DNA BARCODING OF MACROFUNGI FROM THE 2018 SMITH FORAY: NEW FUNGAL RECORDS FOR WISCONSIN AND THE UNITED STATES OF AMERICA

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ABSTRACT

For accurate evaluation of fungal conservation, modern biodiversity surveys based on vouchered specimens and DNA barcoding are needed to augment records of fungal distribution and phenology. Even relatively well studied and populated regions of the United States lack comprehensive information on fungal biodiversity, which hampers our ability to swiftly respond to fungal population decline due to habitat loss, climate change, or other anthropogenic stressors. During the 2018 Smith Foray in Dane County, Wisconsin, we vouchered and DNA barcoded 63 specimens of macrofungi. Three species constituted first records for the United States, and 14 additional species were reported for the first time from the state of Wisconsin. Furthermore, eight species were new reports just at the county level, and barcode data for two species represented first records in GenBank, the national public repository for genetic information. Twenty-four specimens were assigned informal placeholder names due to the lack of similar references in GenBank and are fertile ground for future taxonomic studies. While sequence-based identification requires caution due to inaccuracies in reference databases, the prevalence of multilocus genetic data in contemporary taxonomy facilitates global linkages in fungal distribution and increasingly traceable biodiversity assessments.

KEYWORDS: Dane County, fungal biodiversity inventory, mushrooms, North American Mycoflora Project, sequence-based identification

INTRODUCTION

Despite their enormous importance for global ecosystem functioning and as reservoirs of genetic resources, fungi are one of the most understudied groups of organisms, especially in the context of conservation (Hawksworth 2004; Heilmann-Clausen et al. 2014; Willis 2018). Mycologists estimate that the total number of fungi on Earth may exceed 5 million species, yet we have described only approximately 150,000, or about three percent of the estimated total (Blackwell 2011; Willis 2018). Many regions, including ones with a long history of profes-

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sional mycological activity, lack comprehensive data on fungal biodiversity (Haelewaters et al. 2018). Such knowledge gaps in local funga (Kuhar et al. 2018)—undescribed fungal biodiversity and poorly understood distributions and phenology—hamper our ability to assess and respond to population declines. Anthropogenic disturbances such as climate change, habitat destruction, and excess nitrogen deposition threaten the well-being of fungi and the ecosystems whose functioning depends on them (Mueller 2017; Andrew et al. 2018; van der Linde et al. 2018), thereby lending urgency to increased biodiversity surveys for the accurate evaluation of the conservation status of fungal species. High-quality vouchers of mushrooms, ideally paired with detailed photography and DNA barcode data, serve as evidence of reproducing populations of macrofungi and as benchmarks for future assessments of fungal range shifts and population declines (Andrew et al. 2018).

In this paper, we report on macrofungi that were vouchered and DNA barcoded from the 2018 A.H. Smith Lake States Foray (commonly known as the "Smith Foray") on October 4–7, 2018 near Mazomanie, Wisconsin. This was the 44th annual Smith Foray, which has been held each year since 1975 to honor the mycological accomplishments of Dr. Alexander Smith (1904–1986) (Thiers 1987) and to foster interactions among mycologists in the Great Lakes region. Past foray locations have included sites in Indiana, Illinois, Michigan, Minnesota, and Wisconsin (Mycological Society of America 2019). Attendees typically consist of professional mycologists from the upper Midwest and their students, as well as other amateur and professional scientists who are interested in fungi. This annual event is an opportunity to enhance our knowledge of macrofungal biodiversity from the Great Lakes states, and ultimately to contribute our understanding of the response of fungi to global change (Andrew et al. 2018), by augmenting records of occurrence, distribution, and phenology of local mushroom taxa.

MATERIALS AND METHODS

Study-site Description

Fungi were collected from four areas in Dane County, Wisconsin, during the 2018 Smith Foray (Figure 1)—Festge County Park, Mazomanie Bottoms State Natural Area, Mazomanie Oak Barrens State Natural Area, and Walking Iron County Park—as well as opportunistically from several other locations around Dane County. These locations exist on the eastern edge of the extensive Driftless Area, a region surrounding the upper Mississippi River in Wisconsin, Minnesota, Iowa, and Illinois. As indicated by a lack of glacial till, the Driftless region was never glaciated during the last Ice Age (Hobbs 1999). It is characterized by a topology of rolling hills that contrasts with an otherwise smooth Midwestern landscape. Special geological features and ecosystems such as algific talus slopes harbor unique and speciose funga (Hawksworth 2010; Thompson and Colbert 2020). A short description of each of the four primary collecting sites is as follows:

Festge County Park in Cross Plains (43.121744 N, –89.6829076 W): Mature *Carya* spp. (hickory) and *Quercus* spp. (oak) are the primary tree species here. The steep topography of Festge County Park is evidence of the unglaciated history of this region and provides an overlook of the Black Earth Creek Valley and Blue Mound State Park in the distance.

