bernese Alps)	Vančurová et al. 2020	Thallus where the Sanger sequencing method failed
S. alpinum	fru.	*Coccomyxa viridis*, *Elliptochloris* sp.	2	Sanger	Switzerland (Bernina Range)	Vančurová et al. 2020	
S. canariense	fru.	*Asterochloris* sp., *Vulcanochloris symbiontica*	2	Sanger	Canary Islands (La Palma)	Vančurová, unpublished	
S. nanodes	fru.	*Chloroidium ellipsoideum*, *C. lichenum* B	2	Cultivation	Czech Rep.	Vančurová et al. 2018	
S. vesuvianum s.l.	fru.	*Chloroidium ellipsoideum*, *C. lichenum* B	2	Sanger	Guatemala	Vančurová et al. 2018	

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
S. vesuvianum s.l.	fru.	*Chloroidium lichenum A*	3	Sanger, Cultivation	Czech Rep.	Vančurová et al. 2018	*Chloroidium lichenum* A (three genotypes)
S. vesuvianum s. str.	fru.	*Asterochloris mediterranea*, *Chloroidium lichenum* A	2	Sanger	Canary Islands (La Palma)	Vančurová et al. 2018	
S. vesuvianum s. str.	fru.	*Chloroidium ellipsoideum*, *C. lichenum* A	2	Sanger	Canary Islands (Tenerife)	Vančurová et al. 2021	
S. vesuvianum s. str.	fru.	*Chloroidium ellipsoideum*	3	Sanger	Canary Islands (Tenerife)	Vančurová et al. 2021	*Chloroidium ellipsoideum* (three genotypes)
Tephromela atra	cru.	*Trebouxia* spp.	NA	Cultivation, Sanger, TEM	Globally (high altitudes)	De Carolis et al. 2022	
T. atra	cru.	*Trebouxia jamesii* (former TR1), *T. lynnae* (former TR9)	2	NA	Iberian Peninsula	Del Campo et al. 2013 (Del Campo unpublished)	
T. atra	cru.	*Asterochloris* sp., *Trebouxia* (12 lineages)	NA	Cultivation, Sanger, SSCP, TEM, LM	Globally	Muggia et al. 2014	
***Thamnolia* sp.**	fru.	*Trebouxia* spp.	NA	Sanger, Ion Torrent	Sweden (Öland), Iceland	Onuț-Brännström et al. 2018	
Umbilicaria antarctica	fol.	*Asterochloris erici*, lineages related to *Pseudochlorella*, *T. jamesii* (related sp.)	min 3	454	Antarctica (King George Island)	Park et al. 2015	
U. decussata	fol.	*Trebouxia jamesii*	2	Sanger	Antarctica (Lagoon Island, Rothera)	Romeike et al. 2002	*T. jamesii* (different genotypes)
U. hispanica	fol.	*Trebouxia* clade S	up to 6	Illumina, (Sanger)	Spain	Paul et al. 2018	
U. hispanica	fol.	*Trebouxia* spp.	2	Illumina	Spain (Sierra de Gredos)	Dal Grande et al. 2018	
U. phaea	fol.	*Trebouxia* spp.	NA	Sanger	USA (Mount San Jacinto, Sierra Nevada)	Rolshausen et al. 2020	
U. pustulata	fol.	*Trebouxia* clade S	up to 4	Illumina, (Sanger)	Spain	Paul et al. 2018	
U. pustulata	fol.	*Trebouxia* spp.	2	Illumina	Spain (Sierra de Gredos)	Dal Grande et al. 2018	
U. pustulata	fol.	*Trebouxia* spp.	NA	Sanger	Italy (Mount Limbara), Spain (Sierra de Gredos)	Rolshausen et al. 2020	
Usnea aurantiaco-atra	fru.	*Trebouxia jamesii*, other unspecified sp.	NA	454	Antarctica (King George Island)	Park et al. 2015	
Verrucaria aethiobola	cru.	*Pleurococcus* sp., *Stichococcus* sp.	2	Cultivation	NA	Ahmadjian 1967	*V. aethiobola* marked as *Staurothele catalepta*
Xanthoparmelia* aff. *chlorochroa	fol.	*Trebouxia* clade A	3	Illumina	USA (Nevada, Utah, Wyoming)	Smith et al. 2020	
X. aff. *mexicana*	fol.	*Trebouxia* clade A	3	Illumina	USA (Arizona)	Smith et al. 2020	

Table 1 (continued)

