Relative symbiont input and the lichen symbiotic outcome
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The term symbiosis was first used in biology to describe the ‘living together’ of fungi and algae in lichens. For much of the 20th century, the fungal partner was assumed to be invested with the ability to produce the lichen body plan in presence of a photosynthesizing partner. However, studies of fungal evolution have uncovered discordance between lichen symbiotic outcomes and genome evolution of the fungus. At the same time, evidence has emerged that the structurally important lichen cortex contains lichen-specific, single-celled microbes, suggesting it may function like a biofilm. Together, these observations suggest we may not have a complete overview of symbiotic interactions in lichens. Understanding phenotype development and evolution in lichens will require greater insight into fungal–fungus and fungal–bacterial interplay and the physical properties of the cortex.

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Introduction
Of all the cellular multikingdom symbioses, lichens maintain a unique status. In most symbioses, a single organism acts as structural scaffold: plant roots without mycorrhizae still resemble roots; a cicada without its bacterial endosymbiont still resembles a cicada, though it may be dead; a Euprymna squid without Vibrio bacteria is still a squid. Lichens however, which contain at least a filamentous fungus and a single-celled algal or cyanobacterial partner (photobiont), have no a priori scaffold: neither, in isolation, forms anything resembling a lichen, nor do any of their known relatives or ancestors [1]. They self-construct and self-replicate characteristic body plans, generation over respective symbiont generation. Lichens are basically interactome that you can roll around in your hand (Box 1).

How lichens achieve their characteristic thallus forms has been a major source of disagreement among lichen researchers since the discovery that they are a chimera [2]. At one end of the spectrum is the view, first espoused by Anton de Bary, that lichens are a mutualistic consortium with properties acquired only through cooperation of the symbionts [3]. At the other end is the view that the fungal symbiont parasitizes the photobiont and determines the lichen growth form [1,2]. According to this view, lichen traits are fungal traits, and each lichen has a distinct fungus which forms it (it was implicitly recognized early on that photobionts were promiscuous with respect to the lichens they occurred in [2]). Early experimenters sought to determine the roles of the symbionts by attempting to resynthesize the lichen in vitro, but found them recalcitrant to forming anything that looks like a natural lichen [4,5]. Nonetheless, incipient in vitro and wild symbioses always exhibit traits never produced in isolated fungal symbionts. To distinguish between symbiotic outcome and isolated symbiont, one of the most successful early experimenters began referring to the lichen and its fungal partner with separate nomenclature [6]. Systematists however pushed back. Citing contemporary practice but no actual biological evidence, they blocked the development of parallel nomenclature [7]. In 1950, the Code of Botanical Nomenclature was amended to anchor the name of the lichen to the fungus (Figure 1a). The move was not without criticism: the Italian mycologists Raffaele Ciferri and Ruggero Tomasselli warned that some fungi may occur in more than one lichen species [8], but struggled to find examples to prove this. Other workers similarly warned that traits such as secondary metabolite profiles occurred only in the symbiotic state [9]. Nonetheless the change to the Code and its inherent assumptions were upheld and since then whole lichens have technically remained nameless [10,11**].

It is hard to overstate the effect that the definition of lichens as fungi has had on the study of the lichen symbiosis. Because naming something is everything in a taxonomy-driven discipline, the rule change incentivized the study of the fungal partner and led to much work on its sexual reproduction and molecular evolution. Today, most lichen biologists assume a single symbiont — one fungus — is invested in its DNA with the ability to generate the lichen body plan in presence of
Box 1 The origins and diversity of lichen symbioses.

Fungi enter into several important nutritional symbioses with plants and animals. The two main kinds of symbiosis, in terms of the numbers of species involved, are lichens (approaching 20,000 species [68]) and mycorrhizal associations with vascular plants (about 50,000 species [69]). Lichens are a diverse group of fungal-algal symbioses that evolved multiple times independently within both large divisions of Fungi, the Ascomycota and Basidiomycota, together with photosynthesizing partners from a wide range of chlorophytes and cyanobacteria. Together, two or more partners form a thallus, in which one or more fungi are quantitatively dominant over the photosynthesizing partner(s).

