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Basidiomycete yeasts in the cortex of ascomycete macrolichens

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For over 140 years, lichens have been regarded as a symbiosis between a single fungus, usually an ascomycete, and a photosynthesizing partner. Other fungi have long been known to occur as occasional parasites or endophytes, but the one lichen–one fungus paradigm has seldom been questioned. Here we show that many common lichens are composed of the known ascomycete, the photosynthesizing partner, and, unexpectedly, specific basidiomycete yeasts. These yeasts are embedded in the cortex, and their abundance correlates with previously unexplained variations in phenotype. Basidiomycete lineages maintain close associations with specific lichen species over large geographical distances and have been found on six continents. The structurally important lichen cortex, long treated as a zone of differentiated ascomycete cells, appears to consistently contain two unrelated fungi.

Most definitions of the lichen symbiosis emphasize its dual nature: the mutualism of a single fungus and single photosynthesizing symbiont, occasionally supplemented by a second photosynthesizing symbiont in modified structures (*1*–*4*). Together, these organisms form stratified, often leafy or shrubby body plans (thalli) that resemble none of the symbionts in isolation, a feature thought to be unique among symbioses (*1*). Attempts to synthesize lichen thalli from the accepted two components in axenic conditions, however, have seldom produced structures that resemble natural thalli (*5*, *6*). Notably, a critical structural feature of stratified lichens, the cortex, typically remains rudimentary in laboratory-generated symbioses (*5*). Recently, it has been suggested that microbial players, especially bacteria, may play a role in forming complete, functioning lichen thalli (*7*). However, although culturing and amplicon sequencing have revealed rich communities of microbes (*7*, *8*), including other fungi (*8*–*10*), no new stably associated symbiotic partners have been found.

The recalcitrance of lichens to form thalli in vitro means that characterizing symbiont gene activity (e.g., through transcriptomics) requires an approach that works with natural thalli. We used metatranscriptomics to better understand the factors involved in forming two macrolichen symbioses, *Bryoria fremontii* and *B. tortuosa*. These two species have been distinguished for 90 years by the thallus-wide production of the toxic substance vulpinic acid in *B. tortuosa*, causing it to appear yellowish, in contrast to *B. fremontii*, which is dark brown (*11*). Recent phylogenetic analyses have failed to detect any fixed sequence differences between the two species in either the mycobiont (Ascomycota, Lecanoromycetes, *Bryoria*) or the photobiont (Viridiplantae, *Trebouxia simplex*) when considering four and two loci, respectively (*12*, *13*). We hypothesized that differential gene expression might account for the increased production of vulpinic acid in *B. tortuosa*.

We first selected 15 thalli (six from *B. fremontii* and nine from *B. tortuosa*, all free from visible parasitic infection) from sites across western Montana, USA, for mRNA transcriptome sequencing. An initial transcriptome-wide analysis of single nucleotide polymorphisms (SNPs) for Ascomycota and Viridiplantae transcript subsets showed no correlation between genotype and phenotype in *B. fremontii* and *B. tortuosa*, confirming previous results (*12*, *13*) (Fig. 1, A and B). Next, we estimated transcript abundances by mapping raw reads back to a single, pooled metatranscriptome assembly and binning by taxon. Restricting our analyses to Ascomycota and Viridiplantae revealed little differential transcript abundance associated with phenotype (Fig. 1, C and E). Taken together, these analyses confirm previous conclusions that the two lichen species are nomenclatural synonyms (*12*) but still provide no explanation for the underlying phenotypes (which we shall continue to refer to by their species names for convenience). However, by expanding the taxonomic range to consider all Fungi, we found 506 contigs with significantly higher abundances in vulpinic acid–rich *B. tortuosa* thalli. A majority of these contigs were annotated as Basidiomycota (Fig. 1D). These data suggested that a previously unrecognized basidiomycete was present in thalli of both species but was more abundant whenever vulpinic acid was present in large amounts.

We next sought to determine whether this uncharacterized basidiomycete was specific to the studied *Bryoria* species or could be found in other lichens. From metatranscriptome contigs containing ribosomal RNA (rRNA) basidiomycete sequences, we designed specific primers for ribosomal DNA [rDNA; 18*S*, internal transcribed spacer (ITS), and D1D2 domains of 28*S*) to screen lichens growing physically adjacent to *Bryoria* in Montana forests. Each assayed lichen species carried a genetically distinct strain of the basidiomycete, indicating a high degree of specificity. Furthermore, we found that *Letharia vulpina*, a common lichen species growing intermixed with *Bryoria*, maintained basidiomycete genotypes that were distinct from those in *Bryoria*, not only in Montana but also in northern Europe (fig. S1). When assaying for the basidiomycete across the seven main radiations of macrolichens in the class Lecanoromycetes, we found related basidiomycete lineages associated with 52 lichen genera from six continents, including in 42 of 56 sampled genera of the family Parmeliaceae (fig. S2). As a whole, these data indicate that basidiomycete fungi are ubiquitous and global associates of the world's most speciose radiation (*14*) of macrolichens.

To place the basidiomycete lineages in a phylogenetic context, we generated a 349-locus phylogenomic tree by using gene sequences inferred from our transcriptome data set and other available genomes (table S1). This analysis placed the basidiomycete as sister to *Cystobasidium minutum* (class Cystobasidiomycetes, subphylum Pucciniomycotina) with high support (Fig. 2A). The only previously known lichen-associated members of Cystobasidiomycetes are two species of *Cyphobasidium*, which is hypothesized to cause galls on species of Parmeliaceae (*15*). When incorporated into a broader sample of published cystobasidiomycete rDNA sequence data (*16*–*18*), the majority of our lichen-derived sequences form a strongly supported monophyletic clade with *Cyphobasidium* (Fig. 2B). Using current classification criteria (*18*), the lichen-associated lineages would include numerous new family-level lineages, and we recognize this set of subclades as the new order Cyphobasidiales (*19*). Applying a relaxed molecular clock to our phylogenomic tree (Fig. 2A) shows the *Cystobasidium*-*Cyphobasidium* split occurring around the same time as the origin of three of the main groups of lecanoromycete macrolichens in which Cyphobasidiales species occur, suggesting a long, shared evolutionary history. Two fossil calibrations place this split at around 200 million years before the present (figs. S4 and S5).