Mazomanie Bottoms State Natural Area in Mazomanie (43.219381 N, –89.818698 W): This site encompasses a large area of Wisconsin River floodplain forest. The forest is dominated by *Acer saccharinum* L. (silver maple), *Ulmus* spp. (elm), *Tilia americana* L. (American basswood), and *Fraxinus* spp. (ash) and, in addition, contains occasional individuals of *Quercus bicolor* Willd. (swamp

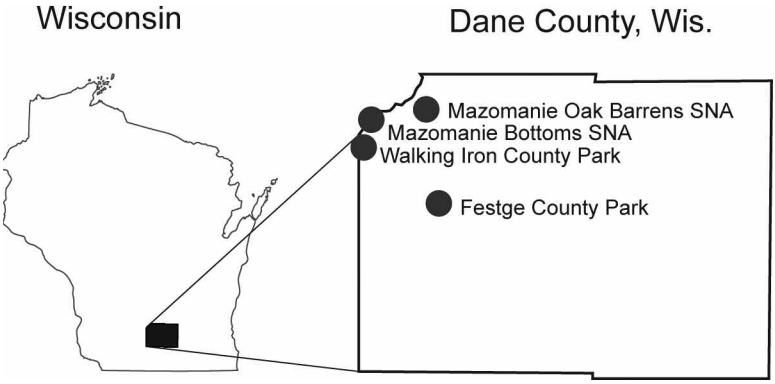

FIGURE 1. Map of the locations of the 2018 Smith Foray collection sites in Dane County, Wisconsin. "SNA" is an abbreviation for "State Natural Area".

white oak), *Populus deltoides* W. Bartram ex Marshall (eastern cottonwood), *Salix* spp. (willow), and *Betula nigra* L. (river birch). Elm mortality has resulted in canopy openings that support a dense understory of native and introduced shrubs. Periodic flooding results in sand bars, ephemeral pools, and river channels running through the forest.

Mazomanie Oak Barrens State Natural Area in Mazomanie (43.242305 N, –89.739786 W): This site is decidedly drier than the Mazomanie floodplain forests surrounding the Wisconsin River. Wisconsin's native cactus, *Opuntia cespitosa* Raf. (eastern prickly pear cactus) is abundant at Mazomanie Oak Barrens. Oak species such as *Quercus macrocarpa* Michx. (bur oak), *Quercus velutina* Lam. (black oak), and *Quercus alba* L. (white oak) interspersed with xeric prairie dominate in this dry, sandy environment.

Walking Iron County Park in Mazomanie (43.187734 N, –89.8246248 W): This park preserves a segment of the extensive prairie that extended from the bottoms area of the Wisconsin River to the surrounding oak savanna. Most of the park is sandy uplands covered by grasslands, including some unplowed, remnant prairie. The north area has a ridge that drops sharply down to Marsh Creek, one example of the many cool, spring-fed streams found in this part of the state.

Specimen Collection, Processing, and Vouchering

Macrofungal sporocarps were opportunistically collected at the foray locations by event participants and brought to a centralized processing area at Hoofbeat Ridge Camps. No effort was made to systematically cover a collecting location. Each specimen was tentatively identified by a local expert and recorded into a central database along with metadata such as collection location. Sixty-three mushrooms from the event were selected for DNA sequencing. These specimens were generally species that were new to the Smith Foray, of particular interest to the foray attendants, or that lacked reference sequence data in public repositories. Each specimen that was selected for DNA sequencing was assigned a collection number, photographed, and uploaded to iNaturalist (2020). The selected specimens were thoroughly dried in a dehydrator (Presto 06301) at 32°C. Dried specimens were deposited in the Kriebel Fungarium (PUL) at Purdue University and were digitally accessioned in the Mycology Collections data Portal (MyCoPortal 2020; Miller and Bates 2017).

