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Morphotyping Fungal Cultures V.1

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Abstract

This protocols describes the process of designating fungal morphotypes.

This protocol is part of the Bark Beetle Mycobiome (BBM) Research Coordination Network. For more information on the BBM international network: Hulcr J, Barnes I, De Beer ZW, Duong TA, Gazis R, Johnson AJ, Jusino MA, Kasson MT, Li Y, Lynch S, Mayers C, Musvuugwa T, Roets F, Seltnann KC, Six D, Vanderpool D, & Villari C. 2020. Bark beetle mycobiome: collaboratively defined research priorities on a widespread insect-fungus symbiosis. *Symbiosis* 81: 101–113 <https://doi.org/10.1007/s13199-020-00686-9>.

The process of designating morphotypes is probably the most important step in distinguishing which fungi are associated with beetles. For this reason, careful assignments should be made from observations collected at different times and DNA data collected from multiple isolates per morphotype and per beetle.

After isolation of fungi from beetles, fungi need to grow to sufficient size to allow for elucidation of macroscopic characteristics (ie color, size comparison/growth rate, texture, etc). Additional methods for differentiation morphotypes may be deployed at this step, including culturing duplicate dilutions in darkness vs. fluorescent (+blue-black-blue) light.

Subculture: 5-10 days post isolation

Before subculturing, create preliminary morphotypes by recording the original isolation plate # and the phenotype on the back of a subculture plate. Indicate which CFU(s) will be sampled on the isolation plate, and photograph them (front & back) without opening. For each morphotype, subculture two different CFUs per plate

DNA extraction and RAPD: 7-14 days post subculture

Once subcultures have grown sufficiently, single colony subcultures should be photographed prior to DNA extraction. In pure culture/at a larger size, subcultured fungi may display slightly different phenotypes than those observed on isolation plates, and may allow for re-assessment of preliminary morphotypes (or grouping order in RAPD gel).

After DNA extraction, perform a RAPD PCR reaction (M13 primer & cycling) by grouping similar morphotypes next to each other on the gel. If the results are unexpected (similar morphotypes give very different RAPD profiles), a single-direction PCR of a more variable locus (or loci) can be used to test the accuracy of the RAPD result.

PCR Master Mix for RAPD PCR:

		per rxn
	Taq	12.5 uL
	M13	2.5 uL
	template	1 uL
	water	9.5 uL

1. Use these cycling conditions on the thermocycler:
2. 94 C for 3:00
3. 94 C for 0:30
4. 52 C for 1:30
5. 68 C for 3:00

6. Repeat steps 2 through 4 thirty times
7. 68 C for 8:00
8. 10 C for ∞

For extra care (ie publications), prepare a replicate PCR for each extraction.

Running the gel:

1. Prepare a 1% agarose gel (careful, it will be fragile). Make sure the gel is level when pouring!
2. Use extra reagents, but make sure the volume will fit in the comb you use for making the wells:

	ladder	9ul
	templat e	9ul
	dye	2ul

1. When loading, arrange samples from similar morphotypes together. If replicate PCRs were prepared, do not group them directly next to corresponding replicate sample.
1. Place both a 100bp and 1000 bp ladder on either side of every 5-10 reactions ran. This will aid in reading the gel, and will help to show if the DNA is moving at different speeds in different parts of the gel.
2. Run for up to 3 hours at 100-110V. Check every 45min-1 hour and take photo. The longer you run the gel, the more differentiated the bands will become, making it easier to read.

Reading the gel:

1. Use a photo editor (ie photoshop) to adjust the contrast/brightness so that all bands can be seen and differentiated between samples.
2. Use a line tool in the photo editor to connect bands in ladders with the same lengths. Use the slope of these lines to gage the distances traveled between samples.
3. Note any differences in fragment location and presence/absence between samples. Same species should be basically identical in their arrangement. Samples that appear different should be sequenced at a variable locus for confirmation.

Database entry and CFU quantification

New or existing morphotypes can now be entered into the database. This will also create a new "final" plate number for subcultures, which needs to be recorded on the plate. CFU counts can be calculated using isolation plate photos as a reference. Relevant isolates should be subcultured to cryo-tube slants and preserved.

