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https://link.springer.com/protocol/10.1007%2F978-1-4939-7804-5_5

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Peng, X, Swift, C.L., Theodorou, M.K. and O'Malley, M.A. 2018. Methods for genomic characterization and maintenance of anaerobic fungi. In de Vries, R.P., Tsang, A. and Grigoriev, I.V. *eds. Fungal genomics: methods and protocols.* 2nd ed. New York: Humana Press.

Methods for Genomic Characterization and Maintenance of Anaerobic Fungi

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Running title: Anaerobic Fungi Genomic Characterization and Maintenance

Abstract (100 word limit)

The rapid development of molecular biology and bioinformatics has fueled renewed interests in anaerobic fungi from the phylum *Neocallimastigomycota*. This chapter presents well-established methods for isolation, routine cultivation, and cryo-preservation of anaerobic fungi. Moreover, detailed nucleic acid extraction protocols are provided, which should enable readers to isolate high quality DNA and RNA from a variety of anaerobic fungal culture media for downstream applications such as next-generation sequencing.

Keywords: Anaerobic fungi, Genomes, Transcriptomes, DNA extraction, RNA extraction, cultivation, isolation, Neocallimastigomycota, next-generation sequencing, consecutive batch culture

1 Introduction

Since the discovery of chitin in the cell wall of Neocallimastigomycota [1], which led to their reclassification from protozoa to fungi, there has been a large body of published literature that describes their morphology, physiology, ecology, and biochemistry [2]. The rapid development of molecular biology and bioinformatics in the past decade has provided powerful tools for scientists and engineers to gain deeper insight into the functional role of these unusual anaerobic fungi.

In particular, next-generation sequencing approaches have opened the way for comparative genomics, transcriptomics, and metagenomic modeling in these systems for the first time [3]. For example, a recent study identified novel enzyme candidates in anaerobic fungi for lignocellulose breakdown by combining transcriptomics, proteomics, and biochemical characterization [4], which are a powerful combination of tools that also promise to unravel the native syntrophy of anaerobic fungi and methanogenic archaea [5]. All of these methods depend upon successful cultivation of the anaerobic fungi and effective extraction of high-quality nucleic acid samples. This chapter builds upon established methods for basic maintenance of anaerobic fungi, and highlights new protocols we have developed in the laboratory to extract high molecular weight genomic DNA, as well as intact RNA for next-generation sequencing applications. We also include helpful tips for troubleshooting these experiments that are not easily found in the literature.

The first part of the methods discussed pertains to routine isolation and maintenance of anaerobic fungi based on the consecutive batch culture technique [6]. We then provide detailed protocols for extraction of RNA from batch cultures of anaerobic fungi or mixed microbial consortia that also include prokaryotic organisms (bacteria and archaea). Finally, we focus on two effective cell disruption methods to rapidly isolate high-quality genomic DNA from anaerobic fungi, which relies on simple-to-implement commercial kits.

2 Materials

2.1 For routine cultivation

1. Clarified rumen fluid: fresh rumen fluid centrifuged at 3220 g (or sufficient speed to sediment all particles, leaving a tan or greenish colored clarified liquid) for 1 hour at 4°C, separated from the resulting cell pellet. Carefully transfer 75 ml of the supernatant into 120-ml serum bottles and store at -20°C. Volumes of one liter to three liters are routinely clarified at one time.
2. Double clarified rumen fluid: autoclave the clarified rumen fluid at 121°C for 40 min and store at 4°C. Before use, the autoclaved rumen fluid is centrifuged at 3220 g (or sufficient speed to sediment all particles, leaving a tan or greenish colored clarified liquid) for 30 min at 4°C. Only use the resulting supernatant for subsequent media making.
3. Mineral Solution I: Dissolve 3.0 g of dibasic potassium phosphate (K_2HPO_4) in 1 L of water. Filter sterilize and store at 4°C.
4. Mineral Solution II: Dissolve 3.0 g of monobasic potassium phosphate (KH_2PO_4), 6.0 g of ammonium sulfate ($(NH_4)_2SO_4$), 6.0 g of sodium chloride (NaCl), and 0.6 g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) in 800 ml of water. Dissolve 0.6 g of calcium chloride dihydrate ($CaCl_2.2H_2O$) in 100 ml of water separately. These two solutions are combined, filter sterilized, and made up to a final volume of 1 L with water. Store at 4°C.
5. Resazurin stock solution (1 mg/ml): Dissolve 0.100 g of resazurin sodium salt (redox indicator) in 100 ml of water and filter sterilize. Store at 4°C.
6. Chloramphenicol stock solution (10 mg/ml): First completely dissolve 0.50 g of chloramphenicol (C1863 Sigma) in minimal amount of molecular biology grade ethanol (20

ml or less). Add water to a final volume of 50 ml. Filter sterilize the solution and store at 4°C.

