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Tami R. McDonald, Ester Gaya & François Lutzoni

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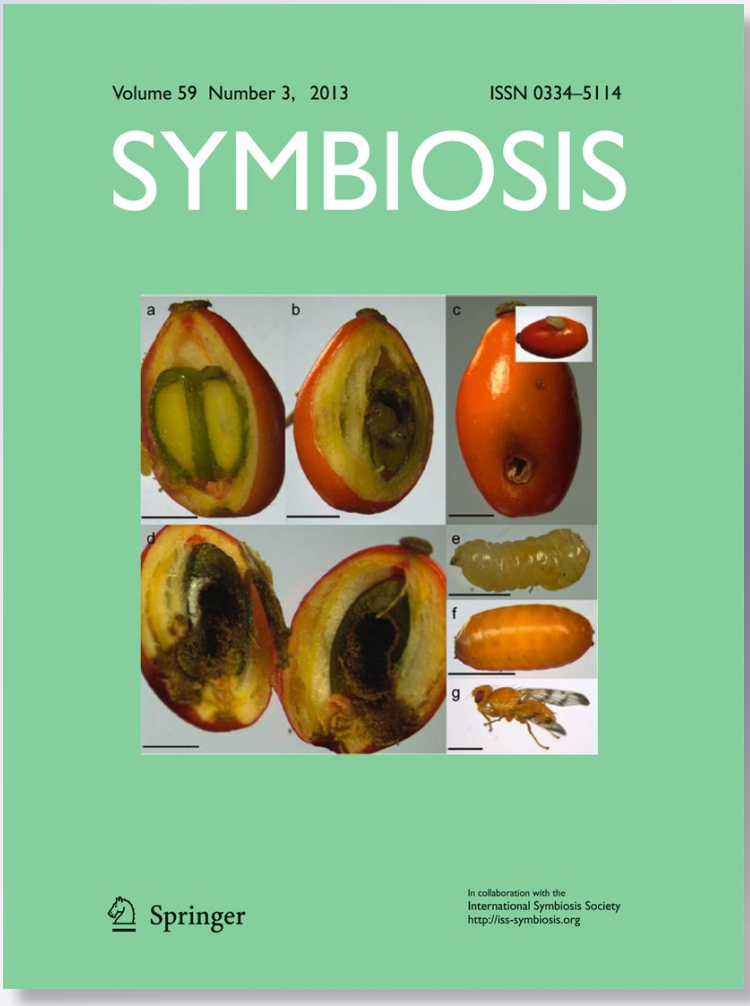

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SHORT COMMUNICATION

Twenty-five cultures of lichenizing fungi available for experimental studies on symbiotic systems

Tami R. McDonald · Ester Gaya · François Lutzoni

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Abstract In this study we describe the techniques used to culture 25 mycobionts spanning three classes and five orders of the leotiomyceta (Ascomycota). We find that five media, including potato-carrot, malt extract-yeast extract (MY), Bold's basal medium with nitrogen (NMBBM), oatmeal, and yeast extract with supplements (YES), are sufficient to induce ascospore germination of many lichenizing fungi and are also suitable for maintaining growth of the culture over the long term. Regular physical disruption of the cultures in liquid media is recommended to stimulate continued growth. Genomes of five of these lichen-forming fungal strains have been sequenced. The identity of each culture was confirmed by sequencing the nuclear ribosomal internal transcribed spacer (ITS) or the mitochondrial small subunit (mitSSU) from each strain. Additionally, the level of sequencing in terms of total number of genes sequenced for each taxon is provided. All fungal cultures have been deposited in public culture collections and, therefore, are available to the scientific community for conducting in vitro experiments.

Keywords Culture · Lichens · Fungi

Tami R. McDonald and Ester Gaya were equally contributing authors.

