Review of: "Fungal Spores in Insect Trapping Fluids: Simultaneous Sampling for Insects and Pathogens"

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Potential competing interests: The author(s) declared that no potential competing interests exist.

The manuscript aims to compare and contrast the recovery of fungal metagenomic communities from slide based spore traps and bycatch of fungi in liquid based insect traps, using metabarcoding for fungal ITS. The authors report amplifying segments of the fungal ITS1 and 5.8S rRNA encoding regions from DNA isolates using the standard primers ITS1F and ITS7G, with Illumina Miseq next-generation sequencing. The authors report isolating 1277 distinct fungal OTUs across 90 samples, taken from 6 sites, with 2 slide spore traps and 2 insect traps installed at each site. Samples were collected weekly or biweekly at the six sites between August 1 and September 13 2018, with a total of 46 slide samples and 44 insect trap fluid samples collected. The authors reported 476 OTUs were detected only in the insect trap fluids, compared with 132 OTUs detected only from the slide samples. The same relative diversity relationship also existed in the restricted set of OTUs assigned to “pathogen and forest pathogen” categories. From these observations the authors draw the inference that insect trap fluid is an under-utilized source for information on fungal population structure in the environment. 669 fungal OTUs were detected in both insect trap fluid and slide trap spore samples.

While the comparisons drawn are valid and insightful within the confines of the experimental design, there remain factors involved in evaluating fungal community composition and structure which the authors do not address. The improvised slide traps described are innately limited by the mechanics involved and do not represent standard spore sampling methods in current use [1]. The theory of their described ‘slide’ aerial spore samplers involves exposing an air permeable filter(grade 50 double layer cheese cloth) treated with a particulate binding coating(Sigma silicone oil #378399) to ambient air for a set period of time, then evaluating the particulates bound in the coating. This only collects, and thereby enables detection of, those particles which passively pass through the apparatus, and only until the coating becomes saturated and ceases to bind particulates. This makes for relatively low sensitivity, reproducibility, and true duration of sampling, compared to the true population of airborne particulates.

It is unclear what the retention capacity of these apparatuses would be from the information provided (60 g/m^2 x 28.27 sq. mm of slide surface[6mm diameter filter punch] = 1.7 mg of silicone oil/sample, viscosity 1,000 cSt at 25 °C[2]), or how much air-borne particulates would have been expected to pass through the samplers in comparison to that retention capacity. Given the materials and methodology described, it seems plausible for these ‘slides’ to reach saturation in a much shorter interval than they were collected, such that rather than reflecting an unbiased aggregation of particulates these

slides would disproportionally reflect particulates from early in the sampling period vs later in the sampling period. More details about the theoretical retention capacity and associated linear sampling period would be required to contextualize these samples.

As the authors noted economic considerations can justify application of suboptimal methodologies, so long as the limitations are recognized and taken into consideration. For applications interested in high abundance community members, and not requiring accurate quantification, the greater ease of use and lower cost compared to more sensitive methods, may make such ‘slide’ samplers appropriate. Based on the authors’ description however their ‘slide’ traps do not seem equivalent to established air-borne particulate spore traps, and further trials would be required to demonstrate the authors’ ‘slide’ traps are valid analogs of established spore sampling methods.

However pathogens are almost always low abundance community components, often with strong dynamic shifts in prevalence over timescale shorter than a week, and the utility of pathogen detection is directly dependent on sensitivity to low abundance community components. Rotorod samplers use a similar principle as the ‘slide’ samplers used, but utilize coated sampling cylinders/rods and mechanically exposed, rotated through a relatively large circumference of air increasing the volume of air sampled, then mechanically sheathed to prevent contamination [1]. Alternatively spore traps such as Burkard samplers siphon air through the apparatus and collect particulates into tubes, allowing for unbiased sampling of much higher volumes of air, for time periods constrained by the volume of the tube rather than the surface area of sampler slide or rod [1]. These more sensitive sampling methodologies are more expensive and more technical to operate, but represent the current standards of sampling used in the aeromycota field [1].

As the authors note in their discussion, conversely insect traps will preferentially collect fungi associated with the insects and debris caught by the trap in addition to fungi deposited from the air passing through the traps, as the authors noted at the end of their introduction. As the authors also note in their discussion, the deposition of spores from the air into an insect trap is expected to follow similar passive physical principles as for the ‘slide’ samplers, but scaled up due to the greater relative surface area and volume of the collection fluid compared to the ‘slide’ coating. Both the rotorod and Burkard type samplers would more actively sample the air than passive slide or insect traps[1], although the larger volume of the insect traps could partially offset this difference. As the authors noted at the end of their introduction, the additional contribution of fungi associated with insects and debris could account for the higher abundance of spores found, above what would be expected based on Brownian diffusion of airborne spores into the insect traps.

The insights into community structures and contributions from these insect and debris associated fungi is valuable for investigating insect borne fungi, but the insect traps are not collecting equivalent communities as air particulate sampling traps. The use of baits/lures also skews the collection of insect borne fungi towards the insects targeted by the baits, integrating a bias/enrichment in fungi sampled into the communities predicted. In the case of fungal pathogens vectored with particular insect hosts, this can

be beneficial by enriching for the vectors and pathogens together, improving sensitivity. Thus fungal communities extrapolated from insect traps are expected to be less quantitatively representative of overall environmental communities, but with skews potentially beneficial for some applications, such as sensitive detection of the presence of a pathogen, particularly insect-borne pathogens.