Lichen	Thallus	Photobionts	No. per thallus	Methods	Locality	Source	Note
X. aff. *plittii*	fol.	*Trebouxia* clade A	3	Illumina	USA (California)	Smith et al. 2020	
X. subcumberlandia	fol.	*Trebouxia* clade A	up to 3	Illumina	USA (Utah, Colorado)	Smith et al. 2020	
Xanthoria parietina	fol.	*Trebouxia decolorans*	NA	Sanger (microsatelites)	Italy, Germany	Dal Grande et al. 2014	*T. decolorans* (different haplotypes)
X. parietina	fol.	*Trebouxia arboricola*, *T. decolorans*	NA	Sanger, RAPD	France, Switzerland	Nyati et al. 2013	*T. arboricola* only in the Swiss population
X. parietina	fol.	*Pleurococcus* sp., *Pseudotrebouxia* sp.	2	Cultivation, SEM	Netherlands, Norway, Sweden	Ott 1987a	

allow the detection of less abundant photobionts and thus to obtain accurate data on their real diversity within the thallus. Results may therefore be biased. In order to obtain accurate data on the presence of multiple species in the thalli, metabarcoding and culture isolations from all parts of the thallus are advisable and seem to become more and more affordable. Also, in the future, it could be suggested to study photobiont plurality using advanced visualization methods, such as fluorescence in situ hybridization (FISH). This method was, among others, used to study bacteria in lichens (e.g. Cardinale et al. 2008; Muggia et al. 2013a). By visualizing individual lineages, reliable information regarding the distribution of species/haplotypes within the thallus and their abundances could be obtained. A pitfall of this method is the poorly permeable cell wall of photobionts for fluorescent molecular probes. Additionally, autofluorescence of chloroplasts is problematic. However, this could be mitigated by choosing fluorescent probes outside the spectrum in which chlorophyll emits. Indeed, FISH has already been applied to photosynthetic organisms, primarily marine algae (e.g. Simon et al. 1995; Not et al. 2002), so protocols for lichen photobionts could be adapted and optimized from the existing ones.

Another topic deserving attention is the study of photobiont plurality in vegetative propagules of asexually reproducing lichens. If we were able to sequence or visualize photobionts in structures such as soredia, it would contribute to a better understanding of how multiple photobionts enter the thallus and spread. Casano et al. (2011) already discussed the possibility that the stability of the proportion in which *Trebouxia jamesii* and *T. lynnae* are present in the sorediate lichen *Ramalina farinacea* is not only due to the high selectivity of the mycobiont but also to its mode of reproduction. However, no comprehensive study focusing on the relationship between plurality and the reproductive strategy of lichens has been published yet.

In studies focusing on photobiont plurality, there is often a lack of data that elucidates the physiological differences between photobionts within the thallus. Such information has only been obtained for the photobionts isolated from the lichens *Ramalina farinacea* (Casano et al. 2011, 2015; Del Hoyo et al. 2011; Álvarez et al. 2012, 2015), *Buellia zoharyi*, and *Cladonia squamosa*, where differences in tolerance to various humidity and temperature levels within one thallus have been described (Noh et al. 2020). Performing similar comparative experiments on other lichen species with multiple photobionts would certainly help to assess the significance of each coexisting photobiont for symbiosis.

The occurrence and abundance of coexisting algae have also been described in terms of their distribution within the thallus (Mansournia et al. 2012; Moya et al. 2017;

Noh et al. 2020; Molins et al. 2021). Again, however, a vastly limited amount of data is available on this subject, and this data is probably, on top of that, influenced by the methodology used. Future studies could focus on whether individual photobionts within the thallus are segregated in different zones correspondingly to cyanobacteria and algae in the photosymbiodemes described in tripartite lichens, or whether they are rather intermixed within the thallus.

The results of this review suggest that the occurrence of photobiont plurality is, in fact, much more common than previously assumed. The number of studies focusing primarily on the topic of photobiont plurality is still relatively small. In most cases, photobiont plurality was discovered as a side effect of the main topic of the research such as the specificity and selectivity of mycobionts or the diversity of photobionts in general (e.g. Helms et al. 2001; Romeike et al. 2002; Muggia et al. 2014). Therefore, more studies focusing directly on the topic of multiple photobionts in a lichen thallus are needed to gain more comprehensive knowledge.

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Author contributions All authors contributed to this review. K.D. has performed the main literature research, the writing of the text, and table and figure preparation. J.S. has participated in the writing of the text and consultation. L.V. and L.M. have revised the text and given their notes on their personal data reviewed in this work. L.V. has performed analyses of the photobionts of *Biatora globulosa*. All authors read, corrected and approved the final manuscript.

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Data availability All data generated or analysed during this study (regarding *Biatora globulosa*) are included in this published article (and its supplementary information file). There are no further datasets to be provided.

Declarations

Competing interests The authors declare no competing interests.

Compliance with ethical standards All authors declare that there are no conflicts of interest, and no research has been performed on humans or animals.

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