Molecular Methods

Mushroom tissue was extracted from the interior flesh or gill tissue of fresh specimens at the foray processing center utilizing sterile forceps. The tissue was placed in 2.0 mL screw-top microcentrifuge tubes containing 600 µL of Promega Nuclei Lysis Solution (Promega Corp., Madison, Wisconsin). Each tube was labeled with the specimen's collection number and transported to the Aime Lab at Purdue University in West Lafayette, Indiana for DNA extraction and amplification. DNA extraction was accomplished by macerating the tissue using a sterile pestle, heating the solu-

tion at 65°C for 15 minutes, and centrifuging the contents of the tube at 21,000 g for three minutes. The supernatant was transferred to a 1.5 mL microcentrifuge tube, 200 µL of Promega Nuclei Lysis Solution was added, and the tube was then vortexed for 20 seconds. The solution was centrifuged again at 21,000 g for six minutes and the supernatant was added to a new, sterile, 1.5 mL microcentrifuge tube. 600 µL of 100% isopropanol was added to the supernatant to precipitate the DNA. The solution was centrifuged for one minute at 21,000 g and the supernatant was poured off, leaving the DNA pellet in the bottom of the tube. 600 µL of 70% ethanol was added to the tube, and the solution was centrifuged a final time for one minute at 21,000 g. The ethanol was poured out and the 1.5 mL microcentrifuge tube was placed upside down on a Kimwipe overnight. The following day, 30 µL of water was added to the tube, resulting in purified DNA for use in PCR amplification.

PCR amplifications of the internal transcribed spacer (ITS) ribosomal DNA (rDNA) region—the universal DNA barcode marker of fungi (Schoch et al. 2012)—were carried out using the ITS1F forward primer and the ITS4 reverse primer (White et al. 1990; Gardes and Bruns 1993). Each PCR reaction contained 12.5 µL Promega PCR Master Mix, 9 µL water, 1.25 µL forward primer, 1.25 µL reverse primer, and 1 µL DNA template for a total PCR volume of 25 µL. The following PCR protocol was used: (i) initial denaturation at 9C for one minute; (ii) 30 cycles of denaturation at 94°C for one minute, annealing at 51°C for one minute, and extension at 72°C for one minute; (iii) hold at 72°C for eight minutes. Electrophoresis with a 1% agarose gel was used to verify successful amplification. PCR amplicons were sent to Genewiz (Genewiz, Inc., Boston, Massachusetts, USA) for sequencing of both the forward and reverse DNA strands. The two reads were assembled using Sequencher 5.0.1 (Gene Codes Corp., Ann Arbor, Michigan) and the consensus sequence was deposited in GenBank (Clark et al. 2016). Raw DNA sequence data (trace files) are available at the 2018 Smith Foray MycoMap project (MycoMap 2018).

Species-level Determination

Identifications were made with a combination of macroscopic, microscopic, and/or ITS rDNA sequence analysis. For sequence-based identifications, consensus sequences were analyzed with the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST®) (NCBI 2020) using the "megablast" option and excluding "uncultured/environmental sample sequences". Species-level assignments were made based on a minimum percent identity threshold of 98.5% and query coverage of 80%. Ambiguous nucleotides in the consensus sequence were regarded as correct if they matched the correct nucleotide in the reference alignment. Species-level identifications were not made if more than one specific epithet in the BLAST search corresponded with the cutoff values above. However, highly similar sequences from type specimens and UNITE species hypotheses took precedence when available (Nilsson et al. 2019). Species names were checked for synonymy and verified using MycoBank (Robert et al. 2013; MycoBank 2020). To facilitate the tracking of operational taxonomic units for future taxonomic research and biodiversity surveys, informal placeholder names were utilized. For specimens with ≥ 98.5% similarity to GenBank references with provisional specific epithets, those names were adopted here (e.g., taxa assigned a *nomen provisorum* by *Amanitaceae* expert Rod Tulloss); in addition, new placeholder labels (designated with the state initials for Wisconsin, WI) were generated for specimens for which no reference sequences had ≥ 98.5% similarity in GenBank (Table 1). These informal names serve to delineate likely taxa at the species level but do not necessarily imply that a given species is taxonomically novel; rather, additional research is required to obtain a sequence-supported identification for that specimen. Finally, to determine whether any identified specimens represented first records for Dane County, Wisconsin, or the United States, the currently known distribution of each species was checked in MyCoPortal (Miller and Bates 2017).