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T. R. McDonald (✉)
Department of Microbiology, University of Minnesota,
MMC 196 420 Delaware St. SE,
Minneapolis, MN 55455, USA
e-mail: mcdo0264@umn.edu

E. Gaya · F. Lutzoni
Biology Department, Duke University, Durham, NC, USA

1 Introduction

Attempts to culture the fungal and algal components of lichens began almost concomitantly with the field of lichenology. As early as the 1880s, Bonnier (1887, 1889) and Stahl (1877) separately reported success in culturing lichen fungi (Stocker-Wörgötter 2001). Nearly a century later, Ahmadjian (1966, 1973) did much to advance culturing techniques for lichenizing fungi, introducing and refining the spore-shot method (Ahmadjian 1973). Additionally, cultures have been successfully isolated from soredia (e.g. Armaleo and May 2009) and thallus fragments (Yamamoto et al. 1985; Armaleo 1991; Yamamoto et al. 1993). However, despite the many reports of cultures of isolated fungal partners of lichens (e.g., Oliver et al. 1989; Jahns 1993; Yamamoto et al. 1993; Crittenden et al. 1995; Stocker-Wörgötter 2001, 2002; Sangvichien et al. 2011), few cultures of lichenizing fungi are publicly available in culture banks compared to the prevalence of yeasts and non-lichenizing filamentous fungi in culture collections (see CBS-KNAW database as an example), with the exception of 38 mycobiont cultures from Ahmadjian in the American Type Culture Collection.

One major reason for the comparative dearth of publicly available cultures of lichenizing fungi is that these fungi are tedious to isolate. Although culture conditions and nutritional requirements have been reported for many lichenizing fungi (e.g., Bubrick 1988; Stocker-Wörgötter 1991; Crittenden et al. 1995; Sangvichien et al. 2011), the culture conditions for most lichenizing fungi, especially those with slow growth rates, remain unknown. In addition, if culturing conditions are known, it is not trivial to establish a pure culture since even when external fungal and bacterial contaminants are scrupulously excluded, within the thallus of a lichen are several endolichenic fungi (Arnold et al. 2009; U'Ren et al. 2010),

which may grow faster than, or as slowly as, lichenizing fungi. These contaminants can grow out of thallus fragments if these are used to initiate the culture, and thus it is possible that some reports of the successful culture of lichenizing fungi were actually describing endolichenic fungi. The great majority of these endolichenic fungi are filamentous ascomycetes in the Pezizomycotina, the same subphylum that includes all lichen-forming Ascomycota species (Arnold et al. 2009). Further, the lichen symbiosis is obligate in nature for most lichenizing fungi as well as for several of the most common lichenizing algae, suggesting that it might not be possible to bring certain lichenizing fungi into pure culture.

Another major reason why so few cultures are available is that once a pure culture is obtained, it may be difficult to maintain. The slow growth rates of lichenizing fungi in culture mean that the lichenizing fungus is readily outgrown by even slow-growing contaminants. In addition, lichenizing fungi grow in liquid culture for only a few weeks before appearing to lapse into a static state. Once cultures have reached this stage, they must be physically disrupted (for example, in a blender) in order to stimulate growth (Ahmadjian 1973). Furthermore, each transfer and every further manipulation presents a possibility of contamination.

We have isolated the mycobionts of 25 lichens in axenic culture. These cultures were obtained in the context of three projects: (1) the Assembling the Fungal Tree of Life (AFToL 2) project; (2) a project at Duke University and Pacific Northwest National Laboratory to sequence the genomes of the fungal and algal partners of three lichens; and (3) a Ph.D. dissertation focusing on the molecular evolution of ammonium transporters and DNA methylation in lichen-forming fungi (McDonald 2011).

The Assembling the Fungal Tree of Life (AFToL 2) project involved the sequencing of 21 new protein-coding genes from 63 species representing all main lineages of lichen-forming fungi and allied non-lichenized fungi. In most cases, amplification of DNA from cultures of lichenizing fungi rather than from thalli was deemed the best option, as it ruled out the possible amplification of DNA from contaminating endolichenic fungi. Additionally, culturing was also necessary to generate sufficient material to allow amplification of all of the 21 genes.

For the second project, Duke University and Pacific Northwest National Laboratory have partnered to sequence the genomes of the fungal and algal partners of three lichens representing the Acarosporomycetidae (*Acarospora strigata*), Lecanoromycetidae (*Cladonia grayi*), and Ostropomycetidae (*Leiorreuma sericeum*) (Lecanoromycetes) to investigate the comparative genomics of lichenization. Comparing and contrasting the expression profiles of the free-living symbionts versus the lichenized symbionts in these phylogenetically distantly related lichens will determine which, if any, genes or pathways are evolutionarily conserved in lichenization, potentially shedding light on how many independent origins of lichenization there have been in the evolution of filamentous ascomycetes. A novel aspect of this project is that the fungal and algal cultures for each lichen are derived from the same thallus, so that the genomes represent the symbiosis as it exists in nature.

