Introducing the Ohio Mushroom DNA Lab: Early Lessons and Contributions from a New Community-Driven Nanopore Sequencing Laboratory

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ABSTRACT

The Ohio Mushroom DNA Lab (OMDL), a motivated group of community mycologists, present an approach to better understand and map fungal diversity via high-throughput DNA barcoding. Operating within a grassroots community science framework, OMDL focuses on widespread DNA barcoding of macrofungal specimens at zero cost to the public. Community scientists submitted 2,304 specimens primarily from North America, for DNA barcoding of the nrITS region using an Oxford Nanopore sequencing platform and the results are presented here. The overall success rate was lower than anticipated, however several methodological challenges were identified. Exactly 1,146 new ITS sequences were generated and deposited to Genbank. The adjusted success rate was approximately 65%, after excluding failures due to preventable issues. Novel and noteworthy results include 94 new provisional species codes and 367 new distribution reports. Approximately 58% of successfully sequenced fungi were identified to an existing validly published species, ~41% to a provisional species code, and 1% require further investigation. This standardized protocol for summarizing results and highlighting discoveries could be a template for other biodiversity projects using high-throughput DNA barcoding.

Keywords: Community Science, Amateur Mycology, Oxford Nanopore Technology, Fungal Diversity, Fungal Distribution, DNA Barcoding, Biodiversity

INTRODUCTION

Recent community science initiatives to document fungal biodiversity in North America benefit from the contributions of a broad network of geographically dispersed amateur mycologists (Cantonwine et al., 2022). Several groups are involved in large-scale, amateur-driven, exploration of fungal diversity utilizing molecular barcoding. The first ever lab to develop a high-throughput nanopore sequencing method for macrofungi was Mycota Lab (Russell, 2023a, see www.mycota.com). Their "Continental Mycoblitzes" are now held quarterly and accept ten collections per participant for DNA barcoding at zero cost. (Russell 2022). There is also the Fungal Diversity Survey (FUNDIS, www.fundis.org), North America's only non-profit committed to fungal conservation. FUNDIS is a fully operational sequencing hub funded by the State of California and the California Institute for Biodiversity. The organization offers DNA sequencing for their Rare Fungi Challenges, project-based California collections (e.g., Fungal Diversity Survey, 2023), and Local Projects around the continent. The most recent player to offer no-fee fungal DNA barcoding is the Ohio Mushroom DNA Lab (OMDL, www.ohiomushroomdnalab.com). The OMDL is a volunteer-run organization that provides unlimited DNA sequencing of fungal specimens for mycologists and enthusiasts located anywhere in the world, during any time of year.

All three players in this space utilize the Oxford Nanopore platform. Nanopore is a third-generation sequencing method with high-throughput capacity and low per-sequence cost compared to traditional Sanger sequencing (Russell, 2023a). Resulting sequences are uploaded to Genbank, a publicly-accessible repository. Through integration with iNaturalist, metadata such as specimen photographs and geolocations are associated with sequences. Publishing DNA sequence data to iNaturalist observations engages experts within the community. This facilitates open peer review and public feedback among professional and amateur mycologists. Using iNaturalist, novel species can be highlighted and geographical distribution reports can be easily obtained (see Appendix A Figures 1–5)). Community-run DNA sequencing labs have been making inroads toward high-throughput barcoding since 2016. In just seven years community

scientists managed to publish 45,000 sequences on iNaturalist and Mushroom Observer, see (Figure 1). The high volume of new data and novel provisional codes across a wide geographic range are evidence that the mycology community is making progress. Amateur mycologists seem well equipped to survey macrofungal diversity in a reasonable time frame to overcome the taxonomic impediment (Cao et al., 2016).

Given the recent surge in sample throughput and data generation, consistent summary metrics and reporting criteria are increasingly necessary. Transparent and detailed discussion of successes and failure rates is not typical. Challenges encountered while developing methods are not often reported. Because OMDL strives to advance community research, we are providing all of our data in order to aid current and future researchers. We pledge to prioritize accurate, reliable, and publically-available fungal diversity data for the benefit of everyone. The OMDL team performs a full barcoding workflow including fungi submission, tissue sampling, DNA extraction and sequencing, data validation, bioinformatics, and scientific communications. The mission of OMDL is to increase knowledge of fungal diversity and distribution with a long term goal of aiding multidisciplinary research. We already published early findings from our first nanopore run (Canan et al., 2023; Ostuni et al., 2023). Yet no comprehensive review of our progress has been presented until now.

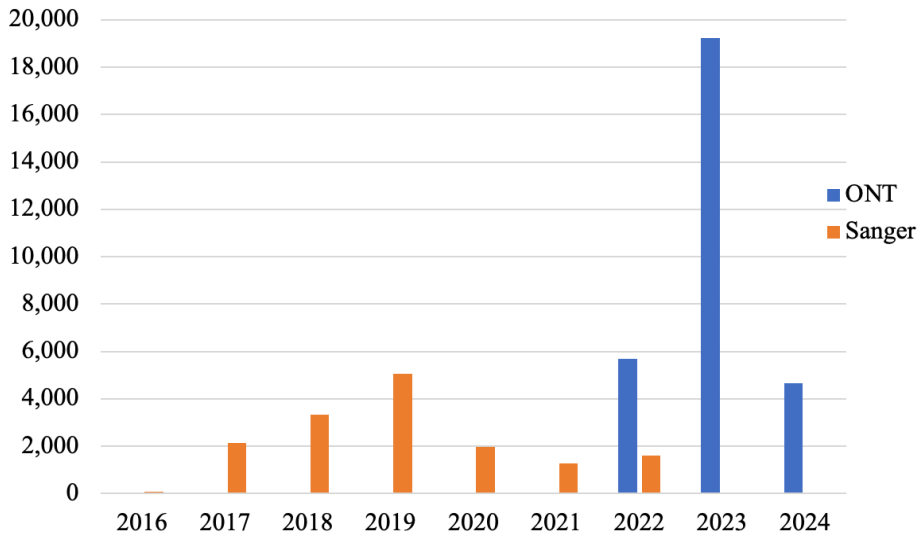


Figure 1. Total yearly fungal DNA barcodes published to MycoMap by sequencing technology as of January 31, 2024. ONT is Oxford Nanopore Technology.

MATERIALS AND METHODS

Sample Collection, Submission and Receipt. Samples were collected, dehydrated, and mailed to OMDL by individual contributors within the mycology community, including the authors. While the methods used by each individual to collect, dry, and send could not be controlled, detailed recommendations were provided (https://fundis.org/sequence/collect-dry). Upon receipt by OMDL, specimens were stored in their original containers until ready for sampling. While many types of containers were received, storage of specimens in ziplock bags with the iNaturalist number clearly visible from the exterior is the ideal method to streamline both storage and processing (Figure 2A).

Sample Processing. Collections were visually examined for proper drying and labeling. Any specimens showing signs of moisture-induced microbial decomposition or lacking clearly associated iNaturalist/Mushroom Observer numbers were discarded. During sampling, an OMDL master spreadsheet was populated containing all observation numbers tied to an OMDL record number and the corresponding 96-well plate position. Forceps were used to subsample a very small piece (e.g., a ~2 mm or less portion of the hymenium) of each specimen which were carefully deposited in the corresponding well location (Figure 2B). Forceps were flame sterilized initially and cleaned between samples.

DNA Extraction. The methods of Russell (2023b) were followed with a few notable exceptions. Briefly, 25 µL of X-Amp DNA Reagent (IBI Scientific, Dubuque, Iowa) was added to each well using a multichannel pipette, the plate was sealed using transparent sealing films (ZhiBang Automation Technology, China), and the volume was collected in the bottom of the wells via centrifugation at 10,000 rpm. Labeled plates were loaded into a single or dual-block GeneAmp® PCR System 9700 (Applied Biosystems, Waltham, MA) and heated to 80 °C for one hour followed by hold phase at 4 °C. After removal, 100 µL molecular grade water was added to each well and the plates were transferred to the freezer for storage at -20 °C until ready for dual-indexed PCR (Figure 2C).