RESULTS

The identities and accession data of the 2018 Smith Foray macrofungi are listed in Table 1. Of the 63 specimens that were vouchered and sequenced, 35 (56%) were identified to officially described species, 24 (38%) were assigned

species-level informal names, and the remaining four specimens (6%) could only be identified to the genus level due to conflicting reference information. Taxonomically, these collections were spread across the phyla Ascomycota (five specimens) and Basidiomycota (58 specimens) and included 44 different genera. Twenty-five species represented new records for Dane County, 17 represented new records for the state of Wisconsin, and three represented new records for the United States. New records are indicated in Table 1.

DISCUSSION

A small portion of the total estimated number of fungal species are currently described, and even fewer have been evaluated for their conservation status (Hawksworth and Lücking 2017; Mueller 2017). More surveys of macrofungi are desperately needed to advance our understanding of fungal biodiversity and distribution, especially in the tropics (Aime and Brearley 2012). In turn, these data aid global change biologists in assessing fungal range shifts and population declines. By opportunistically sequencing specimens collected during the 2018 Smith Foray in Mazomanie, Wisconsin, we significantly expanded the known ranges of 17 fungal species, including three species that had not been previously reported from the United States, and provided novel genetic barcode data for 11 specimens of uncertain species-level taxonomic affinity (those with newly assigned "WI" informal placeholder labels) .

While great care is required in accurately interpreting sequencing results (Haelewaters et al. 2018; Hofstetter et al. 2019), modern biodiversity surveys of macrofungi using DNA barcoding routinely result in significant range expansions and uncover potentially novel species. For example, Haelewaters et al. (2018) discovered four new taxa, new fungal records for North America and Massachusetts, and a novel ecological interaction between a cheese mold (*Chrysosporium sulfureum* (Fiedl.) Oorschot & Samson) and woodlice (Crustacea: Malacostraca: Isopoda: Oniscidea) at a popular urban-island national park outside of Boston. Hofstetter et al. (2019) documented the polypore *Antrodiella stipitata* H.S. Yuan & Y.C. Dai for the first time in Europe and recorded four other macrofungal species for the first time in Switzerland, not to mention numerous very rare and indicator taxa. Together, these studies reveal the great paucity of information on fungal biogeography and the fact that fungi, even terrestrial macrofungi in populated areas, are an understudied reservoir of biodiversity. In the following paragraphs, we discuss several of the most interesting collections that were first records for the state of Wisconsin or for the entire United States.

Highlighted New Records for Wisconsin

Cortinarius dolabratus Fr. has been previously documented from Europe and North America, but in the United States it had only been collected in Alaska, California, and Washington (Liimatainen et al. 2017). Our collection (iNaturalist

TABLE 1. List of vouchered and DNA-barcoded fungal specimens from the 2018 Smith Foray in Mazomanie, Wisconsin. Each row corresponds to a single specimen and lists that specimen's determination as well as its accession numbers for iNaturalist (photos and metadata), MyCoPortal (fungarium information), and GenBank (ITS rDNA sequence). Informal placeholder names are enclosed by quotation marks; these names have either been propagated from other sources (in which case the name is followed by a citation) or are new labels from this study (all WI labels). The last column indicates whether a specimen was a new geographic record for just Dane County (DC), for Wisconsin and Dane County (WI), or for the United States, Wisconsin, and Dane County inclusive (US), or whether the ITS rDNA sequence that was generated for that specimen was the first reference for that species on GenBank; if the specimen was not novel in any of these regards, the column is marked with a hyphen.