Lichens are thought to have arisen in early terrestrial life from a biofilm-like association of fungi, cyanobacteria and other microbes [70]. A majority of lichen species, the microlichens, form thalli closely molded to the shape of their substrate. The lichen association also gave rise on multiple occasions to novel, three-dimensional architectures adapted to optimize photosynthesis, unique for a fungal-dominated organism [71**]. These ‘macrolichens’ include foliose lichens, which form dorsiventral structures superficially similar to a plant leaf, and fruticose lichens, which encompass both phototropic, radially symmetrical, shrub-like lichens, as well as hair lichens, which are composed of thin thallus filaments that hang from tree branches. Some lichens, such as members of the species-rich cosmopolitan genus Cladonia, are dimorphic, with leaf-like basal squamules bearing complex, tower-like or shrub-like structures called podetia. How lichen symbionts went from inhabiting biofilms to acquiring self-replicating architectures is one of the unsolved mysteries of lichen evolution. To achieve a specific three-dimensional motif, fungal and algal cells form differentiated meristem-like regions [71**], termed pseudoparenchym, in which rapidly dividing fungal and algal cells are aggregated. In branching and anastomosing lichen thalli, the location and shape of these aggregations determine branching pattern, and increasing thallus size is supported in turn by diffuse or intercalary growth [71**,72]. However, as discussed in this review, it is unlikely thallid would maintain rigidity and their characteristic shapes without an extracellular polysaccharide layer that cements the fungal ‘wadding’ in place, with tension and flexibility of its own.

Studies of the lichen species as fungal species

The first published DNA sequences of lichen fungi were used to demonstrate that two different lichens could be formed by the same fungus [13]. However, this was considered to be a curious induced by photobiont switching, and subsequent studies showed similar lichens did indeed cluster according to their fungal DNA. As more data became available, questions shifted from broad-scale relationships to delimitation of individual lichen species. Sampling intensity and phylogenetic resolution has varied widely, but broadly speaking, three types of results have been obtained (Figure 1b). For the purpose of discussion, lichen species here refer to long-standing morphological or chemical circumscriptions, and fungal species refer to entities delimited from multilocus molecular data.

(Result 1) Lichen phenotypes are reflected in fungal gene evolution. This kind of result has been taken as support of fungal concordance [14]. It is frequently obtained for crust lichens, which form only thin, substrate-hugging body plans and are predominantly classified according to fungal traits. However, species-level concordance has also been reported for lichens with three-dimensional architecture (macrolichens) such as Cladonia [15], often alongside reports of non-concordance for other species (see below).

(Result 2) More fungal genetic species can be distinguished than there are matching lichen phenotypes. In many cases, the sequence data are only part of a process of discovery of traits with which the species can later be distinguished [16–18]. In others, no distinguishing characters are found and the resulting taxa are called cryptic species [19].

(Result 3) More lichen phenotypes exist than are reflected in fungal gene evolution. This phenomenon—which I will call phantom phenotypes—is most common among macrolichens. In some cases, lichen species that have been distinguished since 1810 have been found to be formed by the same fungal species, despite differing in secondary chemistry, ecology, geographic range and thallus traits [20–25]. Sometimes the phylogenetic pattern is messy, lending no support for a connection of evolution to phenotypes [26–29].

For many lichen biologists, the third set of results has been the least intuitive, because it requires downweighting or disregarding well-documented traits, ecologies and geographic ranges. Though most recognizable at the leading edge of evolutionary differentiation—speciation—discordance with lichen phenotypes is evident throughout fungal evolution in the form of wholesale body plan changes between sister lineages [30,31]. One by one, workers have reassessed ‘taxonomic value’ of anatomical traits [32] and secondary metabolite chemistry [27] in favor of traits that match clades [33,34]. Not accepting that obvious phenotypes are unsupported by fungal phylogenies, at least one group has flipped the script, searching for loci that form clades matching the phenotypes [33]. In recent years, many workers have begun referring to lichen-forming fungi as opposed to lichens, but still map traits that only exist in the symbiotic state to a fungal tree [34,35]. The morphological discordance crisis is not subtle: it recently led Lumbsch and Leavitt to ask if the era of lichen morphology is over altogether, using the title ‘goodbye morphology?’ [36**].