7. Sodium bicarbonate, yeast extract, and Bacto™ Casitone (or tripticase peptone).

8. Plant material: should be air dry and milled to provide millimeter-sized pieces (generally 2-4 mm). Some examples include reed canary grass, switchgrass, alfalfa stems, and corn stover.

9. A static incubator set at 39°C, weighing boats, spatulas, beakers, graduated cylinders, 2-L microwave flask (must fit in a microwave), carbon dioxide (CO_2), gas manifolds to distribute CO_2, pipettes, 5-mL syringes connected to blunt-end needles, and autoclave are also required. Hungate tubes with butyl rubber stoppers and/or serum bottles outfitted with butyl rubber stoppers and crimp seals are adequate vessels for culturing the anaerobic fungi. It is also advantageous to have access to a pressure transducer manifold for quantifying fungal growth [7].

2.2 For nucleic acid (DNA/RNA) extraction

1. Biospec Mini-Beadbeater-16
2. Gel-loading pipet tips
3. 2-ml screw-cap tubes and caps with O-ring
4. 0.5 mm zirconia/silica beads (Biospec)
5. Microcentrifuge
6. Vortexer
7. RNAlater
8. Centrifuge

3 Methods

3.1 Media preparation

Anaerobic media is required for isolation and maintenance of anaerobic fungi as detailed in this section. Many of the recipes and culture techniques used in rumen microbiology were first described by Hungate [8], followed by a number of modifications [9–11]. Liquid media is typical for routine maintenance; media supplemented with agar (1% w/v) for solidification in roll tubes are often used for fungal isolation procedures.

The following is a recipe for preparing 1 liter of Medium C (use amounts shown in Table 1) or "Medium C Minus" ("MC-", use amounts shown in Table 2; *see* **Note 1**) dispensed in 9-ml aliquots. Alternatively, media can also be dispensed in larger volumes into serum bottles with crimp seals. All media should be prepared and aliquoted under a stream of CO_2 to minimize the introduction of oxygen.

1. Weigh out yeast extract, Bacto™ Casitone, and sodium bicarbonate into a 2-L flask.
2. Add 150 ml of Mineral Solutions I and II, and clarified rumen fluid.
3. Microwave for 20 minutes (*see* **Note 2**).
4. Purge with CO_2 for 10 minutes (*see* **Notes 3 - 4**).
5. Transfer into a 1-L bottle with 1 g of cysteine in it.
6. Close the lid and let it cool to below 39°C. (Optional: To speed up the cooling, place the media bottle into an ice bath.)
7. Dispense 9 ml of media into 16-ml Hungate tubes with substrates pre-weighed and aliquoted in them. For roll tubes, dispense 5 ml of media into 20-ml Balch tubes with 1% (w/v) agar pre-weighed in them. a) Use a 3-way gas manifold for CO_2 supply during media dispensing

(Figure 1). One of them is placed in the media bottle, and the other two are placed in Hungate tubes. Use blunt-end needles (14 gauge, 6 inches long, Cadence Inc.) at the end of the manifold. For the two needles purging Hungate tubes, bend the ends at about 1 inch length, so that they are not directly blowing at the carbon substrate at the bottom of the Hungate tubes. a) Use a 10-ml serological pipet to dispense 9 ml of media into a Hungate while it is purged with CO_2. c) Place a septum to cover the top of the Hungate tube, and cover it completely as the blunt-end needle is pulled out of this Hungate tube and placed into another one. Perform this step carefully to minimize introducing any air into the headspace of the Hungate tube. d) Seal the Hungate tube with a plastic screw cap (*see* **Note 5**).