Indexed PCR. The methods of Russell (2023b) were followed. Unique indexed forward and reverse primers were diluted in water and added to a 96-well plate or 8-strip tubes for a final

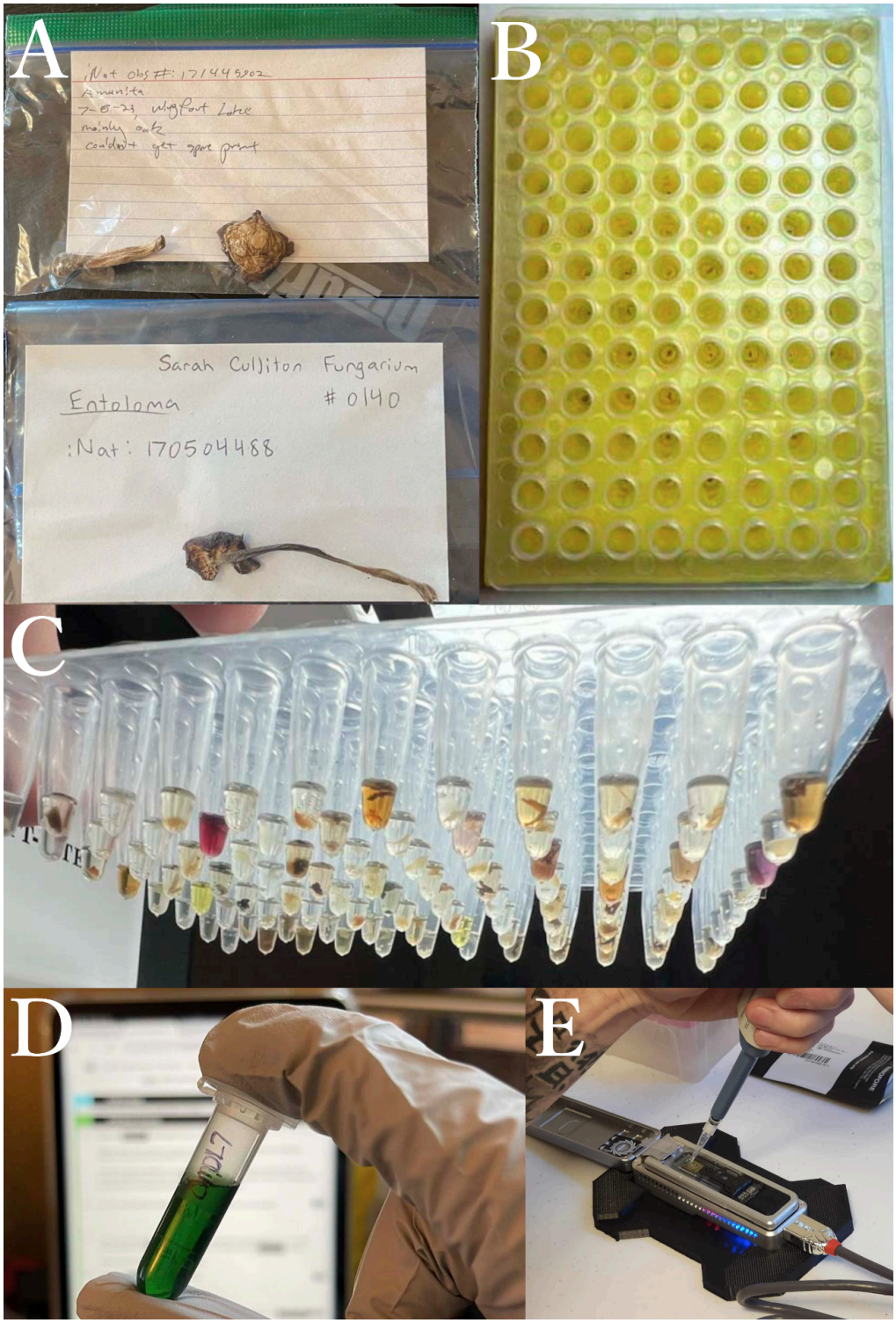


Figure 2. A. Optimally labeled specimens sent in for sampling. B. Tissue samples for analysis in a 96-well PCR plate. C. Plate with fungal specimen, after addition of X-Amp extraction reagent. D. Combined purified PCR products pending preparation for nanopore sequencing. E. Loading a single pipette tip containing several hundred fungal amplicons onto a nanopore Flongle.

(cont. from pg. 4) concentration of 10 µM with 6.25 µL (Taq 2X MeanGreen, Empirical) mastermix and 1.1 µL DNA extract for a final volume of 12.5 µL per reaction. The plate with sealing film (OMDL1) or strip tubes with caps (OMDL 2-5) fit into a single or dual-block GeneAmp® PCR System 9700 (Applied Biosystems, Waltham, MA). They were heated to 94 °C for one minute follow by 30 cycles of a one minute denaturation step at 94 °C, a one minute annealing step at 51 °C, and a one minute elongation step at 72 °C before a final eight minute 72 °C elongation step and indefinite cooling to 4 °C. Samples were stored in the freezer at -20 °C until ready for downstream processing.

Nanopore Sequencing. The methods of Russell (2023b) were followed with a few exceptions. A 2 µL subsample of every PCR product was pooled (Figure 2D), cleaned with magnetic beads (HighPrep PCR, MagBio) using an ethanol wash, and then eluted in molecular grade water. This pooled product was not quantified or adjusted. The pool was dA-tailed using the NEBNext® Ultra™ II End Repair/dA-Tailing Module and bead cleanup was performed on the dA-tailed product. (New England BioLabs, Ipswich, MA). Adapters were added to the tailed product using the Ligation Sequencing Kit V14 (Oxford Nanopore Technologies, Oxford, UK) and a final bead cleanup was performed. Clean ligated product was diluted with 50 µL water. After priming the R10.4.1 Flongle flowcell utilizing the Q20+ (V14) Ligation Sequencing Kit (Oxford Nanopore Technologies, Oxford, UK), a 5 µL subsample was combined with sequencing buffer and library beads and dispensed into the sampling port with care to not introduce air bubbles (Figure 2E). The MinION device was run for 24 hours using default parameters for the flow cell and ligation kit using the MinKNOW software.

Primary and Secondary Sequence Analysis. The methods of Russell (2023b) were followed. The MinION sequencer generates raw signal data as a multiplexed pool containing all sequenced samples. Guppy basecaller software Version 6.5.7 processed signal data into nucleotide sequences using the ‘super-accurate’ (SUP) parameter. The portion of samples with duplex reads, those with both the template DNA sequence and its reverse complement, were identified by the Duplex Tools package (Version 0.2.19) and re-basecalled using the duplex function of the Guppy tool. These duplex reads provide an inherently higher sequence quality and are manually substituted for the corresponding lower-quality “simplex” reads within the pooled samples.

MinION run quality is evaluated with the MinIONQC R software package (Version 1.4.2; Lanfear et al., 2019). Pooled sequence data is demultiplexed by the Minibar python package (Version 0.24; (Krehenwinkel et al., 2019) into individual samples by identifying the dual synthetic nucleotide sequences flanking either end of the template DNA. The NGSpeciesID software pipeline (Version 0.3.0; Sahlin et al., 2021) generates a consensus sequence for each sample, serving as support for existing bioinformatic tools such as isONclust, SPOA, and Medaka. Due to the propensity for contaminating sequences within each sample's data, reads are first clustered based on similarity to establish a limited number of distinct ITS sequences predicted for each sample. NGSpeciesID then performs a consensus-building step using potentially hundreds of sequences to refine and polish the quality of these predicted ITS sequences. The number of reads used for each final consensus sequence is reported as 'Reads in Consensus' (RiC) by NGSpeciesID. The RiC metric has been identified as a critical indicator of confidence in the sequence quality. We use a threshold of 30 RiC as a QC flag to determine which samples require closer examination by an expert sequence analyst. Finally, a purpose-built python script is used to organize and summarize the consensus sequences before they are uploaded to MycoMap for subsequent analysis. MycoMap is a publicly accessible online information management platform that aids data organization, links sequences to iNaturalist and Mushroom Observer observations, and provides additional sequence reference data in a local modified BLAST search including metadata from the web platforms UNITE, iNaturalist, Mushroom Observer, amongst other internal and external research data.

Validation and Classification. DNA barcodes passing the quality filters were analyzed according to the methods of Russell (2023b) using MycoMap. The quality filters that we conceived and utilized include whether the macromorphology matches other collections that can be viewed with mycoBLAST results, whether the RiC was greater than the minimum cutoff of 30, along with no chimeric signal, no sequencing artifacts, and no highly suspect or high proportion of indels identified. Sequences of contaminating fungi were excluded from the records. Chimeric or otherwise erroneous sequences were excluded based on query coverage, sequence length, and identity. Identifications based on sequence similarity were made based on the expert opinion of trained sequence validators. Validators would view the generated sequence in comparison to the reference datasets in MycoMap and GenBank using the MycoBLAST and NCBI BLAST tools.

They would consider morphology of the observation using the photos and geographic distribution of the observation and compare these with the list of potential BLAST results. The ITS sequence along with all of the metadata, raw data, and the purported identity were published to Genbank. The ITS sequence was also uploaded to the individual iNaturalist and/or Mushroom Observer observations. If an observation was positively identified to a previously known species, MycoPortal (Miller and Bates, 2017) was used to identify new distribution reports. If an observation was identified to a provisional code, MycoMap BLAST results and iNaturalist records were searched for conspecific distribution records. Sequence (not species) novelty was classified according to the number of MycoMap adjusted pairwise similarity *matches* as defined by Cantonwine et al. (2022) and a modified version called Cantonwine 99 for runs OMDL2 - OMDL5 (Table 1). All data was processed collaboratively using a shared spreadsheet (Supplementary Table 1).

Table 1. Percent identity and quantity criteria for sequence classification.