Our initial microscopic imaging failed to reveal any cells that we could assign to Basidiomycetes with certainty. Furthermore, attempts to culture the basidiomycete from fresh thalli were unsuccessful. We therefore developed protocols for fluorescent in situ hybridization (FISH) targeting specific ascomycete and cystobasidiomycete rRNA sequences. Cystobasidiomycete-specific FISH probes unambiguously hybridized round, ~3- to 4-μm-diameter cells embedded in the peripheral cortex of both *B. fremontii* and *B. tortuosa* (Fig. 3 and movie S1). Consistent with the transcript abundance data, these cells were more abundant in thalli of *B. tortuosa* (Fig. 3), where they were embedded in secondary metabolite residues (movie S1). Imaging of other lichen species likewise revealed cells of similar morphology in the peripheral cortex (fig. S6). Composite three-dimensional FISH images from *B. capillaris* show the cells occurring in a zone exterior to the lecanoromycete (Fig. 4 and movie S2) and embedded in polysaccharides (Fig. 4C), explaining why these cells are not observed in scanning electron microscopy (Fig. 4A). In some species, such as *L. vulpina*, the abundance of hybridized living cells was low, but selective removal of the polysaccharide layer through washing revealed high densities of collapsed, apparently dead cells within the cortex (fig. S7). These dead cells may explain the paucity of the FISH signal in some experiments. The mononucleate single cells (fig. S8C), evidence of budding, and absence of hyphae or clamp connections are consistent with an anamorphic or yeast state in Cystobasidiomycetes. FISH imaging of *Cyphobasidium* galls on the lichen *Hypogymnia physodes*, obtained from Norway, confirmed the link to the sexual or teleomorphic state (fig. S8), which appears to develop rarely (*15*). These data suggest that the gall-inducing form of *Cyphobasidium* completes its life cycle entirely within lichens.

It is remarkable that *Cyphobasidium* yeasts have evaded detection in lichens until now, despite decades of molecular and microscopic studies of the Parmeliaceae (*20*–*22*). It seems likely that the failure to detect *Cyphobasidium* in both Sanger and amplicon sequencing studies (*8*) is due to multi-template polymerase chain reaction bias. The most widespread clade of *Cyphobasidium* possesses a 595–base pair group I intron inserted downstream of the primer binding site ITS1F, doubling the template length of ITS, a popular fungal barcode (*23*). This, combined with low background abundance, can push a template below detection thresholds (*24*). Also, we cannot rule out that *Cyphobasidium* yeasts have actually been sequenced and discarded as presumed contaminants.

The lichen cortex layer has long been considered to be key for structural stabilization of macrolichens, as well as for water and nutrient transfer into the thallus interior (*6*, *25*). Most macrolichens possess a basic two-layer cortex scheme consisting of conglutinated internal hyphae and a thin, polysaccharide-rich peripheral layer (*25*). However, the internal cellular structure is not uniform across lichens (*26*), and the composition of extracellular polysaccharides is poorly known (*27*). In *Bryoria*, the layer in which *Cyphobasidium* yeasts occur has not been recognized as distinct from the cortex (*11*), although in other parmelioid lichens, a seemingly homologous layer has sometimes been referred to as the "epicortex" (*20*). The discovery of ubiquitous yeasts embedded in the cortex raises the prospect that more than one fungus may be involved in its construction, and it could explain why lichens synthesized in vitro from axenically grown ascomycete and algal cultures develop only rudimentary cortex layers (*5*).

In many lichens, the peripheral cortex layer in which *Cyphobasidium* yeasts are embedded is enriched with specific secondary metabolites (*25*), the production of which often does not correlate with the lecanoromycete phylogeny (*28*). The assumption that these substances are exclusively synthesized by the lecanoromycete must now be considered untested. In *B. fremontii,* differential transcript and cell abundance data, along with physical adjacency to crystalline residues, implicate *Cyphobasidium* in the production of vulpinic acid, either directly or by inducing its synthesis by the lecanoromycete. Confirming a link by using transcriptome or genome data is impossible until the enzymatic synthesis pathway of vulpinic acid is described. However, related pulvinic acid derivatives are synthesized by other members of Basidiomycota (*29*).

The assumption that stratified lichens are constructed by a single fungus with differentiated cell types is so central to the definition of the lichen symbiosis that it has been codified into lichen nomenclature (*30*). This definition has brought order to the field, but may also have constrained it by forcing untested assumptions about the true nature of the symbiosis. We suggest that the discovery of *Cyphobasidium* yeasts should change expectations about the potential diversity and ubiquity of organisms involved in one of the oldest known and most recognizable symbioses in science.