The fungal and algal genomes of the lichen *Cladonia grayi* have revealed several interesting aspects of lichenization. The possibility of horizontal gene transfer between the fungus and alga was explored by examining the algal genome for genes of fungal origin and the fungal genome for genes of putative algal origin. No obvious horizontal gene transfer from the fungus to the alga was evident in the algal genome, but two plant-like genes were discovered in the fungal genome. Both of these genes encoded ammonium transporters (McDonald et al. 2012). Several additional lichenizing fungi were surveyed for the presence of these ammonium transporters to pinpoint the evolutionary timing of this horizontal gene transfer event. Cultures of the lichen mycobionts were necessary to ensure that the plant-like ammonium transporters amplified were indeed from the fungal genomes and could not be attributed to the lichen alga.

In the DNA methylation project, the report of widespread DNA methylation of the fungal genome exclusively in the symbiotic state of *Cladonia grayi* (Armaleo and Miao 1999) was explored by bisulfite sequencing of the free-living and symbiotic genomes of both the fungus and the alga comprising the lichen *Cladonia grayi*.

Here, we describe the methods used to culture lichen mycobionts from 25 lichens in three classes of fungi and five orders, including the Acarosporales, Lecanorales, Ostropales (Lecanoromycetes), Pyrenulales (Eurotiomycetes), and Trypetheliales (Dothideomycetes) to produce large-scale cultures suitable for providing the requisite amount of DNA for genome sequencing. The main goal of this note is to report the strains from these three studies that have been successfully isolated and deposited in publicly available culture banks for future research on lichen symbiotic systems. We also report the number of genes sequenced or average genome coverage for each culture.

2 Materials and methods

2.1 Mycobiont cultures

Single apothecia or fragments of thallus with multiple apothecia were washed, blotted and affixed to the lid of an inverted petri dish with double-stick tape or petroleum jelly, allowing the spores to be shot up onto media. Germinated spores were subcultured onto solid media and maintained at 20 °C or room temperature. Cultures were then transferred

to solid slants and maintained at room temperature or 4 °C for long-term growth. For large-scale tissue production for DNA extraction, a plug of solid culture was homogenized with liquid media in a blender and grown at 20 °C with shaking. To prevent liquid shake cultures from going dormant, cultures were periodically re-homogenized every 2 – 8 weeks. Media used were: malt extract-yeast extract (MY) (Ahmadjian 1993), nutrient medium based on Bold's basal medium with nitrogen (NMBBM) (Trembley et al. 2002), oatmeal (10 g/L homogenized oatmeal flakes, Keller et al. 1994), potato-carrot (as in Simmons 1992, except made as a 10x stock, and centrifuged rather than sieved, Dyer and Crittenden, personal communication), and yeast extract plus supplements (YES) (MP catalog #4101-532). A total of 20 g/L agar was added to all solid media and omitted for liquid media. After germination, well-established cultures were transferred to slants of NMBBM or MY, which were sufficient to support continued growth, in some cases for a year or longer between transfers. Vouchers of cultures were preserved as water stocks in which a slice of agar from a growing culture was fitted into a 2 ml tube completely filled with water and maintained at room temperature (Arnold et al. 2009). Frozen stocks are maintained at −80 °C on nitrocellulose disks (following D. Armaleo, personal communication).

2.2 DNA extraction

Tissue from liquid culture was frozen with liquid nitrogen, pulverized in a mini beadbeater with glass beads for three 30-second sessions followed by cooling in liquid nitrogen, or in an Eppendorf tube with a metal spatula. DNA was extracted following the alkali lysis method outlined in Zolan and Pukkila (1986) as modified by Gueidan et al. (2007) with the further modification that after addition of extraction buffer, a plastic micropestle was used to further homogenize tissue if necessary.