Classification	**Cantonwine (≥ 97%)**	**Cantonwine 99 (≥ 99%)**
Novel	0	0
Uncommon	1–3	1–5
Common	4–9	6–14
Very Common	≥10	≥15

RESULTS

Pass/Fail Summary Results

OMDL's grassroots community project has contributed 1,146 ITS barcodes into open-source databases over the course of the first five sequencing runs. There was an 'edge effect' in OMDL1 where samples on the edges of the plates suffered from evaporation. The edge effect wells showed a ~11% lower success rate compared to interior wells. Otherwise we noted no obvious relationship between plate position and success rate. In both OMDL3 and OMDL4, a single plate failed in each run due to plate rotation 180 degrees relative to the order these samples were entered into the OMDL master spreadsheet. The specimens in edge effect wells in OMDL1 and the specimens in the rotated plates in OMDL3 and OMDL4 are therefore excluded from the adjusted totals, along with select specimens that were otherwise disqualified (duplicates, incorrect iNat numbers provided, etc.). After excluding samples with identifiable errors in

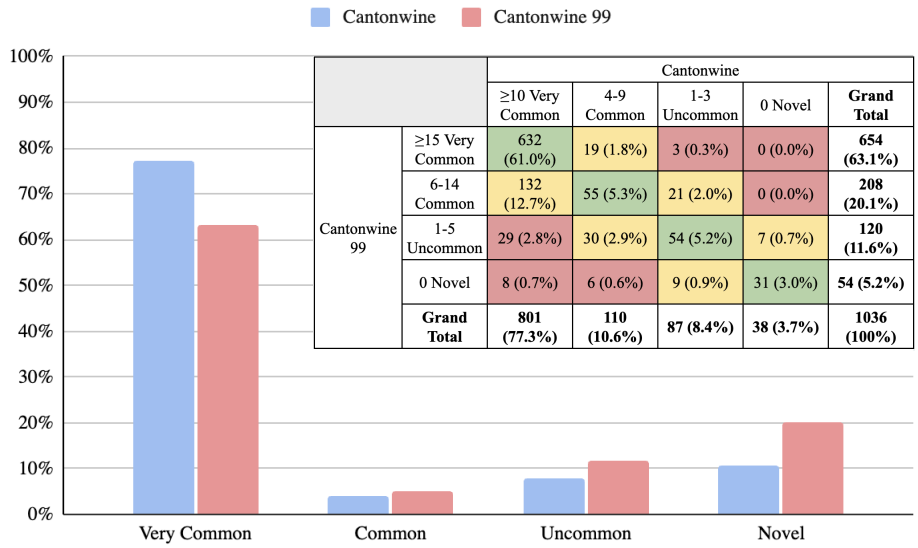
methodology, the resulting success rate over five runs increased to ~65%. See Table 2 for a summary of the results from all runs, OMDL1–OMDL5, containing both the included and excluded data.

Table 2. Summary results from each run and the adjusted total, accounting for excluded samples.

Run	OMDL1		OMDL2		OMDL3		OMDL4		OMDL5		**Adjusted Total**	
Metric	n	%	n	%	n	%	n	%	n	%	**n**	**%**
Excluded	0	0	1	0.2	97	20.2	115	24.0	3	0.8	**216**	**9.4**
Failed	370	77.1	132	27.5	150	31.2	132	27.5	158	41.2	**572**	**31.4**
Pass	110	22.9	347	72.3	233	48.5	233	48.5	223	58.1	**1,146**	**56.8**
Adjusted Pass		22.9		72.4		60.8		63.8		58.5		**64.4**
Total	480	100	480	100	480	100	480	100	384	100	**1,824**	**100**

Sequence Novelty

Cantonwine and Cantonwine 99 categories matched approximately 75% of the time. The most frequent category was "Very Common" while the number of specimens in each subsequent category decreased. Classification was more evenly spread using Cantonwine 99, though both skew to "Very Common." Using Cantonwine 99, 169 sequences classified as "Very Common" according to Cantonwine are now "Common," "Uncommon," or "Novel" (Figure 3 inset).



		Cantonwine				
		≥10 Very Common	4-9 Common	1-3 Uncommon	0 Novel	**Grand Total**
Cantonwine 99	≥15 Very Common	632 (61.0%)	19 (1.8%)	3 (0.3%)	0 (0.0%)	**654 (63.1%)**
	6-14 Common	132 (12.7%)	55 (5.3%)	21 (2.0%)	0 (0.0%)	**208 (20.1%)**
	1-5 Uncommon	29 (2.8%)	30 (2.9%)	54 (5.2%)	7 (0.7%)	**120 (11.6%)**
	0 Novel	8 (0.7%)	6 (0.6%)	9 (0.9%)	31 (3.0%)	**54 (5.2%)**
	Grand Total	**801 (77.3%)**	**110 (10.6%)**	**87 (8.4%)**	**38 (3.7%)**	**1036 (100%)**

Figure 3. Proportion of barcodes assigned to each sequence novelty category using the Cantonwine (blue) and Cantonwine 99 (red) criteria analyzed in OMDL2-5. Inset Table: Percent

overlap between the two classification criteria. Green cells show the percentage of sequences in the same category according to both criteria, yellow cells show the percentage in an adjacent category between the two criteria, and red cells show those two or more categories off.

Barcodes by State

Of the successfully barcoded specimens, the slim majority were from Ohio (~51%), with Washington and Florida at ~13% and ~7%, respectively. The rest were received from 19 other states along with a few international submissions (Figure 4).

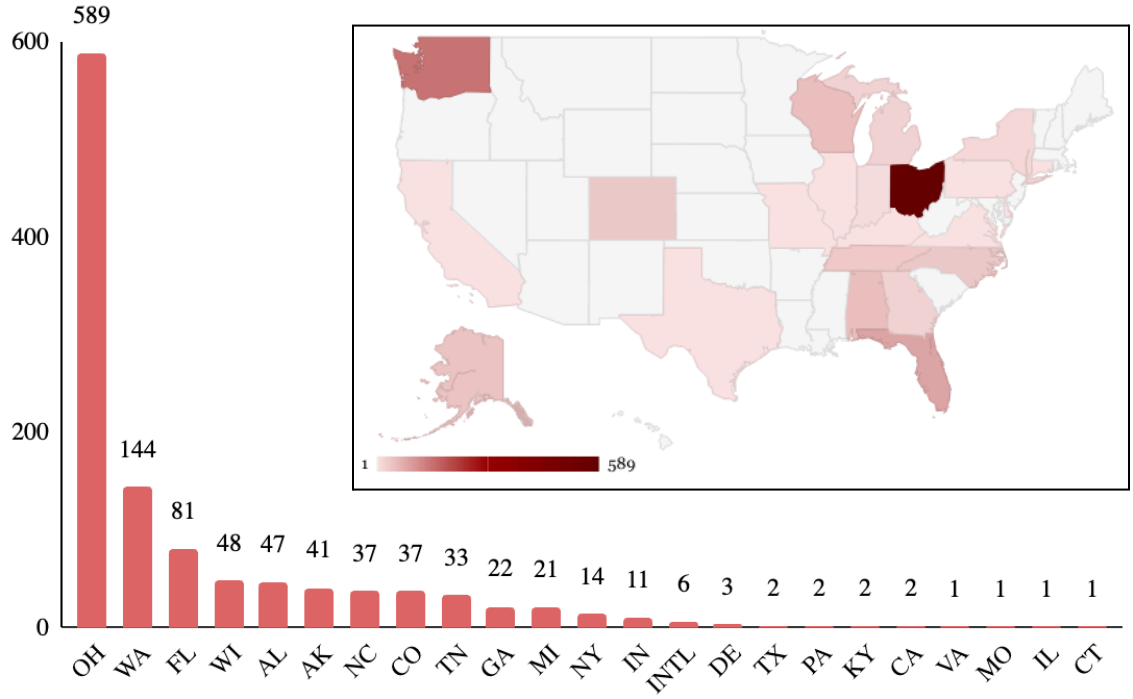


Figure 4. Barcodes generated by state of origin with standard US state abbreviations; INTL is international. Inset: heat map with more collections by deepness of red.

Temporary Codes

Temporary codes have been in use for several years by amateur mycologists on platforms such as Mushroom Observer and iNaturalist. Yet, a full explanation of temporary codes and justification of their use has never been presented in the scientific literature. To provide a long-awaited explanation to the community, the designer of the MycoMap code system (SDR) brings clarity to temporary codes in this section.

Identification of taxa based solely on ITS barcodes, while sometimes straightforward, is often difficult or impossible due to the limitations of NCBI's GenBank. Just a few examples of the difficulties caused by GenBank are type specimens without sequences, lack of well-validated reference material, misidentifications, and cryptic species causing confusion (Meiklejohn et al., 2019; Nilsson et al., 2006). This has led Hofstetter *et al.* to conclude that "prospects for a more reliable sequence-based identification of fungi remain quite dim," (Hofstetter et al., 2019). One reason for the negative outlook is that a significant number of North American species do not yet have reference data in GenBank, and some have conflicting reference data. In these cases, traditional sequence-based identification techniques are not possible. Because of the limitations, new models to circumscribe biodiversity must be considered. To make progress toward understanding extant biodiversity within a reasonable time frame, it is critical these models do not depend upon traditional Latin binomial nomenclature.

One functional model that worked particularly well for the Amanitaceae has been utilized by Rod Tulloss for decades (Tulloss & Yang, 2024). Based primarily on macro- and micromorphology until the advent of DNA sequencing, this three-stage identification system allows for communication about unpublished species-level units. Having a systematic 'language' for this family has enabled the study of its members' morphology, ecology, geographic range, and levels of diversity, even in cases where species were not formally described. Operating by the methods of Tulloss and Yang, when a novel species is encountered standard practice assigns the species a temporary code name and publishes the known information about this species online at Amanitaceae.org. This temporary code name is frequently generated based on the geography of the original collection followed by a sequential numeral e.g. *Amanita* sp-IN01 (Tulloss, 2024). The species is then re-collected throughout time, so more photographs become available, and more macroscopic/microscopic analysis is accomplished. As data aggregation continues, a clear picture of the species begins to emerge. Only then is the species assigned a provisional name following the conventions and standards of nomenclature according to the ICN (Turland et al., 2018). The provisional name must then pass peer-review and scrutiny by taxonomic experts. If a community agreement is reached, this marks the third stage and ultimate goal of the process. The name will then serve as the latin binomial for the species from that time forward.