Species	iNaturalist	MyCoPortal	GenBank	New Record
Agaricus kriegeri Kerrigan	17333117	6596294	MK573882	WI
Agaricus pallens L.A. Parra	17232000	6596228	MN989986	WI
Amanita solaniolens H.L. Stewart & Grund	17338999	6596241	MK573911	WI
Amanita sp. "longicuneus" (Tulloss and Rodríguez Caycedo 2020)	17333556	6596284	MK573886	-
Amanita sp. "texasorora" (Tulloss et al. 2020)	17231768	6596227	MK573879	-
Byssocorticium atrovirens (Fr.) Bondartsev & Singer	17333142	6596293	MN989989	-
Chalciporus piperatus (Bull.) Bataille	17338127	6596254	MK573906	DC
Clitocella sp. "WI-01"	17340579	6596231	MK573922	-
Clitocybe sp.	17340117	6596236	MK573913	-
Clitopilus abortivus Berk. & M.A. Curtis	17340459	6596234	MK573919	-
Collybia cookei (Bres.) J.D. Arnold	17231324	6596225	MK573873	DC
Coprinellus sp.	17334230	6596275	MK573891	-
Coprinellus sp. "IN-01" (Russell 2020)	17231181	6596224	MK573872	-
Coprinellus sp. "IN-01" (Russell 2020)	17339226	6596244	MK573918	-
Cortinarius dolabratus Fr. (Russell 2020)	17336721	6596258	MK573902	-
Cystoagaricus sp. "WI-01"	17332756	6596282	MK573876	-
Cystodermella sp. "IN-01" (Russell 2020)	17335122	6596272	MK573895	-
Cystodermella sp. "IN-01" (Russell 2020)	17339256	6596243	MK573917	-
Cystolepiota sp.	17334037	6596278	MK573889	-
Echinoderma sp. "IN-01" (Russell 2020)	17335043	6596283	MK573894	-
Entoloma psammophilohebes Vila & J. Fernández	17337073	6596255	MK573905	US
Flammula sp. "WI-01"	17338285	6596251	MK573909	-
Fuscopostia fragilis (Fr.) B.K. Cui, L.L. Shen & Y.C. Dai	17333313	6596290	MK573885	-
Galerina sp. "WI-01"	17339978	6596238	MK573914	-
Galerina triscopa (Fr.) Kühner	17338265	6596252	MK573908	WI
Gerronema subclavatum (Peck) Singer ex Redhead	17333993	6596279	MK573888	WI

Hygrocybe cantharellus (Schwein.) Murrill	17340623	6596230	MK573923	-
Hygrophorus sordidus Velen.	17333057	6596296	MK573880	DC
Hymenoscyphus fructigenus (Bull.) Gray	17332929	6596297	MK573877	-
Hymenoscyphus immutabilis (Fuckel) Dennis	17334099	6596277	MK573890	WI
Hypomyces sp. "WI-01"	17338188	6596253	MK573907	-
Inocybe ericetorum Vauras & Kokkonen	17323865	6596229	MK573874	US
Inocybe griseoscabrosa (Peck) Earle	17336824	6596256	MK573904	WI
Lactarius imperceptus Beardslee & Burl.	17339056	6596247	MK573912	DC
Lactarius sp. "IN-06" (Russell 2020)	17338937	6596250	MN989993	-
Lentinellus ursinus (Fr.) Kühner	17335268	6596266	MK573897	-
Lepiota castanea Quél.	17334272	6596273	MK573893	DC
Lepiota clypeolaria (Bull.) P. Kumm.	17336609	6596260	MK573900	DC
Lepiota umbrosa Morgan	17339931	6596239	MK573915	WI
Lepista sp.	17333187	6596292	MK573883	-
Limacella sp. "CMP0152" (Tulloss 2020)	17336802	6596257	MK573903	WI
Lycoperdon marginatum Vittad.	17231495	6596226	MK573878	-
Lycoperdon sp. "IN-01" (Russell 2020)	17340541	6596233	MN989996	-
Mycena griseoviridis A.H. Sm.	17336556	6596261	MK573899	GenBank
Mycena olida Bres.	17332707	6596281	MK573875	WI
Mycena sp. "WI-01"	17339784	6596242	MK573916	-
Mycena sp. "WI-02"	17332978	6596280	MN989988	-
Mycetinis sp. "WI-01"	17339146	6596245	MN989995	-
Neofavolus sp. "SAV-10" (Seelan Sathiya Seelan et al. 2015)	17333225	NA	MK573884	-
Neottiella vivida (Nyl.) Dennis	17333411	6596288	MN989990	WI
Otidea rainierensis Kanouse	17335858	6596263	MK573898	WI
Phleogena faginea (Fr. & Palmquist) Link	17333436	6596287	MN989991	GenBank
Pholiota highlandensis (Peck) Quadr. & Lunghini	17333602	6596286	MK573887	DC (WI, since 1967)
Pholiota highlandensis (Peck) Quadr. & Lunghini	17334245	6596274	MK573892	DC (WI, since 1967)
Ramaria sp. "WI-01"	17339094	6596246	MN989994	-
Rhodocollybia badiialba (Murrill) Lennox	17335179	6596271	MK573896	WI
Russula sp. "WI-01"	17340565	6596232	MK573921	-
Singerocybe adirondackensis (Peck) Zhu L. Yang & J. Qin	17336637	6596259	MK573901	DC
Tephrocybe sp. "WI-01"	17334189	6596276	MN989992	-
Tephrocybe sp. "WI-02"	17332892	6596285	MN989987	-
Tricholoma hemisulphureum (Kühner) A. Riva	17340511	6596237	MK573920	US
Tricholoma saponaceum (Fr.) P. Kumm.	17333116	6596295	MK573881	-