8. Sterilize by autoclaving.
9. Liquid media are ready for use after they are pre-warmed to 39°C. Roll tubes are prepared by melting the solid media with agar (1% w/v) in boiling water and allowing tubes to cool in a water bath at 55°C. Roll the tubes under a cold water stream to evenly distribute a thin layer of solid media on the inner wall of Balch tubes (*see* **Note 6**).

3.2 Isolation of anaerobic fungi

Multiple methods have been used to isolate anaerobic gut fungi from rumen digesta and fecal materials, such as those published by Orpin 1975 [12], Bauchop and Mountfort 1981 [13], Lowe et al. 1985 [14], Joblin 1981 [15]. Here, we describe a straightforward method for isolating anaerobic fungi starting from the fresh fecal materials of large mammalian herbivores, but these methods can be readily adapted to isolate fungi from other sources.

1. Prepare Medium C without plant substrates and Medium C with reed canary grass (or another lignocellulosic substrate). Include chloramphenicol in these media with a final concentration of 0.1 mg/ml in the media.

2. Collect fresh fecal material and transport to laboratory facilities (keep as anaerobic as possible).
3. Prepare the initial inoculum by physically breaking down fecal material and transferring them into Medium C without plant substrates under a stream of CO_2. The final concentration of fecal material in Medium C should be approximately 10% w/v.
4. Using a wide-bore needle (0.2 mm or larger) and syringe, prepare 1:10, 1:100, and 1:1000 dilution of the initial inoculum with Medium C without plant substrates.
5. Inoculate Medium C containing reed canary grass with the initial inoculum and the three serial dilutions, with a final inoculum concentration of 10% v/v. Inoculate three to five replicate tubes for each dilution.
6. Examine growth daily and select enrichment cultures from tubes which show fungal growth. Fungal growth is easily observed by "bubbling" of the grass substrate and/or floating of the grass substrate within the culture tube.
7. Inoculate and evenly distribute 0.1 ml of selected liquid cultures into each roll tube.
8. Examine the growth of fungal colonies over time and select at least three colonies.
9. Under a CO_2 stream, pick the selected fungal colonies and inoculate them into liquid media supplemented with chloramphenicol.
10. To ensure axenic cultures are obtained, repeat Steps 6 – 9 at least twice more.
11. Putative axenic cultures should be examined using microscopy, and their phylogeny can be determined by sequencing their ITS region [16].

3.3 Maintenance of anaerobic fungi

Once axenic cultures of anaerobic fungi have been established, they are easily maintained in small batch cultures prepared in gas-tight glass vessels (as detailed in the "Media Preparation" section).

These fungal cultures reach exponential phase of growth typically three to four days after inoculation, and are ready to be transferred into fresh medium. The following procedure adapted from Theodorou and colleagues [17] is used for transferring growing fungal cultures into fresh medium.

1. Flame the rubber stoppers of both the inoculum culture and the fresh tube to be inoculated with 100% ethanol.
2. Shake the inoculum culture vigorously to disperse the fungal material.
3. Invert the tube and insert a needle with syringe into the tube and withdraw 1 ml. If the needle is clogged by particles, try clearing the clog by pushing the plunger gently up and down.
4. Inject the 1 ml inoculum into the recipient tube with 9 ml of fresh medium. Invert the recipient tube several times.
5. Incubate cultures at 39°C.

3.4 Cryo-preservation of anaerobic fungi

For long-term maintenance of anaerobic fungi, cultures are flash-frozen in liquid nitrogen and stored at -80°C using glycerol as a cryoprotectant. The following procedure is based on the method published by Solomon et al. [18].

1. Prepare Medium C containing 15% glycerol (Table 1). This solution should be as anaerobic as possible.
2. Grow fungal culture in Medium C for three to four days with excess plant substrate (3% w/v) in Hungate tubes.
3. Using a syringe and needle, remove all liquid (~10 ml) in the culture.

4. Inject 10 ml of Medium C containing 15% glycerol into the Hungate tube with the residual plant substrate. Shake gently to mix well.
5. Open the Hungate tube under a stream of CO_2. Using pipet tips with tips cut off, transfer 1.8 ml into 2 ml screw-top cryovials.
6. Immediately freeze in liquid nitrogen and store at -80°C.