2.3 DNA amplification and sequencing

Amplification of the Internal Transcribed Spacer (ITS) region and mitochondrial small subunit (mitSSU) of the ribosomal RNA-coding genes for confirmation of the identity of the cultures was performed using primers ITS1F (Gardes and Bruns 1993) with ITS4 (White et al. 1990), and mitSSU1 with mitSSU3R (Zoller et al. 1999), respectively. PCR was performed as in McDonald et al. (2012) and Gaya et al. (2012). After examination with gel electrophoresis, PCR products were purified using ExoSAP-IT® (USB Corporation, Cleveland, OH) or the Montage PCR filter units (Millipore, Billerica MA, USA). Faint PCR products were cloned using the Topo TA cloning kit (Invitrogen™, life technologies, Carlsbad, CA). Sequencing was carried out in 10 μL reactions using: 1 μL of ITS1F, ITS4, mitSSU1, and mitSSU3R separately, 1 μL of purified PCR product, 0.75 μL Big Dye (Big Dye Terminator Cycle sequencing kit, ABI PRISM version 3.1; Perkin-Elmer, Applied Biosystems, Foster City, CA), 3.25 μL of Big Dye buffer, and 4 μL of double-distilled water. Automated reaction cleanup and visualization was performed at the Duke Genome Sequencing & Analysis Core Facility of the Institute for Genome Sciences and Policies. Cleanup reactions were performed over Sephadex G-50 DNA grade columns, eluting in water. Samples were then injected directly on an ABI 3730xl DNA analyzer (PE Applied Biosystems, Foster City, CA) utilizing a 22 s injection time, and a 50 cm capillary array.

2.4 Deposition of strains in culture banks

Strains reported here were submitted to CBS-KNAW (Fungal Biodiversity Centre) after the ITS or the mitochondrial SSU of each strain was sequenced one additional time before shipment. Only if BLAST scores confirmed the expected identity were the cultures sent to the culture collections.

3 Results

Overall, the best media for spore germination were oatmeal, potato-carrot agar, and YES. Although relative growth rates were not taken into account, crustose tropical lichens were the most successful and fastest-growing in axenic cultures. Lichens in the Teloschistales grew well on oatmeal and potato-carrot agar. Cyanolichens in the Peltigerales and likewise *Peltula* did not grow on any of the media described here.

Once spores had germinated and the cultures had grown to a sufficient mass, plugs of the fungi were homogenized and transferred to liquid shake culture. To minimize the risk of contamination, MY and NMBBM were used almost exclusively in liquid cultures. Most cultures that were lost were lost after this point, due to contamination, over-homogenization or under-homogenization. In general, we found that homogenization once a month was sufficient to keep a culture alive, whereas more frequent homogenization, while occasionally tolerated, ultimately led to the death of the culture. Liquid cultures left for more than three months between homogenizations rarely recovered. One strategy employed as a safeguard against over-homogenization was to remove a small portion of the liquid culture before homogenization, to be maintained either in liquid or on solid media. Likewise, maintaining several back-up plates or slants proved important. It may also be advisable to periodically switch the liquid culture directly to solid media or to a filter disk supported on solid media to maintain culture health, although this strategy was not tested here.

A list of axenic cultures of lichenizing fungi obtained for this work with their respective culture bank accession

number is presented in Table 1, including taxonomical information, Genbank accession numbers for the new sequences generated in this study, and the total number of genes that were sequenced for a specific strain here and in other studies (Armaleo and May 2009; Armaleo et al. 2011; McDonald 2011; McDonald et al. 2012. Additionally, representative photographs illustrating the diversity of some of the cultures included in this study are shown in Fig. 1. Voucher information for the thalli from which the cultures were derived is found in Online Resource 1.

Spores that germinated but failed to grow beyond one to five cell divisions, or that germinated, grew to sufficient size to be transferred to liquid culture, but then were lost are shown in Online Resource 2 along with the reason for loss. Representative photographs are shown in Online Resource 3.