#17338950) is a 100.0% match to the epitype collection from Sweden (GenBank #KX964309) and thus is the first representative east of the Mississippi River. *Agaricus kriegeri* Kerrigan was described in 2016 from Pennsylvania (Kerrigan 2016). Our specimen (iNaturalist #17333117) is a 99.72% match (with 94% query coverage) to the type collection and is only the second vouchered record of this species aside from the Pennsylvania type collections. In regard to *Mycena olida* Bres., even though numerous collections of *M. olida* were made by Alexander Smith, an expert on *Mycena* and other genera of agarics, these collections were restricted to Michigan. Nomenclature databases do not agree on the current name of this taxon. Index Fungorum (Index Fungorum Partnership 2020) lists *Phloeomana minutula* (Sacc.) Redhead as the currently accepted name for *M. olida*, but MycoBank does not list them as synonyms. In naming our collection (iNaturalist #17332707), we follow the lead of MycoBank and Telfer et al. (2015), as our specimen is a 100% identity match with 100% query coverage to their specimen under this name (GenBank #KT695358). Lastly, we used microscopy to identity a specimen growing in moss at a xeric oak barren (iNaturalist #17333411) as *Neottiella vivida* (Nyl.) Dennis. Sequence data later showed a 99.28% match to GenBank accession #MF066095 from the Czech Republic, which was identified to the same species. Microscopic details for the Wisconsin specimen can be found at Mushroom Observer (2020).

New Records for the United States

In addition to being new records for Wisconsin, three species with vouchered collections and DNA sequence data are believed to be first records for the United States. *Entoloma psammophilohebes* Vila & J. Fernández was described in 2013 from a collection made in the Basque region of Spain. Our specimen (iNaturalist #17337073) is a 99.37% match (91% query cover) to the type collection (GenBank #JX454912). Additional specimens with a matching ITS region were also collected from Indiana a few weeks after the Wisconsin collection and again from Indiana in the fall of 2019. Images and metadata for these collections can be found at iNaturalist under accession #18030457 and #34805034, respectively. *Inocybe ericetorum* Vauras & Kokkonen was described in 2012 from Finland (Kokkonen and Vauras 2012) and had previously only been documented in eastern Canada. The ITS region of our specimen is a 99.09% match (86% query coverage) to the type collection (GenBank #NR_119994), expanding the range of this species into the Midwest. Finally, *Tricholoma hemisulphureum* (Kühner) A. Riva ex Bofelli was first described as *Tricholoma sulphureum* var. *hemisulphureum* Kühner in 1988 from France. Our Wisconsin specimen is a 99.84% match (87% query coverage) to a specimen with this name from Estonia, where the identity was determined to be appropriately applied for the morphological characters and sequence data present from the specimen (Heilmann-Clausen et al. 2017). There are two matching sequences from Florida which may represent the same species (GenBank #MF153041, #MF153084); however, they are currently listed under the name *Tricholoma sulphureum* in MyCoPortal.

CONCLUSION

Many of the new records reported in this study represent species that were described only recently. Indeed, the increasing prevalence of multilocus genetic data in taxonomic studies facilitates biodiversity assessments by augmenting the number of type specimens in reference databases. In turn, species that were thought to be isolated to confined geographic regions are discovered to exist across continents with high genetic similarity. In addition to the positively determined specimens from the 2018 Smith Foray, the 24 specimens that were assigned informal placeholder names constitute fertile avenues for future taxonomic investigations. They may represent previously described species for which no ITS barcode data exist in GenBank or novel species that await detailed analysis. We hope that informal placeholder names will make species associations traceable across time, allowing for the increased elucidation of the hidden biodiversity that is so prevalent in Wisconsin's macrofungi.

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