3.5 Reviving cryo-preserved fungal stocks

1. Thaw cryo-preserved fungal stocks at 39°C.
2. Under CO_2 streams, or in an anaerobic chamber, remove the liquid media containing 15% glycerol. Leave the plant material behind.
3. Transfer 1 ml of fresh medium into the cryo-vial.
4. Use cutoff pipet tips to transfer the re-suspended culture into a pre-warmed culture tube.
5. Add chloramphenicol to prevent bacterial contamination (final concentration 0.1 mg/ml).
6. Incubate at 39°C and check the growth of the culture daily.

3.6 Determining growth curves using a pressure transducer

It is often necessary to determine the relative stage of growth of anaerobic fungal cultures depending to assist in experimental design and analysis. However, due to the heterogeneity, filamentous nature, and intimate association with plant biomass particles, it is not possible to determine their growth by monitoring the optical density of fungal cultures. Alternatively, measuring the pressure in the headspace of the culture tubes/serum bottles provides a straightforward approximation of the growth of anaerobic fungi, because fermentation gases

(predominantly CO_2 and H_2) accumulate as a consequence of growth. This inexpensive and non-destructive method [19] requires a simple pressure transducer (Figure 2), and is described below. All pressure measurements should be performed at 39°C due to pressure sensitivity to temperature fluctuations. Before introducing a needle into a sample tube/bottle, the rubber stopper is typically sterilized by flaming.

1. Immediately after inoculating a fresh medium tube, release excess pressure in the headspace so that the headspace pressure equals atmospheric pressure on the pressure gauge.
2. Every six to eight hours, measure, record, and release the headspace pressure.
3. Plot accumulated pressure against time. It generally takes at least six days to reach stationary phase (Figure 3).

3.7 Preparation of RNA

Perform protocol using standard best practices for an RNase-free environment (*see* **Note 7**).

1. Invert the culturing vessel (Hungate tubes or serum bottles) several times to break apart the plant substrate with fungal mat.
2. Transfer the culture media including the plant substrate into centrifuge tubes (15-ml or 50-ml, *see* **Note 8**).
3. Centrifuge at 3220 g for 7 min at 4°C with a swinging bucket rotor (*see* **Note 9**).
4. Decant and discard the supernatant.
5. Add 1 ml of RNAlater and store at -80°C if not proceeding to extraction immediately.
6. Thaw samples preserved in RNAlater, or use fresh samples.
7. Centrifuge at 3220 g for 7 min at 4°C with a swinging bucket rotor.
8. Decant and discard the supernatant.

9. Transfer all of the substrate and fungal mat into an autoclaved 2-ml screw-cap tube filled with 450 µL of buffer RLT (QIAGEN) and 1.0 mL of 0.5 mm zirconia/silica beads (*see* **Notes 10 - 11**).
10. Briefly vortex to mix the beads, buffer, and sample (*see* **Note 12**).
11. Bead beat samples for one minute using Mini-Beadbeater-16.
12. Centrifuge at 13,000 g for 3 min.
13. Transfer up to 650 µL of lysate using gel loading pipet tips onto a QIAGEN RNeasy spin column.
14. Follow the protocol "Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi" from the RNeasy Mini Handbook (QIAGEN, *see* **Note 13**).
15. RNA yields can be measured using Qubit fluorometric quantitation, and RNA quality can be assessed using a TapeStation or Bioanalyzer (Agilent). For next-generation sequencing, we recommend using RNA with a RNA Integrity Number (RIN) > 9.0 (Figure 4).

3.8 Genomic DNA extraction from fungal cultures grown on soluble substrates

1. The preparation of genomic DNA from fungal cultures depends on the main carbon substrate used in the culture media. If media contain soluble substrates (e.g. cellobiose, glucose), then a gentle bead beating step is used to lyse the fungal cells [18]. Invert the culturing vessel (Hungate tubes or serum bottles) several times to break apart the plant substrate with fungal mat.
2. Transfer the culture media including the plant substrate into centrifuge tubes (15-ml or 50-ml).
3. Centrifuge at 3220 g for 7 min at 4°C with a swinging bucket rotor (*see* **Note 9**).