REFERENCES AND NOTES

1. A. De Bary, *Die Erscheinung der Symbiose* (Verlag Karl Trübner, 1879).
2. A. Gargas, P. T. DePriest, M. Grube, A. Tehler, Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science* **268**, 1492–1495 (1995). Medline doi:10.1126/science.7770775
3. F. Lutzoni, M. Pagel, V. Reeb, Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* **411**, 937–940 (2001). Medline doi:10.1038/35082053
4. D. L. Hawksworth, The variety of fungal-algal symbioses, their evolutionary significance, and the nature of lichens. *Bot. J. Linn. Soc.* **96**, 3–20 (1988). doi:10.1111/j.1095-8339.1988.tb00623.x
5. V. Ahmadjian, *The Lichen Symbiosis* (John Wiley & Sons, 1993).
6. R. Honegger, Developmental biology of lichens. *New Phytol.* **125**, 659–677 (1993). doi:10.1111/j.1469-8137.1993.tb03916.x
7. I. A. Aschenbrenner, T. Cernava, G. Berg, M. Grube, Understanding microbial multi-species symbioses. *Front. Microbiol.* **7**, 180 (2016). Medline doi:10.3389/fmicb.2016.00180
8. S. T. Bates, D. Berg-Lyons, C. L. Lauber, W. A. Walters, R. Knight, N. Fierer, A preliminary survey of lichen associated eukaryotes using pyrosequencing. *Lichenologist* **44**, 137–146 (2012). doi:10.1017/S0024282911000648
9. O. Petrini, U. Hake, M. M. Dreyfuss, An analysis of fungal communities isolated from fruticose lichens. *Mycologia* **82**, 444–451 (1990). doi:10.2307/3760015
10. J. M. U'Ren, F. Lutzoni, J. Miadlikowska, A. E. Arnold, Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. *Microb. Ecol.* **60**, 340–353 (2010). Medline doi:10.1007/s00248-010-9698-2
11. I. M. Brodo, D. L. Hawksworth, *Alectoria* and allied genera in North America. *Opera Bot.* **42**, 1–164 (1977).
12. S. Velmala, L. Myllys, P. Halonen, T. Goward, T. Ahti, Molecular data show that *Bryoria fremontii* and *B. tortuosa* (Parmeliaceae) are conspecific. *Lichenologist* **41**, 231–242 (2009). doi:10.1017/S0024282909008573
13. H. Lindgren, S. Velmala, F. Högnabba, T. Goward, H. Holien, L. Myllys, High fungal selectivity for algal symbionts in the genus *Bryoria*. *Lichenologist* **46**, 681–695 (2014). doi:10.1017/S0024282914000279
14. G. Amo de Paz, P. Cubas, P. K. Divakar, H. T. Lumbsch, A. Crespo, Origin and diversification of major clades in parmelioid lichens (Parmeliaceae, Ascomycota) during the Paleogene inferred by Bayesian analysis. *PLOS ONE* **6**, e28161 (2011). Medline doi:10.1371/journal.pone.0028161
15. A. M. Millanes, P. Diederich, M. Wedin, *Cyphobasidium* gen. nov., a new lichen-inhabiting lineage in the Cystobasidiomycetes (Pucciniomycotina, Basidiomycota, Fungi). *Fungal Biol.* 10.1016/j.funbio.2015.12.003 (2015).
16. M. C. Aime, P. B. Matheny, D. A. Henk, E. M. Frieders, R. H. Nilsson, M. Piepenbring, D. J. McLaughlin, L. J. Szabo, D. Begerow, J. P. Sampaio, R. Bauer, M. Weiss, F. Oberwinkler, D. Hibbett, An overview of the higher level classification of Pucciniomycotina based on combined analyses of nuclear large and small subunit rDNA sequences. *Mycologia* **98**, 896–905 (2006). Medline doi:10.3852/mycologia.98.6.896
17. Q.-M. Wang, M. Groenewald, M. Takashima, B. Theelen, P.-J. Han, X.-Z. Liu, T. Boekhout, F.-Y. Bai, Phylogeny of yeasts and related filamentous fungi within Pucciniomycotina determined from multigene sequence analyses. *Stud. Mycol.* **81**, 27–53 (2015). Medline doi:10.1016/j.simyco.2015.08.002
18. Q.-M. Wang, A. M. Yurkov, M. Göker, H. T. Lumbsch, S. D. Leavitt, M. Groenewald, B. Theelen, X.-Z. Liu, T. Boekhout, F.-Y. Bai, Phylogenetic classification of yeasts and related taxa within Pucciniomycotina. *Stud. Mycol.* **81**, 149–189 (2015). Medline doi:10.1016/j.simyco.2015.12.002
19. Cyphobasidiales T. Sprib. & H. Mayrhofer, ord. nov. (MB 816120); diagnosis is the same as type family Cyphobasidiaceae T. Sprib. & H. Mayrhofer, fam. nov. (MB 816119); embedded in lichen thalli; teleomorph filamentous, rarely observed; when fertile, basidia develop thick-walled probasidium and thin-walled, cylindrical meiosporangium; anamorph is the prevalent known form, consisting of budding yeast with round, thin-walled cells, 2.5 to 4.5 μm in diameter, embedded in the upper cortex of lichens, especially Parmeliaceae; exogenous compound utilization not characterized; cell wall constituents unknown. Type genus, *Cyphobasidium* Millanes, Diederich, and Wedin, Fungal Biology doi:10.1016/j.funbio.2015.12.003, p. 4 (2015).
20. M. E. Hale Jr., Pseudocyphellae and pored epicortex in the Parmeliaceae: Their delimitation and evolutionary significance. *Lichenologist* **13**, 1–10 (1981). doi:10.1017/S0024282981000030