The Tulloss model was highly influential when designing our current system. In order to be assigned a temporary code, the species-level unit must 1.) lack reference data in public DNA repositories, 2.) have incomplete reference data, or 3.) have conflicting reference data. This basic model was broadly expanded to macrofungi outside of the Amanitaceae starting in 2016 (Russell, 2022). The benefits of creating provisional codes include streamlining the identification process and gaining the power to link unnamed specimens together for future research, ultimately providing a reliable sequence-based identification regime for macrofungi. Novel individual species-level sequence clusters can be delimited and assigned a temporary code name as sequences are generated. Our temporary code names are roughly equivalent to the UNITE species hypothesis (Nilsson et al., 2018; Abarenkov et al., 2022). Our system allows for new codes to be immediately generated as novel sequences are encountered, without the need to wait for periodic database updates. Further, our code names are not solely sequence-based identifications, but also take morphology, ecology, and phenology into account when circumscribing a putative species-level delimitation. Once species are delimited and a temporary code name is assigned, this method allows quick sequence-based identifications for all future collections of the species. These can be quickly linked to all other collections in the dataset.

Importantly, assigning a temporary code name to a collection is not meant to imply that the species is undescribed or novel. The temporary code represents the fact that we are unable or unwilling to make a definitive sequenced-based identification due to incomplete reference data in public DNA repositories. The temporary code is simply a flag implying the sequence validator believes more work needs to be done before a latin binomial can be reliably assigned. Other options historically utilized by the mycological community include appending cf. (*confer*) or aff. (*affinis*) within a latin binomial e.g. *Amanita* aff. *canescens*. This convention has significant drawbacks if the goal is to outline total biodiversity present in a geographic area. In that case, the same name is used for multiple species-level units and species cannot be easily disaggregated to assess total biodiversity. We also do not believe the term OTU (Operational Taxonomic Unit) is synonymous with the temporary code name methodology outlined here. An OTU typically represents a statistical cluster of environmental sequences based on an arbitrary sequence similarity value, such as 97%. Our temporary code names are dynamically clustered based on the intraspecific and interspecific variation present within the available DNA reference data (i.e., a

barcode gap). Our temporary codes are then backed up and validated by similarities in ecology, phenology, and morphology typically supported by multiple color images *in situ*. One goal for temporary code names is for them to be truly temporary. In this way, they quickly deprecate as more type species are sequenced and more novel species are described. A second and proximate goal is to allow better communication as we assess what is currently known about a species. We can continue aggregating new information about the species in a systematic and methodical manner until the species-level unit is assigned an existing name or formally described.

The process for creating and registering new temporary code names is straightforward. Once a sequenced specimen can be delimited, you can register the name on MycoMap (www.mycomap.com/taxonomy/add/). Two name generation formats are most broadly utilized. The first involves the geographic location of the first collection and a sequential number as mentioned above, e.g. *Amanita* "sp-IN01." The second naming convention is used in cases where 1.) the species has a close relative it is likely to be confused for in the field, 2.) it is a known member of a species cluster, or 3.) if the name commonly used for the species is outdated e.g. *Hygrocybe* "conica-IN04." Once a candidate name is selected, it can be searched in the "Copy From" field at the link above to see if there have been other names registered. For example, type in '*Hygrocybe* "conica' and examine the dropdown to see the other names within this cluster that have been previously registered. Selecting one of the names in the "Copy From" dropdown will copy the classification - kingdom, phylum, class, order, family, and genus from that species record and apply it to your new name. Next, select the rank for the name you will be creating, this will almost always be "Species." Finally enter the name you would like to register into the "Name" field. After submitting, the name is registered to MycoMap and available for use.

In the OMDL project, 9% of successfully barcoded specimens (99) were assigned new temporary codes. Multiple specimens per run were occasionally assigned the same code, thus 99 samples were matched with 94 new temporary codes. The majority of new temporary codes were required for species in the Agaricales, with many in *Cortinarius, Entoloma, Inocybe,* or *Russula* (Table 3). The most new codes were generated for Ohio collections, though these represent a small portion of specimens from that state (Table 4, Figure 5). For states with moderate sampling (>20 specimens), Florida had the highest percentage of new temporary codes (23.5%).

Table 3. New codes by Order, Family, Phylum and Total Fungi.

Order	Family	New Codes
Agaricales	Inocybaceae	9
	Hygrophoraceae	7
	Entolomataceae	6
	Marasmiaceae	6
	Mycenaceae	6
	Cortinariaceae	5
	Strophariaceae	5
	Agaricaceae	4
	Bolbitiaceae	4
	Psathyrellaceae	4
	Tricholomataceae	4
	Hymenogastraceae	3
	Pleurotaceae	3
	Amanitaceae	2
	Clavariaceae	2
	Omphalotaceae	2
	Crepidotaceae	1
	Pluteaceae	1
	Tubariaceae	1
	Total	**75**

Order	Family	New Codes
Russulales	Russulaceae	6
Pezizales	Helvellaceae	1
	Pezizaceae	1
	Sarcosomataceae	1
Boletales	Boletaceae	2
Cantharellales	Clavulinaceae	1
Trechisporales	Hydnodontaceae	1
Thelephorales	Thelephoraceae	1
Polyporales	Polyporaceae	1
Hypocreales	Cordycipitaceae	1
Hymenochaetales	Hymenochaetaceae	1
Gloeophyllales	Gloeophyllaceae	1
Atractiellales	Phleogenaceae	1
Grand Totals	**Basidiomycota**	**90**
	Ascomycota	**4**
	All Fungi	**94**

Table 4. Breakdown of new provisional codes in each state.

U.S. State or International	Fungi with New Codes	Total Fungi Sequenced	% New Codes Each State	% of Total New Codes
OH	36	589	6.3%	37.4%
FL	16	81	23.5%	19.2%
AL	8	47	19.1%	9.1%
WI	5	48	10.4%	5.1%
WA	5	144	3.5%	5.1%
TN	5	33	15.2%	5.1%
NY	5	14	35.7%	5.1%
NC	3	37	8.1%	3.0%
International	3	6	50.0%	3.0%
CO	3	37	8.1%	3.0%
AK	3	41	7.3%	3.0%
GA	1	22	4.5%	1.0%
DE	1	3	33.3%	1.0%

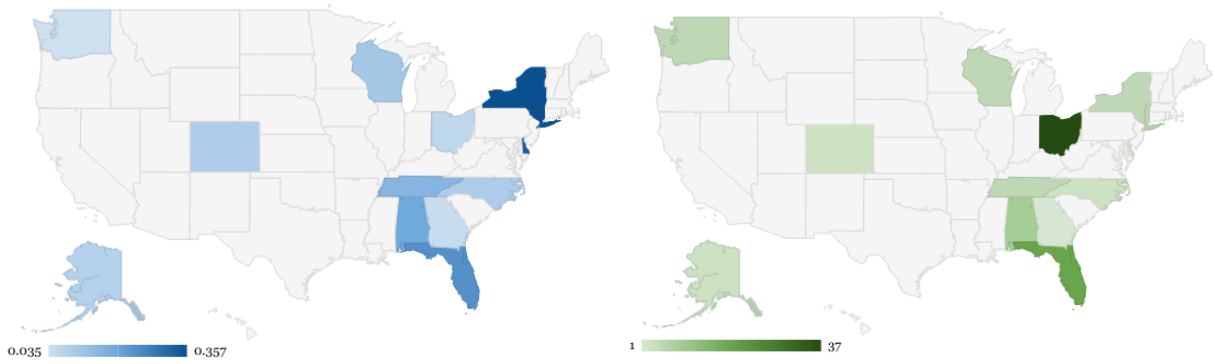


Figure 5. Geographic distribution of new provisional species codes. Left: New provisional codes as numbers. Right: Percent of the total number of specimens representing new provisional codes.

New Distribution Reports

A total of 408 barcoded specimens had 367 new distribution reports, excluding multiple specimens of the same species and distribution expansion. The majority were state records with one country-wide and four continental new distributions. For states with at least moderate sampling (>20 specimens), Alabama had the most new distributions (Figure 6). New distribution reports are nearly evenly split between described taxa and provisional species codes (Figure 7).

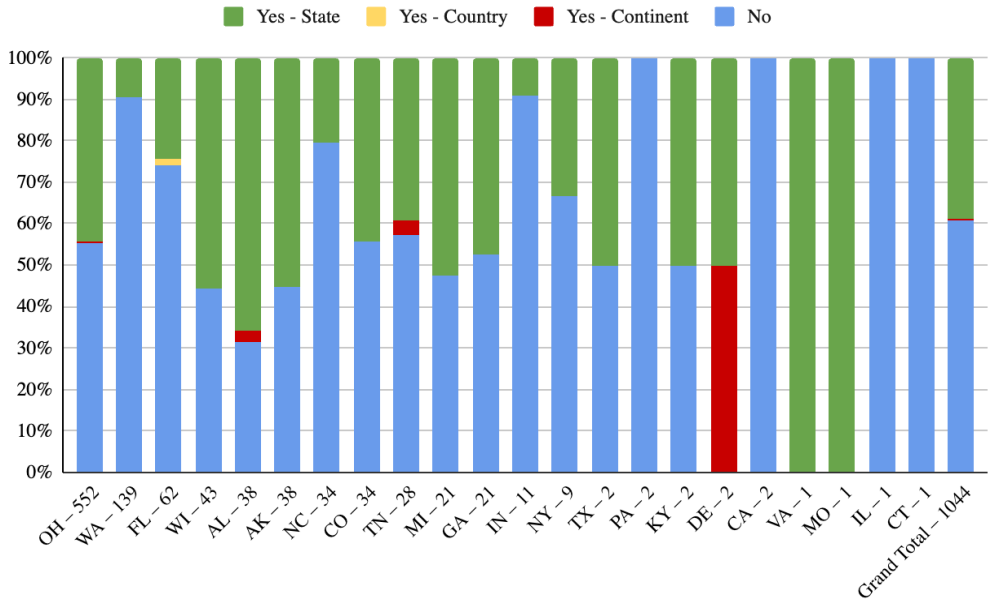


Figure 6. Percent of barcoded specimens (not distribution expansions) from each state by extent of range expansion. Blue: Previously Recorded, Red: New Continent Record, Yellow: New Country Record, Green: New State Record.