Applying new tools to the lichen symbiosis

When the Code of Nomenclature was amended to define the lichen as identical with its sole fungus, the structure of
DNA had yet to be elucidated, and ribosomes had not even been discovered. Ciferri and Tomaselli would doubtless have found the preponderance of lichen species that do not match evidence from fungal DNA sequences useful to their argument that morphologically distinct lichens can share one fungal species. Taxonomy and nomenclature aside, documented patterns of discordance raise two fundamental questions about the nature of the lichen symbiosis. Why are traits long thought to be adaptive for the lichen symbiosis not recorded in the genome evolution of the fungus? And what causes these traits?

It may help to first refresh some of the built-in assumptions of lichen fungal DNA studies. As in the study of molecular evolution of other organisms, the application of DNA sequencing to lichen fungi was based on the polymerase chain reaction (PCR). In the early days of PCR, primers were built from a small pool of known sequences, typically of ribosomal DNA. Subsequent, newly obtained sequences were checked against previously obtained sequences, in a kind of validation loop, that built the databases we have today. Previous to whole genome sequencing, this validation loop began anew with every new locus sequenced. Few researchers have questioned the emerging sequence-scape. That said, it is known that other fungi can occur in lichens, either as parasites or as ‘asymptomatic’, ‘endolichenic’ fungi [37**], and mistakenly amplified DNA sequences are sometimes deposited under the name of the expected lichen fungus [38*].
Recent studies have confirmed high diversities of fungal DNA sequences from lichen thalli and even fruiting bodies, as well as multiple lichen-forming fungi in one lichen [39,40]. Although it is probable that a majority of DNA sequences in public databases were amplified from the dominant fungus, the cellular source of this DNA is almost never unambiguously identified.

Paralleling developments in the study of microbial diversity [41], the application of random shotgun genomic sequencing technologies to lichens can expand the census of participating symbionts. Together with my collaborators, I studied the cause of phantom phenotypes in the species long recognized as Bryoria fremontii and B. tortuosa. B. tortuosa is enriched with a toxic substance, vulpinic acid, forms coarser, more twisted thalli, and occurs in drier forests and further south than B. fremontii, yet the two have been found to contain identical fungal and algal partners [42,43]. Using whole lichen metatranscriptomes, we asked whether differential gene expression in these two species could explain their differences or whether the conclusion that they were identical was simply a result of lack of phylogenetic resolution. We found no evidence to support either hypothesis. Instead, we found that both lichens contained hitherto overlooked yeasts from a previously unknown order of basidiomycetes, the Cyphobasidiales [44**]. The yeasts, which were localized to the lichen cortex layer, were about 10 times more abundant in B. tortuosa than in B. fremontii and occur in close proximity to concentrations of vulpinic acid crystals, though it is not known if they produce them. The discovery of these yeasts does not seem to be luck of the draw: we found lichen species-specific strains of Cyphobasidiales in most macrolichens we surveyed. Nor do they always occur alone: in the macrolichen Letharia vulpina, Cyphobasidiales co-occur with a yeast-forming species of Tremella in about 95% of thalli tested in a global survey [45].