21. A. Thell, A. Crespo, P. K. Divakar, I. Kärnefelt, S. D. Leavitt, H. T. Lumbsch, M. R. D. Seaward, A review of the lichen family Parmeliaceae – history, phylogeny and current taxonomy. *Nord. J. Bot.* **30**, 641–664 (2012). doi:10.1111/j.1756-1051.2012.00008.x
22. A. Crespo, F. Kauff, P. K. Divakar, R. del Prado, S. Pérez-Ortega, G. Amo de Paz, Z. Ferencova, O. Blanco, B. Roca-Valiente, J. Núñez-Zapata, P. Cubas, A. Argüello, J. A. Elix, T. L. Esslinger, D. L. Hawksworth, A. Millanes, C. Molina, M. Wedin, T. Ahti, A. Aptroot, E. Barreno, F. Bungartz, S. Calvelo, M. Candan, M. Cole, D. Ertz, B. Goffinet, L. Lindblom, R. Lücking, F. Lutzoni, J.-E. Mattsson, M. I. Messuti, J. Miadlikowska, M. Piercey-Normore, V. J. Rico, H. J. M. Sipman, I. Schmitt, T. Spribille, A. Thell, G. Thor, D. K. Upreti, H. T. Lumbsch, Phylogenetic generic classification of parmelioid lichens (Parmeliaceae, Ascomycota) based on molecular, morphological and chemical evidence. *Taxon* **59**, 1735–1753 (2010).
23. C. L. Schoch, K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chen, E. Bolchacova, K. Voigt, P. W. Crous, A. N. Miller, M. J. Wingfield, M. C. Aime, K.-D. An, F.-Y. Bai, R. W. Barreto, D. Begerow, M.-J. Bergeron, M. Blackwell, T. Boekhout, M. Bogale, N. Boonyuen, A. R. Burgaz, B. Buyck, L. Cai, Q. Cai, G. Cardinali, P. Chaverri, B. J. Coppins, A. Crespo, P. Cubas, C. Cummings, U. Damm, Z. W. de Beer, G. S. de Hoog, R. Del-Prado, B. Dentinger, J. Dieguez-Uribeondo, P. K. Divakar, B. Douglas, M. Duenas, T. A. Duong, U. Eberhardt, J. E. Edwards, M. S. Elshahed, K. Fliegerova, M. Furtado, M. A. Garcia, Z.-W. Ge, G. W. Griffith, K. Griffiths, J. Z. Groenewald, M. Groenewald, M. Grube, M. Gryzenhout, L.-D. Guo, F. Hagen, S. Hambleton, R. C. Hamelin, K. Hansen, P. Harrold, G. Heller, C. Herrera, K. Hirayama, Y. Hirooka, H.-M. Ho, K. Hoffmann, V. Hofstetter, F. Hognabba, P. M. Hollingsworth, S.-B. Hong, K. Hosaka, J. Houbraken, K. Hughes, S. Huhtinen, K. D. Hyde, T. James, E. M. Johnson, J. E. Johnson, P. R. Johnston, E. B. G. Jones, L. J. Kelly, P. M. Kirk, D. G. Knapp, U. Koljalg, G. M. Kovacs, C. P. Kurtzman, S. Landvik, S. D. Leavitt, A. S. Liggenstoffer, K. Liimatainen, L. Lombard, J. J. Luangsa-ard, H. T. Lumbsch, H. Maganti, S. S. N. Maharachchikumbura, M. P. Martin, T. W. May, A. R. McTaggart, A. S. Methven, W. Meyer, J.-M. Moncalvo, S. Mongkolsamrit, L. G. Nagy, R. H. Nilsson, T. Niskanen, I. Nyilasi, G. Okada, I. Okane, I. Olariaga, J. Otte, T. Papp, D. Park, T. Petkovits, R. Pino-Bodas, W. Quaedvlieg, H. A. Raja, D. Redecker, T. L. Rintoul, C. Ruibal, J. M. Sarmiento-Ramirez, I. Schmitt, A. Schussler, C. Shearer, K. Sotome, F. O. P. Stefani, S. Stenroos, B. Stielow, H. Stockinger, S. Suetrong, S.-O. Suh, G.-H. Sung, M. Suzuki, K. Tanaka, L. Tedersoo, M. T. Telleria, E. Tretter, W. A. Untereiner, H. Urbina, C. Vagvolgyi, A. Vialle, T. D. Vu, G. Walther, Q.-M. Wang, Y. Wang, B. S. Weir, M. Weiss, M. M. White, J. Xu, R. Yahr, Z. L. Yang, A. Yurkov, J.-C. Zamora, N. Zhang, W.-Y. Zhuang, D. Schindel, Fungal Barcoding Consortium, Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 6241–6246 (2012). Medline doi:10.1073/pnas.1117018109
24. E. Kalle, M. Kubista, C. Rensing, Multi-template polymerase chain reaction. *Biomol. Detect. Quantif.* **2**, 11–29 (2014). doi:10.1016/j.bdq.2014.11.002
25. R. Honegger, "The symbiotic phenotype of lichen-forming ascomycetes and their endo- and epibionts," in *Fungal Associations*, B. Hock, Ed., vol. IX of *The Mycota*, K. Esser, Ed. (Springer, ed. 2, 2012), pp. 287–339.
26. D. Anglesea, C. Veltkamp, G. H. Greenhalgh, The upper cortex of *Parmelia saxatilis* and other lichen thalli. *Lichenologist* **14**, 29–38 (1982). doi:10.1017/S0024282982000048
27. E. S. Olafsdottir, K. Ingólfsdottir, Polysaccharides from lichens: Structural characteristics and biological activity. *Planta Med.* **67**, 199–208 (2001). Medline doi:10.1055/s-2001-12012
28. C. G. Boluda, V. J. Rico, A. Crespo, P. K. Divakar, D. L. Hawksworth, Molecular sequence data from populations of *Bryoria fuscescens* s. lat. in the mountains of central Spain indicates a mismatch between haplotypes and chemotypes. *Lichenologist* **47**, 279–286 (2015). doi:10.1017/S0024282915000274