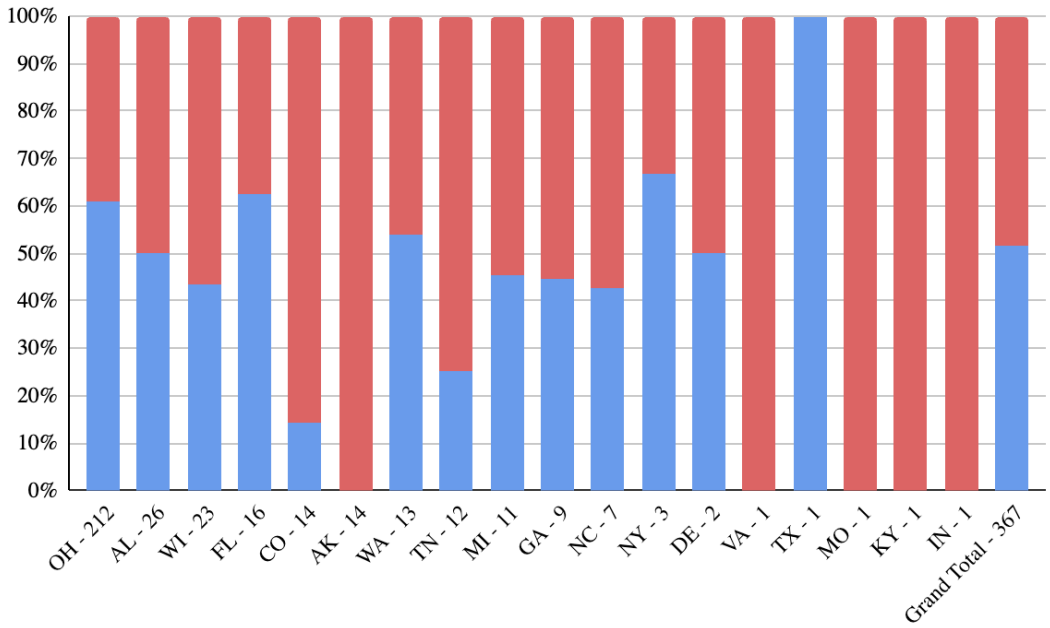


Figure 7. Numbers of new distribution expansions in each state, either representing previously described taxa (in red) or provisional codes (in blue) alongside the grand total geographic expansions.

DISCUSSION

The success rate in our first five runs is lower than those reported by other mycology groups currently utilizing nanopore sequencing (Mycota, FUNDIS). We hypothesize that a significant portion of the overall failure rate is due to two things: the first being evaporation via edge effects in run 1 (OMDL1), and the second being switched plates which necessitated reanalysis. The first issue stemmed from the plastic film used to seal the top of 96-well PCR plates, which did not consistently maintain a proper seal in OMDL1. This caused a reduction in volume along the edge wells of the 96-well PCR plate, ultimately causing PCR failure. We then took advice from the community and initiated use of a rubber mat, cut to size, and placed directly on top of the 96-well plate, which helped to ensure a proper seal in subsequent runs (Harte Singer, FUNDIS, pers. com.). Alternatively, 8-strip PCR tubes with caps can be used instead of the 96-well PCR plates that OMDL has made our laboratory standard. Other issues were identified in OMDL1 - OMDL5 including rotated plates, which can be circumvented by simply pre-labeling the plates before sampling. Another noticeable issue with plate sampling was when this work was outsourced to technicians external to OMDL, the sample amounts in each well were too variable.

Outside technicians had difficulty maintaining necessary minimum tissue sample size requirements, relative to the low volume of each PCR tube. The last issue we were able to identify was DNA concentrations were not quantified post-PCR, which likely contributed to a less-than-optimal success rate during the nanopore library preparation steps. Manifold issues leading to high failure rates have been identified, and failed samples will be reattempted in future runs. We are confident the methodological and analytical optimizations we are making will increase our success rates in the future.

Regardless of future improvements, there will always be a data gap between the outputs of nanopore and Sanger sequencing. Data on success rates generated using nanopore cannot be directly compared to previous Sanger data because of the drastic differences in these technologies and their qualitative methods. With both methods, the basis of 'success' is directly determined by PCR success, and is not an inherent issue with the sequencing technologies themselves. In the past, our low-throughput Sanger sequencing method utilized two PCR attempts before success rates were tallied. Now, several hundred samples can be analyzed on each Flongle flowcell, and a run can be initiated without first checking if the results of PCR were successful. With nanopore, PCR success is determined by a successful sequence being generated, rather than from a band being visible using gel electrophoresis. Another methodological decision to allow high-throughput operations was to use a "quick" extraction protocol. Our method does not involve grinding the fungal tissue, only heating it in an extraction reagent. PCR success for certain groups of macrofungi could be substantially improved if a more robust extraction protocol is employed. The tradeoffs are time and cost. For a second PCR attempt, further dilution of the template should yield success for a substantial number of additional specimens. Further PCR attempts would benefit from a more robust extraction methodology that involves grinding the tissue and cleaning the template.

Most previous research assessing DNA barcodes utilize an arbitrary sequence similarity cutoff, such as 97% or 99%. These cutoffs are typically based on NCBI BLAST identity values to delimit and quantify species (Cantonwine et al., 2022; Fungal Diversity Survey, 2023). Shortcomings of this approach are plentiful and identification of individual specimens can be performed in a more nuanced way based on expertise and experience. While DNA barcoding is

an invaluable component of biodiversity and taxonomic research in light of impediments (Cao et al., 2016), one universal identity threshold does not apply across all taxonomic groups but different thresholds apply to different groups (Wilson et al., 2023). Given that no universal cut-off can be applied, characterizing intra- and inter-specific barcode variation across all taxonomic groups by contributing previously undocumented sequence haplotypes and genotypes is necessary for robust species identification (Phillips et al., 2022). With this in mind, we propose an updated metric based on sequence (not species) novelty, similar to those used by Cantonwine et al. (2022). The Cantonwine categories of DNA sequence novelty was a useful tool screen to summarize, highlight, and compare the uniqueness of results. Yet a modified criterion 'Cantonwine 99' provides a more even distribution of sequence uniqueness. This is particularly evident when accounting for the range between 97% and 99% similarity, the higher end of which is likely more useful when filling space to identify barcoding gaps. While barcode sequence percent similarity can be a very useful line of evidence when seeking identification to species, these criteria should not be used for species identification without additional context e.g., micro- and macroscopic features.

The highest number of new provisional codes were given to specimens collected in Ohio, which is expected given that over half of the successful samples were collected there. Knowledge of fungal distribution is equally poor between previously described/readily identifiable taxa and less well known, provisionally-coded taxa as evidenced by the equal proportion of distribution expansion between the two categories. Ohio had the most new distribution reports while Alabama had the highest percentage of new state records, including two new continent records, when considering states that had ten or more collection records.

We present this data with the aim of tracking our progress over time and encouraging others to compare contributions based on geography, methodology, phenology, ecology, organizational structure, or any other trends of particular interest. In-depth data tracking and periodical strategic reporting could lead to community-wide methodological improvements. The metrics we include quantify the relative uniqueness and novel contributions from each sequencing run. This approach can also bolster the ability to target under-studied locations and taxonomic groups, which can be demonstrated by higher percentages of novel sequences, species codes, and new

distribution reports. Hopefully, highlighting new and noteworthy finds from these runs will encourage further research and engagement between academic and amateur mycologists. In fact, efforts by amateur mycologists are rapidly accomplishing all four strategies proposed by leading mycology experts to realize DNA barcode based molecular identification of fungi, as outlined by Lücking et al. (2020): "(1) broadly document intraspecific and intragenomic variation of barcoding markers; (2) substantially expand sequence repositories, focusing on undersampled clades and missing taxa; (3) improve curation of sequence labels in primary repositories and substantially increase the number of sequences based on verified material; (4) link sequence data to digital information of voucher specimens including imagery."

OMDL is a productive, volunteer group working within a growing community-based initiative focused on fungal diversity in North America and beyond (Appendix B). Using an open-access specimen/metadata tracking system, combined with high-throughput sequencing methods for DNA barcoding, OMDL and its counterparts at Mycota Lab and FUNDIS have all managed to greatly accelerate the pace of discovery and identification of macrofungi. As an astounding result of these community-driven initiatives, and the rapidly accelerating pace of research, almost 20,000 DNA barcodes in 2023 alone were generated from collected specimens and deposited into open-source databases for the benefit of everyone (MycoMap, 2024). The efforts of these cohorts represent a widespread, monumental contribution toward tracking macrofungal biodiversity across North America. An important outcome of biodiversity surveys and taxonomic investigations is the connection to multidisciplinary natural and applied sciences, e.g. evolution, ecology, biogeography, conservation, and more. OMDL has taken our first step in the journey toward broad application of diversity data by observing, sequencing, and mapping the variety of fungi in nature, much of which has been found in our own backyards.