4. Decant and discard the supernatant.
5. Follow the protocol included in the MO BIO PowerPlant Pro DNA Isolation Kit (*see* **Note 14**).

3.9 Genomic DNA extraction from fungal cultures grown on plant substrates

For media contains plant substrates (e.g. reed canary grass, corn stover), use a harsher bead beating step using the Mini-Beadbeater-16 is used for cell lysis, because plant substrates absorb a significant part of the bead beating force. In order to reduce background DNA introduced from rumen fluid, double clarified rumen fluid is recommended (Tables 1 and 2).

We have developed a method to extract RNA and DNA from the same sample based on the QIAGEN AllPrep DNA /RNA/miRNA Universal Kit. This may prove to be advantageous when the quantity of samples is limiting. Follow the second part of this simultaneous preparation of RNA and DNA.

1. Invert the culturing vessel (Hungate tubes or serum bottles) several times to break apart the plant substrate with fungal mat.
2. Transfer the culture media including the plant substrate into centrifuge tubes (15-ml or 50-ml, *see* **Note 8**).
3. Centrifuge at 3220 g for 20 min at 4°C with a swinging bucket rotor (*see* **Note 9**).
4. Decant and discard the supernatant.
5. Add 1 ml of RNAlater and store at -80°C if not proceeding to extraction immediately.
6. Thaw samples preserved in RNAlater, or use fresh samples.
7. Centrifuge at 3220 g for 20 min at 4°C with a swinging bucket rotor.

8. Decant and discard the supernatant.
9. Transfer all of the substrate and fungal mat into an autoclaved 2-ml screw-cap tube filled with 500 µL of buffer RLT Plus (QIAGEN) and 1.0 mL of 0.5 mm zirconia/silica beads (*see* **Notes 10 - 11**).
10. Briefly vortex to mix the beads, buffer, and sample (*see* **Note 12**).
11. Bead beat samples for 1.5 min using a Biospec Mini-Beadbeater-16.
12. Place sample tubes on ice for 1.5 min to lower the temperature.
13. Bead beat samples for another 1.5 min using a Biospec Mini-Beadbeater-16.
14. Centrifuge at 13,000 g for 3 min.
15. Transfer up to 650 µL of lysate using gel loading pipet tips onto a QIAGEN AllPrep DNA Mini spin column (*see* **Notes 15-16**).
16. Follow the protocol "Simultaneous Purification of Genomic DNA and Total RNA, including miRNA, from Cells" from the AllPrep® DNA/RNA/miRNA Universal handbook (QIAGEN).
17. DNA yields can be measured using Qubit fluorometric quantitation, and DNA quality can be assessed using a TapeStation or Bioanalyzer (Agilent). For next-generation sequencing, we recommend using DNA with a minimal degree of shearing (Figure 5).

4. Notes

1. The "Medium C Minus" ("MC-") was developed for experiments that require measurements of media composition using high performance liquid chromatography (HPLC). With reduced concentrations of yeast extract (1/10), casitone (1/20), and rumen fluids (1/2) compared to Medium C, MC- contains lower concentrations of key primary metabolites, such as formate, acetate, and hence lower background signal on the HPLC. Both Medium C and MC- include rumen fluid and are undefined. A defined medium, Medium 2, is described by Lowe and colleagues [14].
2. Boiling for 20 minutes will remove approximately 200 ml of water, so before heating the total volume of the medium solution should be 1200 ml in order to reach a final volume of 1000 ml. After microwaving, the solution should be boiling and pink.
3. It is not necessary to purge with CO_2 for a long time. Slow CO_2 flow rate works better than high flow rate.
4. Alternatively, add a few chunks of dry ice into the solution.
5. Media are usually pink immediately after dispensing into Hungate tubes, but the pink color should disappear after autoclaving.
6. Before rolling the tubes under a cold water stream, avoiding small bubbles in the media will facilitate fungal colony identification.
7. This protocol is equally effective for fungal growth on soluble substrates (e.g. 5 g/L glucose) and insoluble substrates (e.g. 0.1 g reed canary grass).
8. Depending on the purpose of the RNA analysis experiment, it may be necessary to perform this step in an anaerobic environment, such as an anaerobic chamber or a glove bag.