29. N. Arnold, W. Steglich, H. Besl, Zum vorkommen von pulvinsäure-derivaten in der gattung *Scleroderma*. *Z. Mykol.* **62**, 69–73 (1996).
30. W. L. Culberson, Proposed changes in the international code governing the nomenclature of lichens. *Taxon* **10**, 161–165 (1961). doi:10.2307/1216004
31. M. G. Grabherr, B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B. W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman, A. Regev, Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **29**, 644–652 (2011). Medline doi:10.1038/nbt.1883
32. B. J. Haas, A. Papanicolaou, M. Yassour, M. Grabherr, P. D. Blood, J. Bowden, M. B. Couger, D. Eccles, B. Li, M. Lieber, M. D. Macmanes, M. Ott, J. Orvis, N. Pochet, F. Strozzi, N. Weeks, R. Westerman, T. William, C. N. Dewey, R. Henschel, R. D. Leduc, N. Friedman, A. Regev, De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* **8**, 1494–1512 (2013). Medline doi:10.1038/nprot.2013.084
33. C. Stubben, "genomes: Genome sequencing project metadata," R package version 2.16.1 (2015); www.bioconductor.org/packages/release/bioc/html/genomes.html.
34. A. Dobin, C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, T. R. Gingeras, STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013). Medline doi:10.1093/bioinformatics/bts635
35. M. D. Robinson, A. Oshlack, A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol.* **11**, R25 (2010). Medline doi:10.1186/gb-2010-11-3-r25
36. M. D. Robinson, D. J. McCarthy, G. K. Smyth, edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2010). Medline doi:10.1093/bioinformatics/btp616
37. A. McKenna, M. Hanna, E. Banks, A. Sivachenko, K. Cibulskis, A. Kernytsky, K. Garimella, D. Altshuler, S. Gabriel, M. Daly, M. A. DePristo, The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* **20**, 1297–1303 (2010). Medline doi:10.1101/gr.107524.110
38. P. De Wit, M. H. Pespeni, J. T. Ladner, D. J. Barshis, F. Seneca, H. Jaris, N. O. Therkildsen, M. Morikawa, S. R. Palumbi, The simple fool's guide to population genomics via RNA-Seq: An introduction to high-throughput sequencing data analysis. *Mol. Ecol. Resour.* **12**, 1058–1067 (2012). Medline doi:10.1111/1755-0998.12003
39. L. Li, C. J. Stoeckert Jr., D. S. Roos, OrthoMCL: Identification of ortholog groups for eukaryotic genomes. *Genome Res.* **13**, 2178–2189 (2003). Medline doi:10.1101/gr.1224503
40. S. van Dongen, "Graph clustering by flow simulation," thesis, University of Utrecht (2000); www.library.uu.nl/digiarchief/dip/diss/1895620/full.pdf.
41. K. Katoh, D. M. Standley, MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772–780 (2013). Medline doi:10.1093/molbev/mst010
42. J. Castresana, Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* **17**, 540–552 (2000). Medline doi:10.1093/oxfordjournals.molbev.a026334
43. G. Talavera, J. Castresana, Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst. Biol.* **56**, 564–577 (2007). Medline doi:10.1080/10635150701472164
44. A. Stamatakis, RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313 (2014). Medline doi:10.1093/bioinformatics/btu033
45. D. H. Huson, C. Scornavacca, Dendroscope 3: An interactive tool for rooted phylogenetic trees and networks. *Syst. Biol.* **61**, 1061–1067 (2012). Medline doi:10.1093/sysbio/sys062
46. N. Lartillot, N. Rodrigue, D. Stubbs, J. Richer, PhyloBayes MPI: Phylogenetic reconstruction with infinite mixtures of profiles in a parallel environment. *Syst. Biol.* **62**, 611–615 (2013). Medline doi:10.1093/sysbio/syt022
47. N. Lartillot, H. Philippe, A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. *Mol. Biol. Evol.* **21**, 1095–1109 (2004). Medline doi:10.1093/molbev/msh112
48. S. Tavaré, "Some probabilistic and statistical problems on the analysis of DNA sequences," in *Lectures in Mathematics in the Life Sciences* (American Mathematical Society, vol. 17, 1986), pp. 57–86.
49. J. L. Thorne, H. Kishino, I. S. Painter, Estimating the rate of evolution of the rate of molecular evolution. *Mol. Biol. Evol.* **15**, 1647–1657 (1998). Medline doi:10.1093/oxfordjournals.molbev.a025892
50. L. Salichos, A. Stamatakis, A. Rokas, Novel information theory-based measures for quantifying incongruence among phylogenetic trees. *Mol. Biol. Evol.* **31**, 1261–1271 (2014). Medline doi:10.1093/molbev/msu061