ACKNOWLEDGEMENTS

Stephen Russell, Harte Singer, and Danny Miller for their technical, analytical, and directional support. We could not have accomplished this work without all the amazing mycologists and enthusiasts who submitted specimens to the Ohio Mushroom DNA Lab. Listed here by name or iNat/Mushroom Observer handle: YM Wang, Sigrid Jakob, Bruce Welkovich, Alisha Millican, Penny Cooper, Crystal Davidson, James Chelin, Dryw Morehouse, anthonyhazeabrego, Ben

Bohemier, robstafari, James Conway, Eric Hensley, kazaamer, Drew Henderson, Jeff Kentrup, hicks1328, Erin Braley, Evan Adkins, MushDrywM, hmc8522, Cricket Raspet, Tammy Havel Kinney, Flown Kimmerling, Todd, Paul Bailey, Michael Ballou, Matt Pulk, MycoGeeky, Mossy Creek Mushrooms, Kevin Young, Julie Logterman, John Plischke, happihyphae, Fenner Morse, Caroline Wallace, Brian Hunt, Tim Kennedy, Ron Kerner, Patty B, Michael Williams, Michael W. Beug, kkm_myco, Kendra Dedinsky, Jonathan Scooby Bolha, Jed, jacobjanes, Harte Singer, fungiforthefuture, Fungal Foray, Dean Lyons.

SUPPLEMENTARY DATA

https://docs.google.com/spreadsheets/d/1zri97r52X1xUtUufs0pKP4_DvO4tswkKaPqOPmTTB8w/

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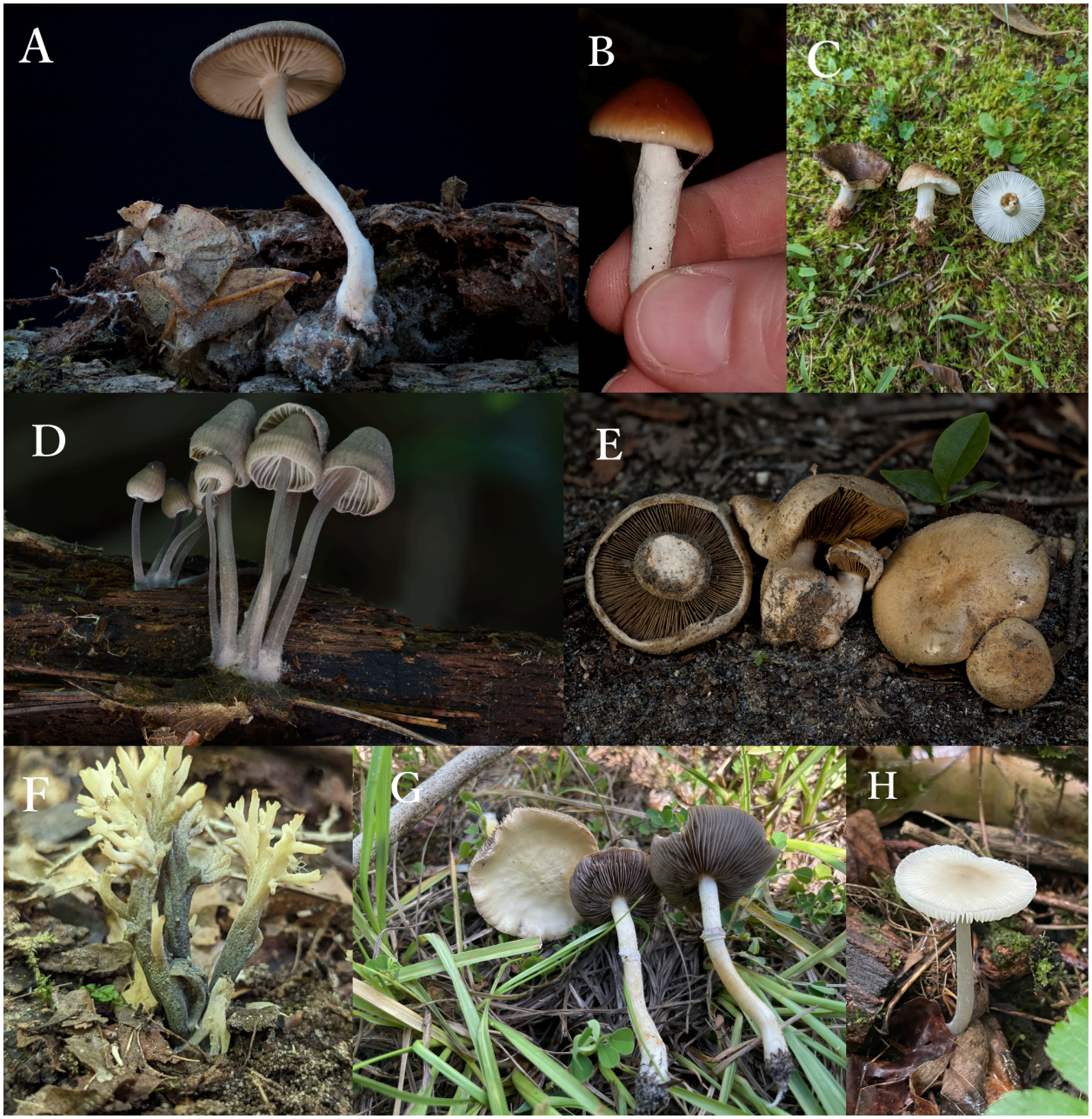
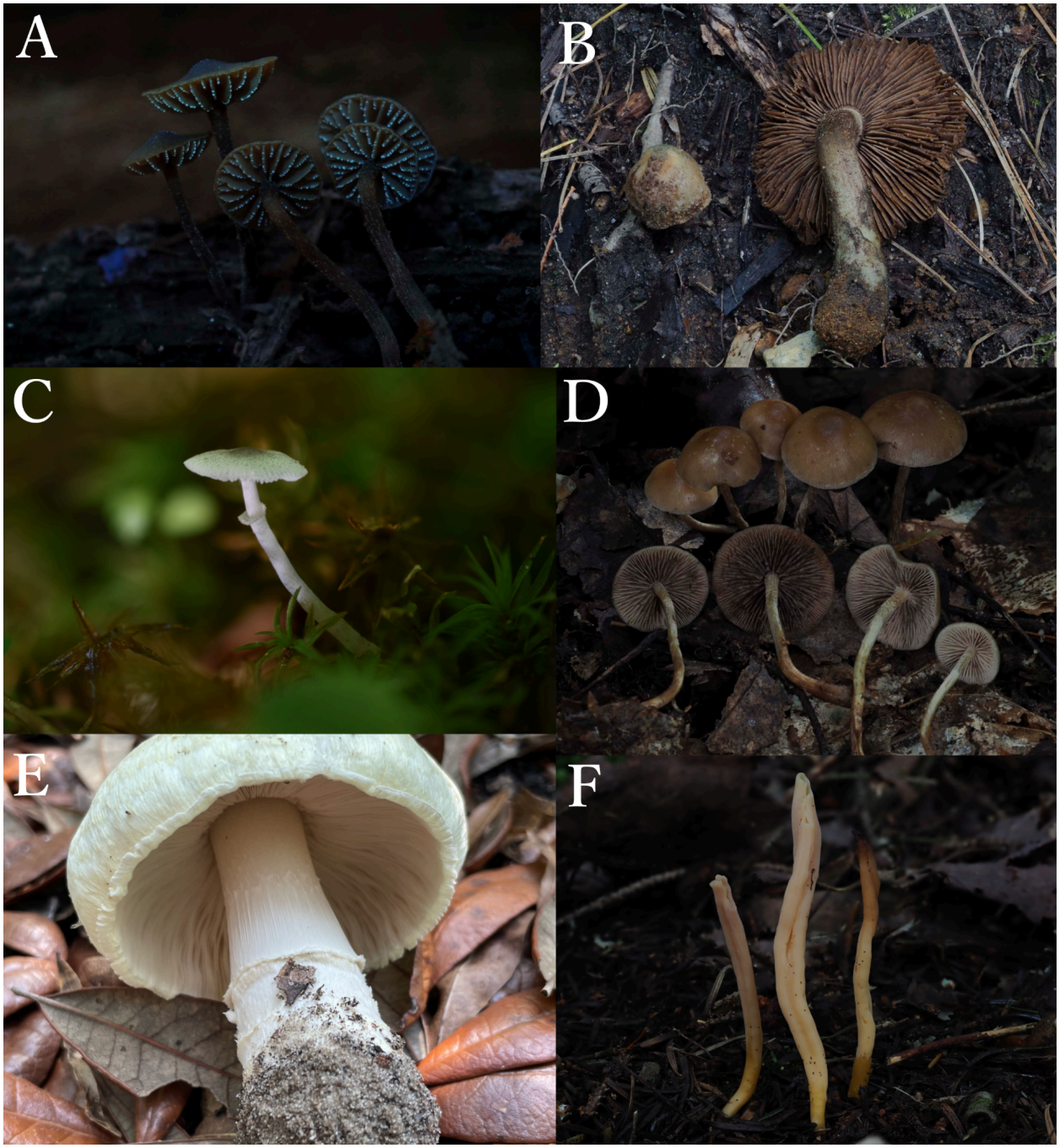
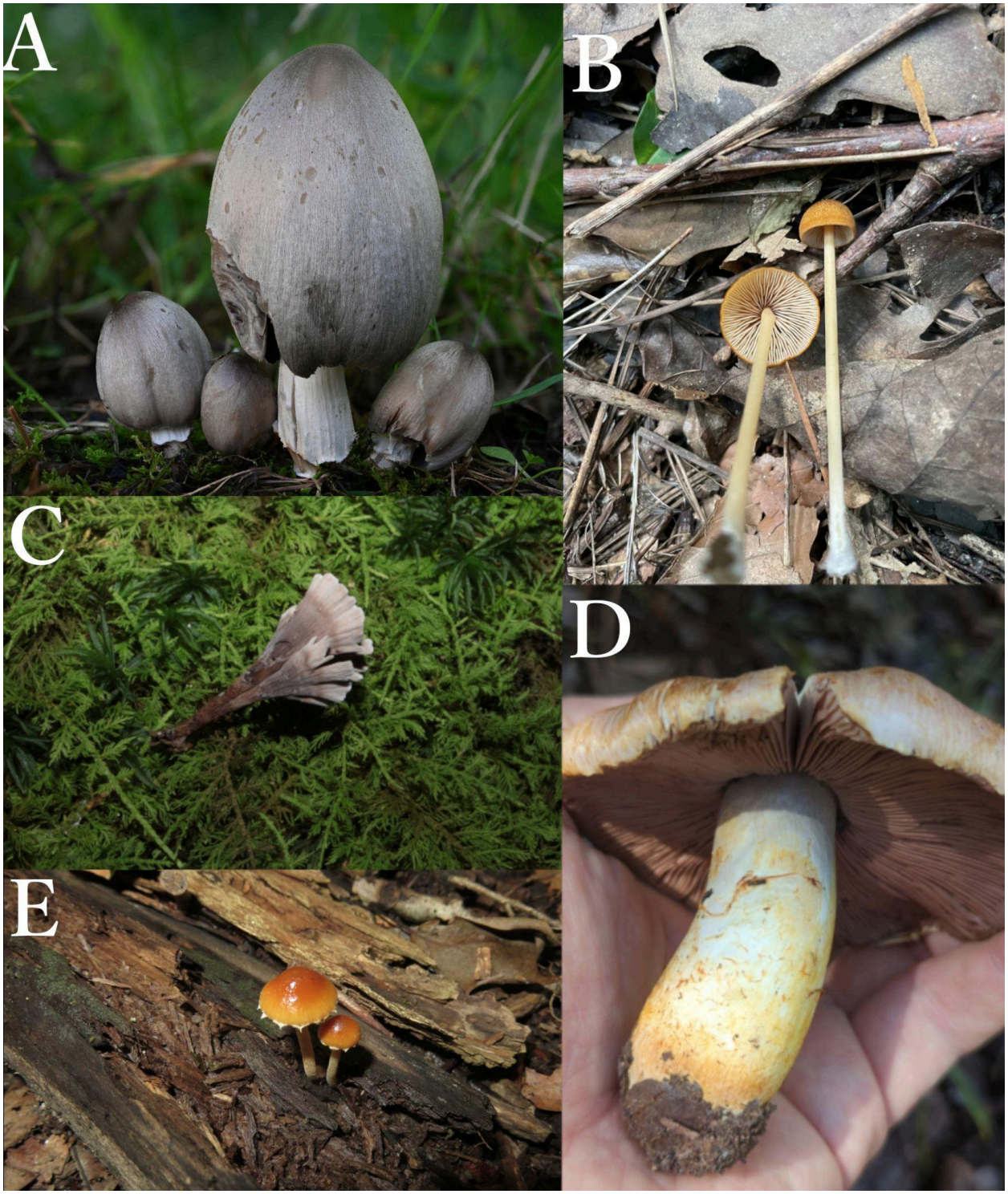
Appendix A