The authors address an interesting and novel question, and overall handle the bioinformatics and statistics well, drawing sounds conclusions within the limitations imposed by some experimental design issues undercutting the strength of their results. I would recommend this manuscript be published with moderate revisions.

Corrections/Clarifications:

1. The authors report using primers ITS1F and ITS7G for amplification. However, ITS1F and ITS7G are both forward primers, so to amplify the region they describe, would require using the reverse complement primer of the ITS7G primer, which is what I infer they actually used. Please correct/clarify this.
2. Primers ITS1F and ITS7G like most ‘universal’ primers complement and amplify with significant taxonomic biases within the groups they attempt to cover, as a by-product of the ingrained taxonomic biases in the training datasets used to design the primers [3, 4]. This can be particularly significant in regards to certain clades of fungal pathogens, such as the order Pucciniales, which tend to be under-represented in published genomes used to generate training sets, due to lower environmental abundance and in some cases genomic properties counter-productive to effective sequencing (repetitive regions, hairpins, indels, etc) [3]. These biases can be difficult to avoid in broad-scale surveillance surveys, but should be acknowledged so as not to give false impressions of proof of absence of taxa of concern, when the reality is the methodologies used may be fundamentally ill suited to detection of those taxa [3].
3. The authors placed their insect traps 30-50 cm above the ground, whereas they placed their slide samplers 2 m above the ground, thus sampling from somewhat different micro-environments, contributing to the distinctive OTUs restricted to one or the other methodology. The shared OTUs reflect those fungi which co-occur in both microenvironments, with the caveat that insect borne fungi may bring spores into the insect traps, which were not as abundant in the air passing through the insect traps. Thus a comparison of air samplers at the same height as the insect traps, sampled as frequently as necessary to prevent saturation, would be more informative to the questions the authors aim to answer. This difference in trap deployment heights also contributes to the insect traps collecting more diversity of fungi, and higher abundances. This is in addition to the much higher retention capacity of the insect traps than the slide based spore traps.
4. Exclusion of low abundance reads, such as the authors’ cutoff at a minimum of 10 reads per OTU per week is an acceptable method of noise reduction in applications focusing on presence of OTUs, but when comparing methodologies for differences in absence it is slightly deceptive. The authors choice to zero OTUs with less than 10 reads/week inflates the apparent differences between the sampling

methodologies. The more conservative approach for accounting for the imprecision of sequencing, would be normalize all OTUs with less than 10 reads/week as 10 reads, and then apply the statistical comparisons. This would underestimate the true divergence between the sampling methods sensitivity, rather than overestimate it as their current approach does. Due to the reality of sequencing noise it is impossible to definitively declare any OTUs absent from any dataset, so it would be more accurate to say 'absent' OTUs are less abundant than their noise reduction cutoff.

5. The use of >97% similar OTUs rather than unique ASVs as units for comparison obscures the true diversity of the reads observed. The authors should ideally address the full information content, features and diversity of their dataset as ASVs, rather than unnecessarily decreasing their information content by abstracting to >97% similar OTUs. The mathematics dictates the more precise and non-ambiguous the training and input data, the more accurately a classifier can correctly predict a categorization. As such classification of ASV data should always theoretically yield as accurate or more accurate classifications than using approximations such as >97% identical OTUs. The use of OTUs rather than ASVs has generally been a historical concession to the limits of computational resources available to analyze these datasets. However the necessary resources to handle data with ASV level precision is now readily available.
6. The authors recognize the differences in filtering methodology between sites could potentially impact fungal distributions recovered, and make reasonable attempts to statistically address this source of variation.
7. The authors unfortunately don't address other methodological differences between their sampling methods, including sampling height, saturation rate, and sample retention capacity. These factors likely contribute significantly to observed differences in species richness, abundance, and diversity.
8. The authors' claim "This is the first comprehensive study to compare insect trap fluids and classical spore collecting devices" is erroneous as they compared insect trap fluids to a novel non-classical spore collecting device. They fail to demonstrate their 'slide' spore traps are analogous to classical spore collecting devices.

References:

1. Crisp, H.C., et al., *A side-by-side comparison of Rotorod and Burkard pollen and spore collections.* Ann Allergy Asthma Immunol, 2013. **111**(2): p. 118-25.

2. Aldritch, S., *silicone oil 378399*, in *Product Specifications*. Sigma Aldritch: 3050 Spruce Street, Saint Louis, MO, 63103, USA. p. 1.

3. Chen, W., D.R. Radford, and S. Hambleton, *Towards improved detection and identification of rust fungal pathogens in environmental samples using a metabarcoding approach.* Phytopathology, 2021.

4. Ihrmark, K., et al., *New primers to amplify the fungal ITS2 region--evaluation by 454-sequencing of artificial and natural communities.* FEMS Microbiol Ecol, 2012. **82**(3): p. 666-77.