4 Discussion

The panel of five media used here seemed to be sufficient to induce germination in most of the lichenizing fungi, with the notable exception of lichenizing fungi symbiotic with cyanobacteria. Spores of the cyanobacterium-associated *Peltula cylindrica* (Lichinomycetes) did not germinate on any of our media. Likewise, *Peltigera* spp., *Leptogium* spp., *Collema* spp., and *Lobaria pulmonaria* (Peltigerales) did not germinate under the conditions tested here, or germinated and

Table 1 Classification of cultures of lichenizing fungi (following Lumbsch and Huhndorf 2010) and their respective culture bank and Genbank accession numbers

Taxon	Isolator	Subclass	Order	Culture Bank Accession no.	GenBank ITS Accession no.	GenBank mitSSU Accession no.	Sequencing Level
Acarospora cf *contigua*	TM	Acarosporomycetidae	Acarosporales	CBS 132361	KC592261	KC592293	2+19(a)+(b)
Acarospora socialis	TM	Acarosporomycetidae	Acarosporales	CBS 132362	KC592262	KC592280	2+(b)
Acarospora strigata	TM	Acarosporomycetidae	Acarosporales	CBS 132363	KC592263		1+19(a)+G(b)
Astrothelium galbineum	EG	Dothideomycetes (Class)	Trypetheliales	CBS 132364			1+8(a)
Cladonia cristatella	TM	Lecanoromycetidae	Lecanorales	CBS 132749	KC592270		1+23(a)+(b)
Cladonia grayi	DA	Lecanoromycetidae	Lecanorales	CBS 132746	KC592272		25(a)+G(c)+(b)
Cladonia peziziformis	TM	Lecanoromycetidae	Lecanorales	CBS 132365	KC592271		1+(b)
Graphis handelii	TM	Ostropomycetidae	Ostropales	CBS 132366		KC592281	1+(b)
Graphis scripta	TM	Ostropomycetidae	Ostropales	CBS 132367	KC592274		1+23(a)+G(b)
Graphis sp.	TM	Ostropomycetidae	Ostropales	CBS 132747		KC592282	1+(b)
Ionaspis alba	TM	Ostropomycetidae	*Incertae sedis*	CBS 132748	KC592264		1+(b)
Leiorreuma sericeum 1	TM	Ostropomycetidae	Ostropales	CBS 132368	KC592265	KC592283	2+(b)
Leiorreuma sericeum 2	TM	Ostropomycetidae	Ostropales	CBS 132369	KC592266	KC592284	2+(b)
Phaeographis elliptica	EG	Ostropomycetidae	Ostropales	CBS 130777	KC592276	KC592285	2
Platygramme sp.	EG	Ostropomycetidae	Ostropales	CBS 130778	KC592277	KC592286	2
Polymeridium subcinereum	EG	Dothideomycetes (Class)	Trypetheliales	CBS 130779	KC592279	KC592287	2
Pyrenula cruenta 1	TM	Chaetothyriomycetidae	Pyrenulales	CBS 132370		KC592288	1+16(a)+(b)
Pyrenula cruenta 2	EG	Chaetothyriomycetidae	Pyrenulales	CBS 132371	KC592267		1
Pyrenula cruenta 3	EG	Chaetothyriomycetidae	Pyrenulales	CBS 132372	KC592268		1
Pyrenula cruenta 4	EG	Chaetothyriomycetidae	Pyrenulales	CBS 132373	KC592269		1
Pyrenula ochraceoflava	EG	Chaetothyriomycetidae	Pyrenulales	CBS 132741	KC592275	KC592289	2
Trypethelium aeneum	EG	Dothideomycetes (Class)	Trypetheliales	CBS 132743	KC592278	KC592290	2+(b)
Trypethelium eluteriae	EG	Dothideomycetes (Class)	Trypetheliales	CBS 132375		KC592291	1+15(a)
Trypethelium virens 2	TM	Dothideomycetes (Class)	Trypetheliales	CBS 132374		KC592292	1+(b)
Usnea strigosa	TM	Lecanoromycetidae	Lecanorales	CBS 132745	KC592273		1+(b)

Initials of the isolator are abbreviated as EG for Ester Gaya, TM for Tami McDonald, and DA for Daniele Armaleo. For those groups without a subclass, the Class is indicated instead. The number of genes sequenced or average genome coverage obtained for each strain is indicated in the last column. Letters in brackets next to numbers refer to the publications in which this sequence data appeared: a. Miadlikowska et al. (unpublished), b. McDonald (2011) and McDonald et al. (2012), c. Armaleo and May (2009) and Armaleo et al. (2011). When the whole genome was sequenced for a strain, this is indicated by the letter G (data available at http://genome.jgi.doe.gov/Clagr2/Clagr2.home.html). Numbers without letters refer to the genes sequenced in this study