51. D. S. Hibbett, P. B. Matheny, The relative ages of ectomycorrhizal mushrooms and their plant hosts estimated using Bayesian relaxed molecular clock analyses. *BMC Biol.* **7**, 13 (2009). Medline doi:10.1186/1741-7007-7-13
52. T. N. Taylor, H. Hass, H. Kerp, M. Krings, R. T. Hanlin, Perithecial ascomycetes from the 400 million year old Rhynie chert: An example of ancestral polymorphism. *Mycologia* **97**, 269–285 (2005). Medline doi:10.3852/mycologia.97.1.269
53. J. W. Taylor, M. L. Berbee, Dating divergences in the Fungal Tree of Life: Review and new analyses. *Mycologia* **98**, 838–849 (2006). Medline doi:10.3852/mycologia.98.6.838
54. H. Dörfelt, A. R. Schmidt, A fossil *Aspergillus* from Baltic amber. *Mycol. Res.* **109**, 956–960 (2005). Medline doi:10.1017/S0953756205003497
55. D. Hibbett, D. Grimaldi, M. Donoghue, Fossil mushrooms from Miocene and Cretaceous ambers and the evolution of Homobasidiomycetes. *Am. J. Bot.* **84**, 981 (1997). Medline doi:10.2307/2446289
56. S. P. Stubblefield, T. N. Taylor, C. B. Beck, Studies of Paleozoic fungi. IV. Wood-decaying fungi in *Callixylon newberryi* from the Upper Devonian. *Am. J. Bot.* **72**, 1765–1774 (1985). doi:10.2307/2443734
57. T. Feuerer, D. L. Hawksworth, Biodiversity of lichens, including a world-wide analysis of checklist data based on Takhtajan's floristic regions. *Biodiversity Conserv.* **16**, 85–98 (2007). doi:10.1007/s10531-006-9142-6
58. J. Miadlikowska, F. Kauff, F. Högnabba, J. C. Oliver, K. Molnár, E. Fraker, E. Gaya, J. Hafellner, V. Hofstetter, C. Gueidan, M. A. Otálora, B. Hodkinson, M. Kukwa, R. Lücking, C. Björk, H. J. Sipman, A. R. Burgaz, A. Thell, A. Passo, L. Myllys, T. Goward, S. Fernández-Brime, G. Hestmark, J. Lendemer, H. T. Lumbsch, M. Schmull, C. L. Schoch, E. Sérusiaux, D. R. Maddison, A. E. Arnold, F. Lutzoni, S. Stenroos, A multigene phylogenetic synthesis for the class Lecanoromycetes (Ascomycota): 1307 fungi representing 1139 infrageneric taxa, 317 genera and 66 families. *Mol. Phylogenet. Evol.* **79**, 132–168 (2014). Medline doi:10.1016/j.ympev.2014.04.003
59. Y. Ohmura, K. Uno, K. Hosaka, T. Hosoya, "DNA fragmentation of herbarium specimens of lichens, and significance of epitypification for threatened species of Japan," *The 10th International Mycological Congress*, Bangkok, Thailand, 4 to 8 August 2014 (2014), p. 151.
60. R. Lanfear, B. Calcott, S. Y. W. Ho, S. Guindon, Partitionfinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Mol. Biol. Evol.* **29**, 1695–1701 (2012). Medline doi:10.1093/molbev/mss020
61. S. Altermann, S. D. Leavitt, T. Goward, M. P. Nelsen, H. T. Lumbsch, How do you solve a problem like *Letharia*? A new look at cryptic species in lichen-forming fungi using Bayesian clustering and SNPs from multilocus sequence data. *PLOS ONE* **9**, e97556 (2014). Medline doi:10.1371/journal.pone.0097556
62. E. Paradis, pegas: An R package for population genetics with an integrated-modular approach. *Bioinformatics* **26**, 419–420 (2010). Medline doi:10.1093/bioinformatics/btp696
63. Y. Yamamoto, R. Mizuguchi, Y. Yamada, Tissue cultures of *Usnea rubescens* and *Ramalina yasudae* and production of usnic acid in their cultures. *Agric. Biol. Chem.* **49**, 3347–3348 (1985).
64. M. del Carmen Molina, A. Crespo, Comparison of development of axenic cultures of five species of lichen-forming fungi. *Mycol. Res.* **104**, 595–602 (2000). doi:10.1017/S0953756299002014
65. M. Gardes, T. D. Bruns, ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **2**, 113–118 (1993). Medline doi:10.1111/j.1365-294X.1993.tb00005.x
66. T. J. White, T. Bruns, S. Lee, J. Taylor, "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sinsky, T. J. White, Eds. (Academic Press, 1990), pp. 315–322.
67. S. Behrens, C. Rühland, J. Inácio, H. Huber, A. Fonseca, I. Spencer-Martins, B. M. Fuchs, R. Amann, In situ accessibility of small-subunit rRNA of members of the domains Bacteria, Archaea, and Eucarya to Cy3-labeled oligonucleotide probes. *Appl. Environ. Microbiol.* **69**, 1748–1758 (2003). Medline doi:10.1128/AEM.69.3.1748-1758.2003
68. J. Inácio, S. Behrens, B. M. Fuchs, A. Fonseca, I. Spencer-Martins, R. Amann, In situ accessibility of *Saccharomyces cerevisiae* 26*S* rRNA to Cy3-labeled oligonucleotide probes comprising the D1 and D2 domains. *Appl. Environ. Microbiol.* **69**, 2899–2905 (2003) .Medline doi:10.1128/AEM.69.5.2899-2905.2003
69. C. Baschien, W. Manz, T. R. Neu, L. Marvanová, U. Szewzyk, In situ detection of freshwater fungi in an alpine stream by new taxon-specific fluorescence in situ hybridization probes. *Appl. Environ. Microbiol.* **74**, 6427–6436 (2008). Medline doi:10.1128/AEM.00815-08
70. B. M. Fuchs, F. O. Glöckner, J. Wulf, R. Amann, Unlabeled helper oligonucleotides increase the in situ accessibility to 16*S* rRNA of fluorescently labeled oligonucleotide probes. *Appl. Environ. Microbiol.* **66**, 3603–3607 (2000). Medline doi:10.1128/AEM.66.8.3603-3607.2000
71. D. Anglesea, G. N. Greenhalgh, C. Veltkamp, The cortex of branch tips in *Usnea subfloridana*. *Trans. Br. Mycol. Soc.* **81**, 438–444 (1983). doi:10.1016/S0007-1536(83)80109-0
72. G. N. Greenhalgh, A. Whitfield, Thallus tip structure and matrix development in *Bryoria fuscescens*. *Lichenologist* **19**, 295–305 (1987). doi:10.1017/S0024282987000264
73. R. Honegger, A. Haisch, Immunocytochemical location of the (1→3) (1→4)-β-glucan lichenin in the lichen-forming ascomycete *Cetraria islandica* (Icelandic moss). *New Phytol.* **150**, 739–746 (2001). doi:10.1046/j.1469-8137.2001.00122.x
74. H. J. Elwood, G. J. Olsen, M. L. Sogin, The small-subunit ribosomal RNA gene sequences from the hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata*. *Mol. Biol. Evol.* **2**, 399–410 (1985). Medline