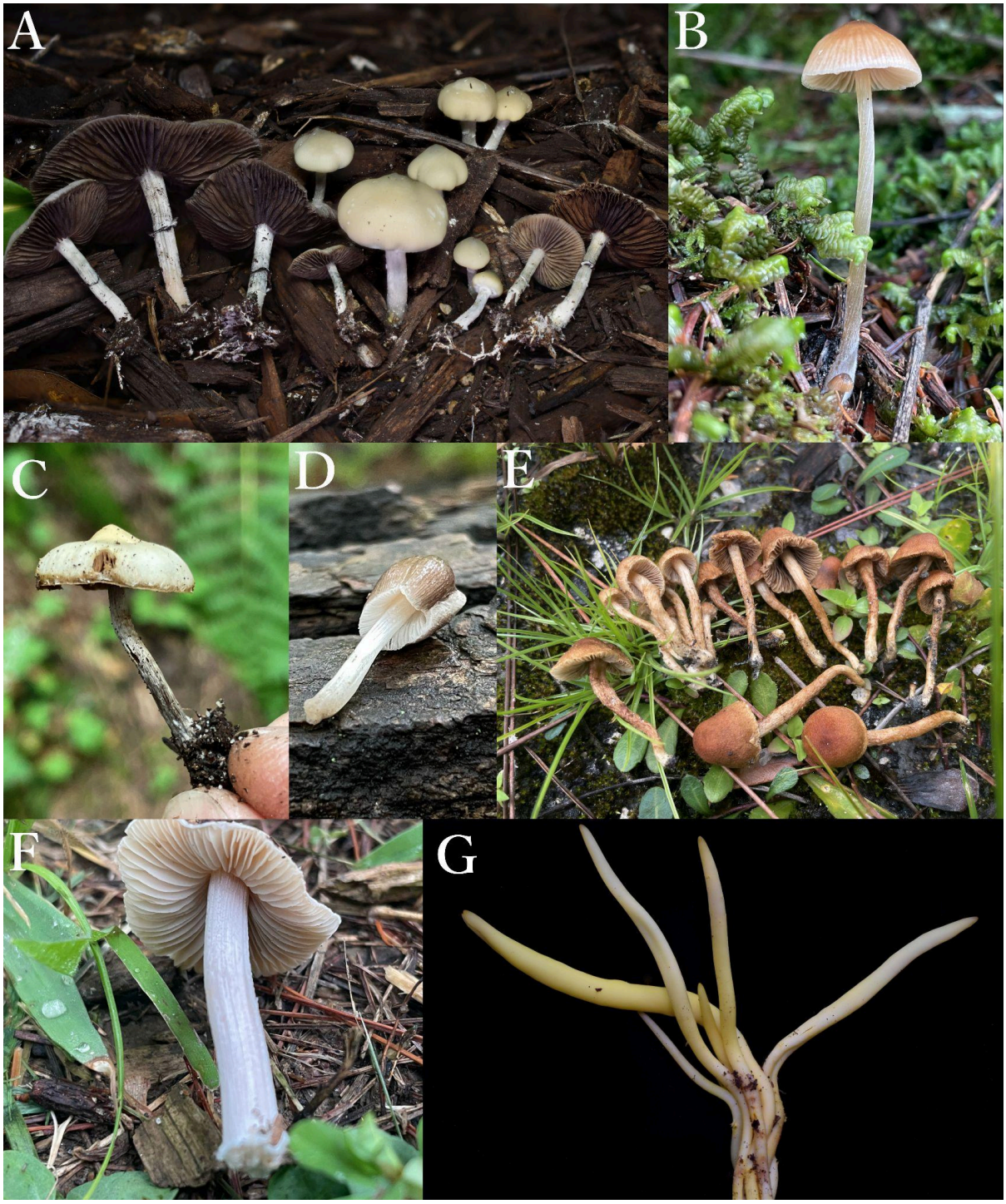
Appendix A Figure 1. Noteworthy fungi submitted and sequenced in OMDL1. A. *Clitocybe* "sp-FL01" MO515621. B. *Cortinarius* "sp-OH01" iNat149262203. C. *Russula* "sp-AL01" iNat168496837. D. *Mycena* "sp-NC01" MO519886 E. *Cortinarius* "sp-IN42" iNat146227121. F. *Clavulina* "sp-AL01" iNat169693109. G. *Stropharia* "sp-FL01" iNat133249975. H. *Mycena niveipes* iNat169200259.



Appendix A Figure 2. Noteworthy fungi submitted and sequenced in OMDL2. A. *Galerina* "sp-NY01" iNat181284206. B. *Inocybe* "glaucescens-NY01" iNat179270646. C. *Leucoagaricus* "sp-NC01" iNat174317433. D. *Psilocybe caerulipes* iNat179755636. E. *Agaricus floridanus* iNat168400858 F. *Clavulinopsis appalachiensis* iNat181275117.



Appendix A Figure 3. Noteworthy fungi sequenced in OMDL3. A. *Coprinopsis pinguispora* iNat183906217. B. *Entoloma* "sp-GA01" iNat172874743. C. *Thelephora regularis* iNat176367801. D. *Agaricus jacobi* iNat132229456. E. *Pholiotina* "sp-OH01" iNat176204927.