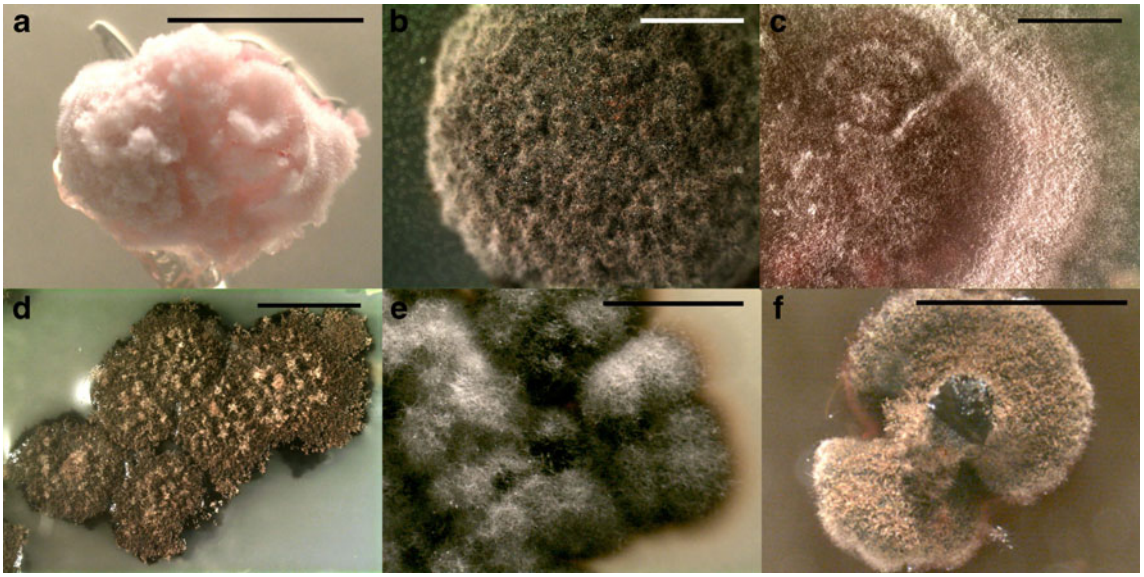

Fig. 1 Cultures of lichenizing fungi. **a**. *Acarospora* cf *contigua* on NMBBM media; **b**. *Astrothelium galbineum* on MY media; **c**. *Phaeographis elliptica* on OAT media **d**. *Polymeridium subcinereum on* NMBBM media; **e**. *Pyrenula cruenta* on NMBBM media; **f**. *Usnea strigosa* on MY media. — All scale bars = 2 mm

grew no more than a few cell divisions. An additional medium made with living or with autoclaved cultures of *Nostoc* (not isolated from a lichen) did not induce germination of *Peltigera* spores. Possibly, germinating the spores on a culture of the cyanobacterium from the same lichen thallus would have induced germination, as previously reported (Stocker-Wörgötter 2001).

One consideration not yet explored is the long-term effects of repeated disruption. The unnatural conditions of liquid culture may be very stressful for lichenizing fungi, and may in fact exert strong selective pressure on the culture. The culture may thus experience large-scale genomic effects such as aneuploidy or genome rearrangements in addition to the accumulation of point mutations. These events may increase with the number of passages through homogenizations and total length of time in culture. To test the effects of culture conditions on genome integrity, it would be interesting to freeze serial isolates of a culture across several years and sequence the genome at various time points. An experiment of this sort is in fact critical to determine if the genomes of the sequenced mycobiont cultures are representative of the genomes of the lichenzing fungi as found in nature. If genome rearrangements or other genomic artifacts of culture are shown to be an issue, new methods of cryostorage will need to be developed. Whereas other fungi may be easily frozen as glycerol stocks of yeast cells or conidia to minimize the number of passages and time in culture during which mutations may accumulate, cultured lichenizing fungi lack the aforementioned cell types and must therefore be stored as hyphal fragments. The effectiveness of freezing hyphal fragments to maintain cultures of lichenizing fungi is largely unexplored.