9. Alternatively, the sample can be centrifuged for 1 hour at 20,000 g using a fixed angle rotor. This results in the separation of the less dense fungal mat on top of the plant substrate. This can be advantageous to reduce the amount of sample to process.
10. We find that a metal spatula with a flat end works best to transfer samples into 2-ml screw-cap tubes for bead beating.
11. We found comparable results in RNA yield by liquid nitrogen grinding compared to bead beating for one minute using Mini-Beadbeater-16.
12. Vortex both orientations of the tube (cap down and cap up) in order to fully mix.
13. Perform “Optional On-Column DNA Digestion with the RNase-Free DNase Set” if performing RT-qPCR.
14. Use the Phenolic Separation Solution at step 1.
15. If the total volume of lysate from a sample was greater than 650 µL, repeat this step until all lysate has passed through the AllPrep DNA Mini spin column in order to maximize DNA yield. Generally the amount of DNA form fungal cultures < 50 ml in volume is not sufficient to overload the AllPrep DNA Mini spin column.
16. Generally RNA yields are high enough from just 650 µL of lysate that it is not necessary to save the flow-through from all of the lysate for RNA purification.

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Table 1: Ingredients in Medium C

Ingredients	**Per 1000 ml final volume**
Yeast Extract	2.5 g
Bacto Casitone	10.0 g
Sodium Bicarbonate	6.0 g
Mineral Solution I	150 ml
Mineral Solution II	150 ml
(Double*) Clarified Rumen Fluid	150 ml
L-cysteine hydrochloride	1.0 g
Resazurin Stock Solution (1 g/L)	1.0 ml
Carbon Substrate (e.g. plant, cellubiose)	1% w/v
Optional: Agar (for roll tube preparation)	*10.0 g*
Optional: Glycerol (for cryo-preservation)	*150 ml*

*Clarified rumen fluid is sufficient for routine maintenance of fungal cultures, whereas double clarified rumen fluid is recommended for cultures from which DNA will be extracted.

Table 2: Ingredients in "Medium C Minus" ("MC-", *see* Note 1)

Ingredients	Per 1000 ml final volume
Yeast Extract	0.25 g
Bacto Casitone	0.5 g
Sodium Bicarbonate	6.0 g
Mineral Solution I	150 ml
Mineral Solution II	150 ml
(Double*) Clarified Rumen Fluid	75 ml
L-cysteine hydrochloride	1.0 g
Resazurin Stock Solution (1 g/L)	1.0 ml
Carbon Substrate (e.g. plant, cellubiose)	1% w/v
Optional: Agar (for roll tube preparation)	*10.0 g*
Optional: Glycerol (for cryo-preservation)	*150 ml*

*Clarified rumen fluid is sufficient for routine maintenance of fungal cultures, whereas double clarified rumen fluid is recommended for cultures from which DNA will be extracted.

Figures

Figure 1. Media dispensing setup with a three-way gas manifold (clear-colored) to supply carbon dioxide simultaneously to the media bottle and two Hungate tubes to be filled. Also shown: a rack of Hungate tubes with pre-aliquoted plant material (center); two one-liter bottles of Medium C (left); serological pipets used for dispensing medium (center); black caps and grey butyl rubber septa for sealing Hungate tubes after medium is dispensed.

Figure 2. A pressure transducer assembly with digital display is used to measure the accumulation of gas pressure in the headspace of a fungal culture in a 60-ml serum bottle.

Figure 3. A typical growth curve of anaerobic fungal culture *Piromyces sp.* (maintained on Medium C supplemented with reed canary grass) determined by monitoring the headspace pressure using a pressure transducer.

Figure 4. An example of high RNA quality isolated (RNA Integrity Number, RIN > 9.0) from anaerobic fungal cultures (upper panel), and an example of poor RNA quality (RIN < 6.0) isolated from anaerobic fungal cultures (lower panel). Samples were measured on the Agilent 2200 TapeStation system (Agilent Technologies).

Figure 5. An example of high DNA quality isolated from fungal cultures (upper panel), and an example of poor, overly sheared DNA isolated from fungal cultures (lower panel). Samples were measured on the Agilent 2200 TapeStation system (Agilent Technologies). The genomic DNA ladders are marked by their sizes on the left, and fungal DNA samples are shown on the right.