Our paper was only the latest to report microbes associated with the lichen cortex. Gabriele Berg, Martin Grube and their colleagues have similarly found structured communities of lichen-specific heterotrophic bacteria, especially alphaproteobacteria [46–54,55**]. The cortex is a thin, stratified zone of extracellular polysaccharides [56]. Bacteria tend to be detected in the outermost layer of this zone; basidiomycete yeasts, when present, occur more deeply embedded, not visible from the outside; and in the innermost zone, branched tips of fungal hyphae from the thallus cavity are cemented into the layer [57,58]. The resulting shell (in some species, an analogous internal layer called the stereome) is pliable when moist and rigid when dry (Figure 2), and determines the shape of most architecturally complex lichen body plans [12]. It is also critical to water uptake and maintaining optimum physiological conditions for the photobiont [1]. It is not clear what triggers the secretion of the extracellular matrix, but it is rarely formed in sterile resynthesis experiments [1]. However, a wide spectrum of biological activity has been traced to both bacteria [53,54,55**,59,60] and unidentified secondary fungi [59,61**] and visually localized to the cortex in MALDI-TOF imaging mass spectrometry of Peltigera hymenina [61**]. The molecular and biophysical properties and diversity of cortex types

![Figure 2](image-url)
are poorly understood, but in sum, cortex properties known thus far are not inconsistent with the attributes of a biofilm.

Outlook

Biofilms and the textbook fungal–algal lichen are both mosaics of unrelated organisms that exchange genetic goods and services, resulting in higher order units that can become the object of natural selection in their own right [62–64]. It may not be too soon to suggest that at least some macrolichens emerged when a fungal–algal matrix began to interact in three dimensions with a biofilm. Confusion arises when different lichens are expected to be analogous. ‘Dust lichens’ of the genus *Lepraria*, for instance, possess no visible biofilm or mucilaginous layer [65]. Crust lichens can possess bacterial biofilms [66] though it is unknown how many also contain yeasts. Over all the body plans formed, one of the challenges for lichen evolutionists will be to ascertain relative symbiont input: to what extent changes in symbiotic outcome relate to changes in proportional input to the goods and services pool over time, or as symbionts come and go (Figure 1c).

The intensive study of the assumed sole fungus in lichens has provided an invaluable roadmap for hypothesis-testing. Phantom phenotypes, cryptic species and evolution-ary saltations in lichen body plans are all promising systems to revisit with shotgun DNA-based, RNA-based, protein-based and metabolite-based approaches. It will be key however to sample with enough depth to capture all players, and to identify the cellular source of DNA. However, even if phenotype modification via a cortical biofilm can be demonstrated, it does not offer a model for why the evolution of the core hyphal fungus should not reflect lichen adaptive traits, or vice versa. It is hard to escape the impression that processes of natural selection for the whole lichen may not be identical to those of the isolated symbionts. If we want to understand these processes, we will have to work with what lichen biology presents us, unfettered by assumptions and rules made in another era.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as: • of special interest •• of outstanding interest

3. de Bary A: *Die Erscheinung der Symbiose*. Verlag Karl Trübner; 1879.

Though neither a research paper nor strictly speaking a review, this essay and the eleven others in the series published between 2008 and 2012 contain extensive interrogation of 20th century assumptions in lichen evolutionary biology that cannot be found elsewhere.


38. Lücking R, Moncada B: Dismantling Marchandiomphalina into *Agonimia* (Verrucariaceae) and Lawreymyces gen. nov. (Corticiaceae): setting a precedent to the formal recognition of thousands of vouchers of fungi based on type sequences. *Fungal Diversity* 2017, 84:119-139.

The authors document two different fungi in one lichen that was first classified as being formed by a hyphal basidiomycete, then by a hyphal ascomycete. It is “Exhibit A” of the kinds of challenges faced when trying to interpret multispecies lichen symbioses without knowing the total number of players present, or the cellular source of the obtained fungal DNA.


Using a shotgun sequencing strategy, we found previously unknown Cyphobasidialean yeasts in over 45 macrolichens genera, and connected these genomic sequences to fungal cells using whole lichen fluorescent microscopy. These results suggest that the assumption that a single constituent fungus is present in the lichen needs to be revisited.


An excellent review of work on lichen-associated bacteria including the history of their study, their taxonomic breakdown and abundance and evidence as to their functions.


The authors studied the distribution of organic molecules within a cross-section of a lichen thallus, mapping orthologous molecules independent of a preconceived census of players.


An excellent, beautifully illustrated and still topical review of the emergence of plant-like morphology and growth patterns in lichen thalli.