As lichenology enters the genomic era, the importance of culturing lichen mycobionts and photobionts increases. Enormous resources have already been invested in sequencing fungi, with more than 179 fungal genomes available through Genbank alone (as of October 2012)—the most of any kingdom of eukaryotic organisms. Now that representatives of the major fungal orders and many important pathogens have been sequenced, attention is turning to underrepresented orders of fungi, particularly fungi with interesting habits and habitats. Lichenizing fungi are of course among these. Sequencing facilities have requested upwards of 150 μg of DNA and 50 μg of RNA to sequence a genome de novo (personal communication to FL, Scott Baker) to publication quality. Ideally, this DNA and RNA are to be from a pure culture. To obtain this quantity of DNA and RNA requires several flasks or plates of culture, which for a lichenizing fungus may represent years of growth. Excitingly, next-generation sequencing platforms such as the HiSeq short-read technology from Illumina allow the sequencing of genomes from as little as 1 μg of DNA. We have achieved 50x and greater coverage of eight genomes of lichenizing fungi multiplexed into three lanes using this platform.

Since common genetic techniques such as gene deletion and transformation have not yet been developed for any lichen system, genomics represents an attractive alternative for exploring the molecular mechanics of lichenization. With a genome available, it will be possible to explore the

gene networks activated or silenced in the aposymbiotic (when the fungus is grown separate from its alga) versus the symbiotic state of a lichenizing fungus. With many lichen genomes, the scientific community can embark on comparative genomics and search for genes shared by very different lichen groups to determine if distantly related lichenizing fungi have the same or different ways of signaling to, and interacting with, their photobionts. It is also possible to search for presence/absence of specific genes, as was done for ammonium transporters by McDonald (2011). One of the greatest benefits of having cultures from both partners isolated from the same lichen thallus is the ability to conduct in vitro resynthesis experiments and conduct transcriptomic and proteomic studies to detect differential gene expression associated with the early stages of the lichen symbiosis (e.g., Joneson et al. 2011). We hope that the availability of these strains in public culture banks will entice researchers to conduct such experiments and encourage them to submit their cultures of lichen symbionts to public culture banks.

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References

Ahmadjian V (1966) Artificial reestablishment of the lichen *Cladonia cristatella*. Science 151:199–201

Ahmadjian V (1973) Methods of isolating and culturing lichen symbionts and thalli. In: Ahmadjian V, Hale ME (eds) The lichens. Academic, New York, pp 653–665

Ahmadjian V (1993) The lichen symbiosis. John Wiley, New York

Armaleo D (1991) Experimental microbiology of lichens: mycelial fragmentation, a novel growth chamber, and the origins of thallus differentiation. Symbiosis 11:163–178

Armaleo D, May S (2009) Sizing the fungal and algal genomes of the lichen *Cladonia grayi* through quantitative PCR. Symbiosis 49:43–51

Armaleo D, Miao VPW (1999) Symbiosis and DNA methylation in the *Cladonia* lichen fungus. Symbiosis 26:143–163

Armaleo D, Sun X, Culberson C (2011) Insights from the first biosynthetic gene cluster for a lichen depside and depsidone. Mycologia 103:741–754

Arnold AE, Miadlikowska J, Higgins KL, Sarvate SD, Gugger P, Way A, Hofstetter V, Kauff F, Lutzoni F (2009) A phylogenetic estimation of trophic transition networks for ascomycetous fungi: are lichens cradles of symbiotrophic fungal diversification? Syst Biol 58:283–297

Bonnier G (1887) La constitution des lichens. J Bot (M Louis Morot) 1:1–5

Bonnier G (1889) Recherche sur la synthèse des lichens. Ann Sci Nat Bot 7:1–34

Bubrick P (1988) Methods for cultivating lichens and isolated bionts. In: Galun M (ed) CRC handbook of lichenology, vol 3. CRC Press, Boca Raton, pp 127–138

Crittenden PD, David JC, Hawksworth DL, Campbell FS (1995) Attempted isolation and success in the culturing of a broad spectrum of lichen-forming and lichenicolous fungi. New Phytol 130:267–297