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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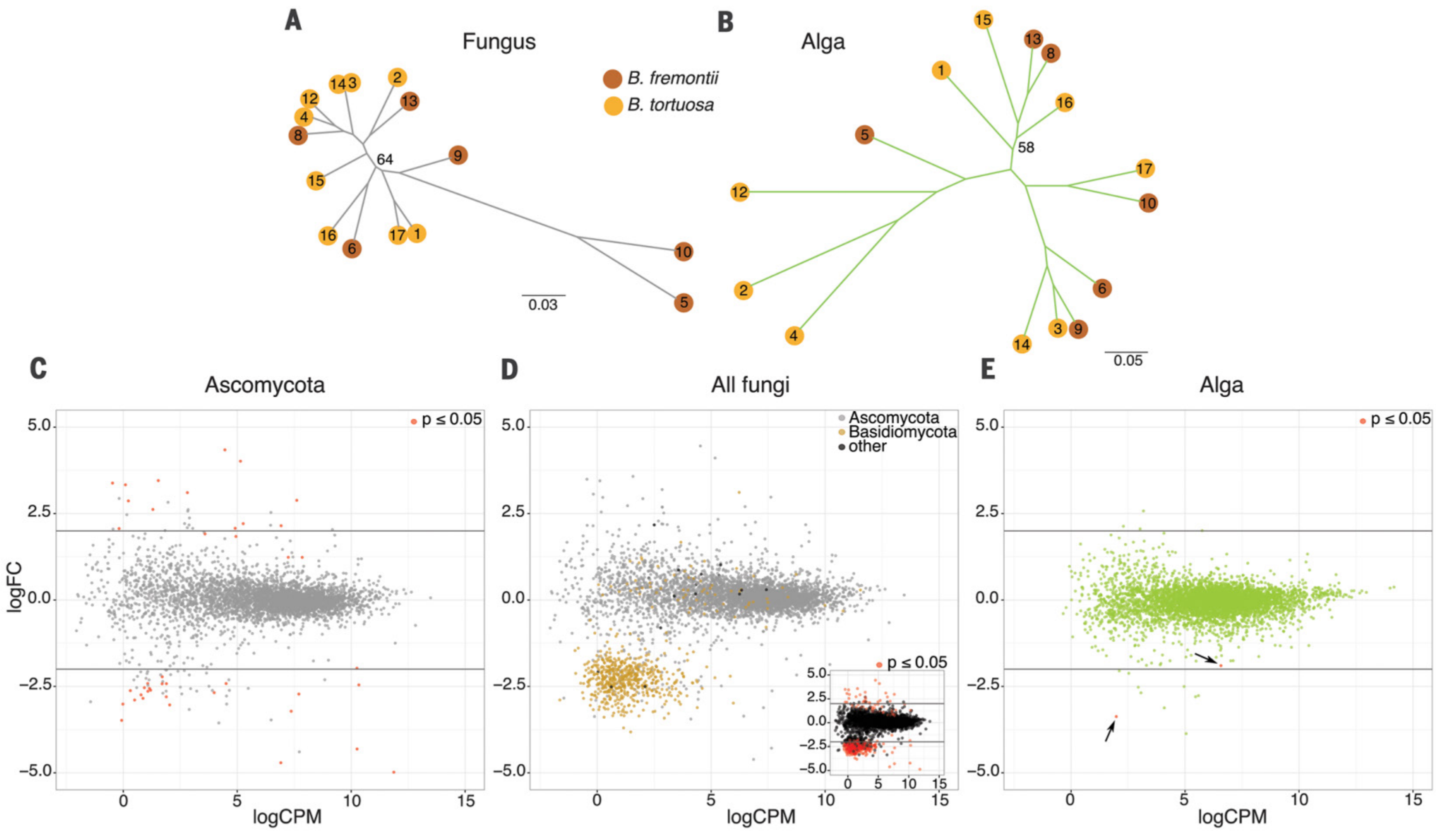