Appendix A Figure 4. Noteworthy fungi sequenced in OMDL4 and OMDL5. A. *Psilocybe niveotropicalis* nom. prov. iNat166495895. B. *Entoloma cetratum* iNat179847663. C. *Hymenogastraceae* PNW01 iNat174787997. D. *Pluteus* “sp-OH01” iNat173103880. E. *Mallocybe* “sp-FL02” iNat180019393. F. *Inocybe grammatoides* iNat178712707. G. *Clavulinopsis* “fusiformis-NC01” iNat177411637.

Appendix A. Table 1. Photograph attribution for Appendix A Figures 1-5.

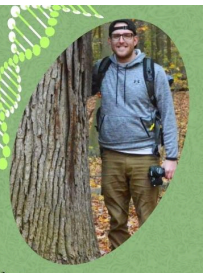
Figure	Panel	Owner	License	Source
1	A	Scott Ostuni (scott)	CC BY-SA 3.0	https://mushroomobserver.org/images/1547596
1	B	Crystal Davidson (squirrely)	CC BY-NC 4.0	https://www.inaturalist.org/observations/149262203
1	C	Alisha Millican (cabracrazy)	©Alisha Millican, all rights reserved	https://www.inaturalist.org/observations/168496837
1	D	Scott Ostuni (scott)	CC BY-SA 3.0	https://mushroomobserver.org/images/1563356
1	E	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/146227121
1	F	Alisha Millican (cabracrazy)	©Alisha Millican, all rights reserved	https://www.inaturalist.org/observations/169693109
1	G	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/133249975
1	H	Kyle Canan - Ohio Mushroom DNA Lab (kylecanan)	CC BY-NC 4.0	https://www.inaturalist.org/observations/169200259
2	A	James Conway (laughingjims)	CC BY-NC 4.0	https://www.inaturalist.org/observations/181284206
2	B	James Conway (laughingjims)	CC BY-NC 4.0	https://www.inaturalist.org/observations/179270646
2	C	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/174317433
2	D	James Conway (laughingjims)	CC BY-NC 4.0	https://www.inaturalist.org/observations/179755636
2	E	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/168400858
2	F	James Conway (laughingjims)	CC BY-NC 4.0	https://www.inaturalist.org/observations/181275117
3	A	Mandie Quark (mandiequark)	CC BY-NC 4.0	https://www.inaturalist.org/observations/183906217
3	B	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/172874743
3	C	Sarah Culliton (verpahh)	CC BY 4.0	https://www.inaturalist.org/observations/176367801
3	D	Alan Rockefeller (alan_rockefeller)	CC BY 4.0	https://www.inaturalist.org/observations/132229456
3	E	Sarah Culliton (verpahh)	CC BY 4.0	https://www.inaturalist.org/observations/176204927
4	A	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/166495895
4	B	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/179847663
4	C	Yi-Min Wang (ym_wang_pnw)	CC BY-NC 4.0	https://www.inaturalist.org/observations/174787997
4	D	Kyle Canan - Ohio Mushroom DNA Lab (kylecanan)	CC BY-NC 4.0	https://www.inaturalist.org/observations/173103880
4	E	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/180019393
4	F	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/178712707
4	G	Scott Ostuni (scottostuni)	©Scott Ostuni, all rights reserved	https://www.inaturalist.org/observations/177411637

Appendix B

Introducing The Ohio Mushroom DNA Lab Team with biographies and photos

Kyle Canan

OMDL Founder
DNA Sequencing Lead



Kyle Canan is an avid naturalist, mushroom enthusiast, and lover of trees. He was born and raised in southwest Ohio where he currently resides. Kyle's top priority is outlining the biodiversity of fungi in the state of Ohio through DNA barcoding. In his free time he loves learning about the properties of fungi; whether edible, medicinal, or psychoactive. Kyle founded OMDL in 2022 and began high throughput nanopore sequencing in 2023. Kyle is a board member of the Ohio Mushroom Society and leads mushroom forays across the state. He is also the Darke County coordinator of the Ohio Old-growth Forest Network. The OMDL Team benefits from Kyle's leadership, with his palpable passion and contagious energy, he naturally encourages everyone around him to thrive.

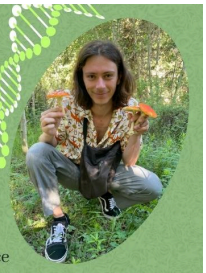
Mandie Quark

Researcher and Communications Lead

Molecular biologist and grant writer by training, Mandie Quark is a researcher, writer, and editor with a passion for the advancement of community mycology. Currently she is devoted to fungal DNA barcoding initiatives, and uses her gift of creative communication to explain the technical complexities of sequencing to her peers. Her work also includes being Communications Lead for the Fungal Diversity Survey. She promotes her own company Mycena LLC which includes her series of classes specifically tailored for mycologists. Mandie organizes international forays and mycology events, and is an active member of the North American Mycological Association DNA Sequencing Committee. She lives and works in the California Bay Area, while continuing to tour the world as a keynote speaker, photographer, and field collector during mushroom season.

Scott Ostuni

Lead Sequence Validator



Scott Ostuni is an autodidact, amateur mycologist, and mushroom photographer who concentrates on fungal taxonomy. In his Sequence Validation role, he specializes in fungal identification based on DNA sequence data, as well as incorporating more traditional methods of ID. Scott lives in Florida and mainly focuses on fungi of the southeastern United States. Yet, as part of his Validator position in the Fungal Diversity Survey, he is also helping to outline California's macrofungi. Some of Scott's accolades include discovering and naming a new species of psilocybin-containing mushroom "*Psilocybe niveotropicalis* nom. prov." as well as increasing understanding of the distribution of various fungal species in the state of Florida. Scott is extremely passionate about recruiting aspiring mycologists to help outline the biodiversity of Florida fungi.

Alan Rockefeller

Sequence Validator

Alan is an expert mycologist in high demand at mushroom events where he teaches workshops on DNA barcoding, field photography, and fungal microscopy. Since 2001 he has photographed more than 2,500 species of fungi. Alan's contributions to community science have been widespread, and his dedication to teaching thousands of people over several years how to DNA sequence fungi is remarkable, and remains unparalleled. Alan has uploaded more than 700 of his own fungal DNA sequences to Genbank, and he is a co-author on several scientific papers, including publications documenting new species of bioluminescent Mycena and Psilocybe. Alan is devoted to the art of macroscopic identification and spends hours each day identifying mushrooms for the general public. Amazingly, he has identified almost 300,000 fungi on websites like iNat and Mushroom Observer.

Continued on the next page…

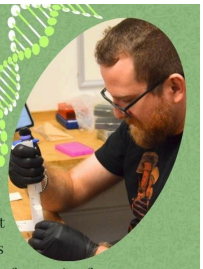
Jessica Williams
Sequence Validator

Jessica Williams is an avid wild mushroom forager and mycologist. She loves her shelf full of mycology books and has an obvious passion for learning as much as she can about the fungal world surrounding us all. In particular, Jessica is enamored with the fungal family Boletaceae and all of the variety contained within that group. She is an active identifier on iNaturalist, and is truly passionate about the potential that community mycology efforts have to advance the collective knowledge of mankind. Jessica lives and works in Ohio, and she is currently employed at Epiphany Mushroom Company as a Lab Assistant and Mushroom Grower, working to produce edible & medicinal mushrooms. During mid-summer in the midwestern woods, you can find her happily sniffing a fist full of *Boletus separans*.

Sarah Culliton
Sequence Validator

Sarah Culliton is a molecular biology student finishing her bachelor's degree part time at Hiram College while working full time for the Ohio Environmental Protection Agency. Sarah has been studying Ohio fungi since 2014, beginning with wild edible mushroom foraging, she slowly expanded her interest to include all things fungi, including genomic research. Sarah was the 2023 recipient of the North Spore Mycology Scholarship and plans to complete independent fungi research at Hiram College's James H. Barrow Biological Field Station in 2024. Sarah is currently cooperating with northeast Ohio park districts and the Cleveland Museum of Natural History, obtaining research permits, and presenting data collected by OMDL to be used by park districts and the public to continue the preservation of ecologically important lands.

Zach Guerin
Bioinformatician
Genomics Consultant



Zach Geurin is a classically trained microbiologist with a BS in Microbiology and Molecular Genetics from Michigan State University and an MS in Bioinformatics from Johns Hopkins University. He has extensive experience in molecular biology, next-generation sequencing, and bioinformatics. Zach recently transitioned from a prokaryote-focused career and is now an avid student of mycology. In addition to his work with OMDL, Zach serves as the Director of Research for Epiphany Mushroom Company while living remotely in Chicago, Illinois. In his role at OMDL, Zach helps the team with sequencing methods, data tracking and analytics, as well as top level molecular biology consulting. He is also currently working on several grant opportunities toward optimization of the entire nanopore sequencing workflow and data outputs.

Joshua Birkebak
Senior Researcher
Principal Investigator

Joshua lives in Knoxville, TN and works as a researcher at Oak Ridge National Laboratories by day. By night he is the Principal Investigator and Senior Researcher at OMDL. Joshua earned his B.S. in Plant Science from the University of Washington in 2009 and received his PhD from the University of Tennessee in 2015 in Ecology and Evolutionary Biology while studying under the legendary Dr. Brandon Matheny. Joshua is keen on mushroom taxonomy and has published work on various fungal groups but has particularly focused on the family Clavariaceae. As a member of the North American Mycological Association and current Chair of the NAMA DNA Sequencing Committee, Joshua is passionate about involving the broader community in amateur mycology and research.