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Gaya E, Högnabba F, Holguin A, Molnar K, Fernández-Brime S, Stenroos S, Arup U, Søchting U, Van den Boom P, Lücking R, Sipman HJM, Lutzoni F (2012) Implementing a cumulative supermatrix approach for a comprehensive phylogenetic study of the Teloschistales (Pezizomycotina, Ascomycota). Mol Phylogenet Evol 63:374–387

Gueidan C, Roux C, Lutzoni F (2007) Using a multigene phylogenetic analysis to assess generic delineation and character evolution in Verrucariaceae (Verrucariales, Ascomycota). Mycol Res 111:1145–1168

Jahns HM (1993) Culture experiments with lichens. Plant Syst Evol 187:145–174

Joneson S, Armaleo D, Lutzoni F (2011) Fungal and algal gene expression in early developmental stages of lichen-symbiosis. Mycologia 103:291–306

Keller NP, Kantz NJ, Adams TH (1994) *Aspergillus nidulans* verA is required for production of the mycotoxin sterigmatocystin. Appl Environ Microbiol 60:1444–1450

Lumbsch HT, Huhndorf, SM (2010) Myconet Volume 14. Part One. Outline of Ascomycota - 2009. Part Two. Notes on Ascomycete systematics. Nos. 4751–5113. Fieldiana: Life Earth Sci. 1:1–64

McDonald T (2011) Genomic insights into the lichen symbiosis: *Cladonia grayi* as a model system. PhD Diss. Duke University, 2011

McDonald T, Dietrich F, Lutzoni F (2012) Multiple horizontal gene transfers of ammonium transporters/ammonia permeases from prokaryotes to eukaryotes: toward a new functional and evolutionary classification. Mol Biol Evol 29:51–60

Oliver E, Crittenden PD, Beckett A, Brown DH (1989) Growth of lichen-forming fungi on membrane filters. Lichenologist 21:387–392

Sangvichien E, Hawksworth DL, Whalley AJ (2011) Ascospore discharge, germination and culture of fungal partners of tropical lichens, including the use of a novel culture technique. IMA Fungus 2:143–153

Simmons EG (1992) *Alternaria* taxonomy: current status, viewpoint, challenge. In: Chelkowski J, Visconti A (eds) Alternaria: biology, plant diseases and metabolites. Elsevier Science Publishers, Amsterdam, pp 1–35

Stahl E (1877) Beitrage zur Entwicklungsgeschichte der Flechten II. Uber Hymenialgonidien. Felix, Leipzig

Stocker-Wörgötter E (1991) Thallus formation of two cyano-bacterial lichens: *Peltigera didactyla* and *Peltigera praetextata*, under laboratory conditions. B Soc Bot Fr-Lett 138:179–187

Stocker-Wörgötter E (2001) Experimental lichenology and microbiology of lichens: culture experiments, secondary chemistry of

cultured mycobionts, resynthesis, and thallus morphogenesis. Bryologist 104:576–581

Stocker-Wörgötter E (2002) Resynthesis of photosymbiodemes. In: Kranner I, Beckett R, Varma A (eds) Protocols in lichenology: Culturing, biochemistry, ecophysiology, and use in biomonitoring. Springer, New York, pp 47–62

Trembley ML, Ringli C, Honegger R (2002) Morphological and molecular analysis of early stages in the resynthesis of the lichen *Baeomyces rufus*. Mycol Res 106:768–776

U'Ren J, Lutzoni F, Miadlikowska J, Arnold AE (2010) Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. Microb Ecol 60:340–353

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. A guide to methods and applications. Academic Press, New York, pp 315–322

Yamamoto Y, Mizuguchi R, Yamada Y (1985) Tissue cultures of *Usnea rubescens* and *Ramalina yasudae* and production of usnic acid in their cultures. Agric Biol Chem 49:3347–3348

Yamamoto Y, Miura Y, Higuchi M, Kinoshita Y, Yoshimura I (1993) Using lichen tissue cultures in modern biology. Bryologist 96:384–393

Zolan ME, Pukkila PJ (1986) Inheritance of DNA methylation in *Coprinus cinereus*. Mol Cell Biol 6:195–200

Zoller S, Lutzoni F, Scheidegger C (1999) Genetic variation within and among populations of the threatened lichen *Lobaria pulmonaria* in Switzerland and implications for its conservation. Mol Ecol 8:2049–2059