Fig. 1. Genome-wide divergence and transcript abundance of fungi and algae, based on symbiont subsets extracted from wild *Bryoria* metatranscriptomes. (**A** and **B**) Unrooted maximum likelihood topologies for (A) the Ascomycota member (lecanoromycete) and (B) the Viridiplantae member (alga) within the lichen pair *B. fremontii* and *B. tortuosa*, based on 30,001 and 25,788 SNPs, respectively. Numbers refer to metatranscriptome sample IDs (table S2). Scale bars indicate the average number of substitutions per site. (**C** to **E**) Logarithm of the fold change (logFC) between vulpinic acid–deficient (*B. fremontii*) and vulpinic acid–rich (*B. tortuosa*) phenotypes in 15 *Bryoria* metatranscriptomes, plotted against transcript abundance (logCPM, logarithm of counts per million reads). Only transcripts found in all 15 samples were included. Ascomycota transcripts only are shown in (C). All fungal transcripts are shown in (D), with taxonomic assignments superimposed; a plot with statistically significant transcript differential abundance is shown as an inset. Viridiplantae transcripts are shown in (E). Red dots indicate a log fold change with $P < 0.05$ in (C), (E) (highlighted with arrows), and the inset of (D).

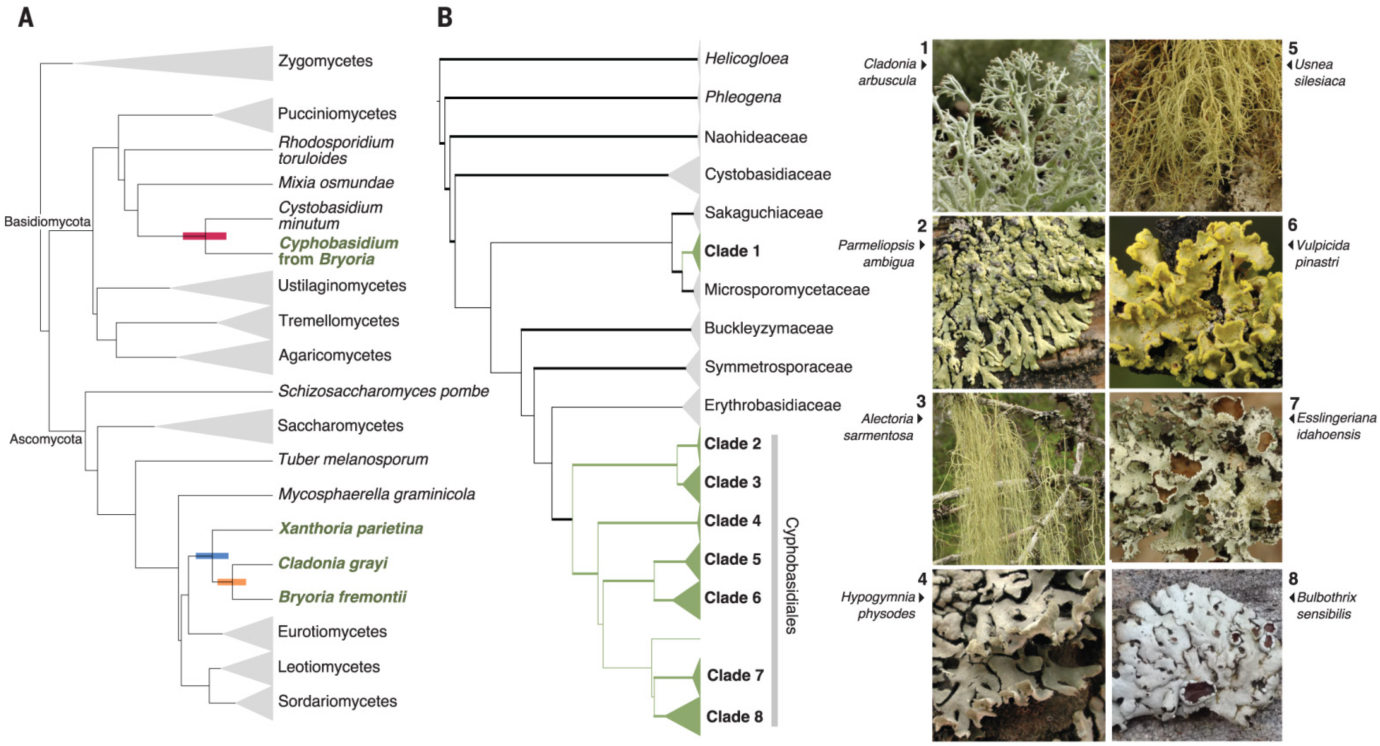

Fig. 2. Placement of Cyphobasidiales members and their diversity within lichens. (**A**) Maximum likelihood phylogenomic tree based on 39 fungal proteomes and 349 single-copy orthologous loci. Dating based on a 58-locus subsample shows relative splits between Cyphobasidiales and *Cystobasidium minutum* and splits leading to the lecanoromycete genera *Xanthoria*, *Cladonia*, and *Bryoria* (colored bars indicate 95% confidence intervals; fungi occurring in lichens are shown in green). (**B**) Maximum likelihood rDNA phylogeny of the class Cystobasidiomycetes, with images of representative lichen species from which sequences were obtained in each clade; thick branches indicate bootstrap support >70%. Shaded triangles are scaled to the earliest branch splits of underlying sequence divergence in each clade. Full versions of the trees are shown in fig. S3.

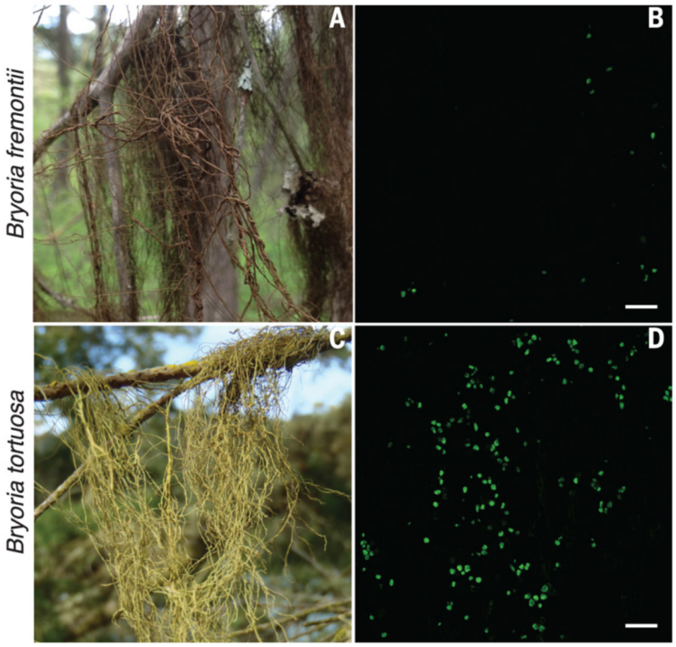

Fig. 3. Differential abundance of Cyphobasidiales yeasts in *B. fremontii* and *B. tortuosa*. (**A**) *B. fremontii*, with (**B**) few FISH-hybridized live yeast cells at the level of the cortex. (**C**) *B. tortuosa*, with (**D**) abundant FISH-hybridized cortical yeast cells (scale bars, 20 μm).

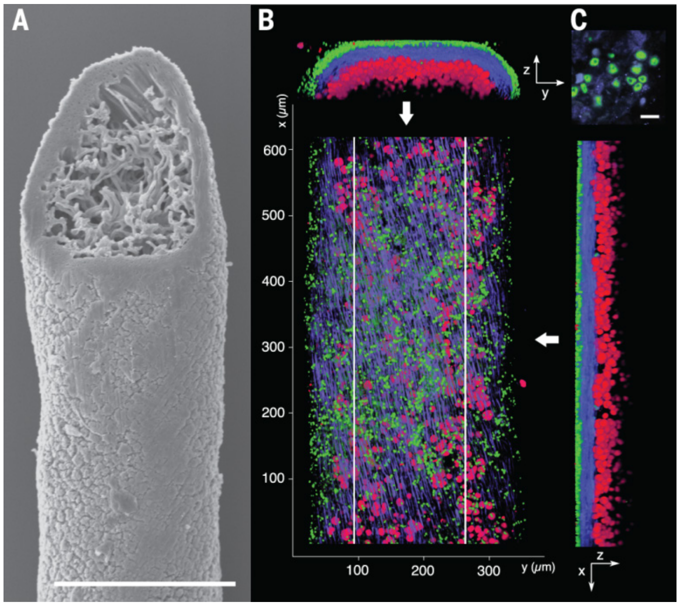

Fig. 4. Fluorescent cell imaging of dual fungal elements in lichen thalli. (**A**) Scanning electron microscopy image of a thallus filament of *B. capillaris* (scale bar, 200 µm). (**B**) FISH hybridization of *B. capillaris* thallus, showing Cyphobasidiales yeasts (green) and the lecanoromycete (blue) with algal chlorophyll A autofluorescence (red). The volume within the two vertical lines is visualized on the right; the unclipped frontal view is shown at the top. Movie S2 shows an animation of the three-dimensional ~100-µm z-stack. (**C**) Detail of yeast cells (scale bar, 5 µm).

Basidiomycete yeasts in the cortex of ascomycete macrolichens

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