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RESEARCH ARTICLE

Mycobiome Traits Associated with Disease Tolerance Predict Many Western North American Bat Species Will Be Susceptible to White-Nose Syndrome

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ABSTRACT White-nose syndrome (WNS), a fungal disease that has caused catastrophic population declines of bats in eastern North America, is rapidly spreading across the continent and now threatens previously unexposed bat species in western North America. The causal agent of WNS, the fungus *Pseudogymnoascus destructans*, can infect many species of hibernating bats, but susceptibility to WNS varies by host species. We previously reported that certain traits of the skin microbiome, particularly yeast diversity and abundance, of bat species in eastern North America are strongly associated with resistance to WNS. Using these traits, we developed models to predict WNS susceptibility of 13 species of western North American bats. Based on models derived from yeast species diversity, only one bat species, *Myotis velifer*, was predicted to be WNS resistant (i.e., may develop the disease, but with low mortality rates). We also screened yeasts found on western bats for *P. destructans*-antagonistic properties by spore germination and growth inhibition/competition assays and found the ability of yeasts to inhibit *P. destructans in vitro* to be strain specific. Similar to results of inhibition assays performed with yeasts isolated from bats in eastern North America, few yeasts isolated from bats in western North America inhibited *P. destructans in vitro*. Continued monitoring of western bat populations will serve to validate the accuracy of the mycobiome analysis in predicting WNS susceptibility, document population and susceptibility trends, and identify additional predictors to assess the vulnerability of naive bat populations to WNS.

IMPORTANCE White-nose syndrome is one of the most devastating wildlife diseases ever documented. Some bat species are resistant to or tolerant of the disease, and we previously reported that certain traits of the skin mycobiome of bat species in eastern North America are strongly associated with resistance to WNS. Predicting which western bat species will be most susceptible to WNS would be of great value for establishing conservation priorities. Based on models derived from yeast species diversity, only one bat species was predicted to be WNS resistant. High susceptibility to WNS would pose a significant conservation threat to bats in western North America.

KEYWORDS bats, commensal yeast, fungal disease, microbiome, predictive modelling, white-nose syndrome, disease resistance, mycology, predictive modeling

White-nose syndrome (WNS) is a devastating disease that has killed millions of hibernating bats in eastern North America (1, 2). The causative fungal pathogen, *Pseudogymnoascus destructans* (3), was first detected in New York in 2006 and has spread rapidly across North America, threatening some bat species with extinction (4, 5). In 2016, *P. destructans* was documented in western North America for the first time (6), and several western bat species have subsequently been found with *P. destructans* on their skin or diagnosed with clinical WNS (7). Western North America has higher bat

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biodiversity than the eastern portion of the continent, and several species are already designated "species of concern" by the U.S. Fish and Wildlife Service due to threats such as habitat loss (8, 9). Despite the substantial conservation impact that WNS has had in eastern North America, the nature and severity of WNS in western bat species remain unclear.

Although *P. destructans* can infect many species of hibernating bats, disease dynamics and population-level impacts of WNS vary by host species (4, 10). Specifically, some bat species in eastern North America are highly susceptible to WNS, others are resistant (develop WNS without experiencing mass mortality), and a few species appear to resist infection altogether (impervious) despite repeated exposure to *P. destructans* (4, 10). Identification of which western bat species are likely susceptible to WNS prior to the onset of disease would help management agencies set conservation priorities. However, mechanisms of resistance have not been elucidated, making such predictions difficult. Regardless of mechanism, we found in a previous study that certain traits of skin mycobiomes (the fungal component of the microbiome) of eastern bat species were strongly associated with resistance to WNS (11). WNS-resistant species of bats had higher abundance of fungi, especially yeasts, compared to WNS-susceptible species of bats, and some fungal taxa were enriched on WNS-resistant species of bats (11). Here, we describe skin yeast assemblages on bats in western North America and use data from our previous analyses with eastern bats to construct predictive models to forecast which western bat species may be susceptible or resistant to WNS.

RESULTS

We isolated a total of 98 yeast operational taxonomic units (OTUs) from 52 genera (see Table S1 in the supplemental material). We found that 57.7% of yeast OTUs were cultured from a single bat, and 86.6% of yeast OTUs were cultured from ≤5 individual bats each (Table S1). The most common yeast genera were *Debaryomyces* spp. (isolated from 27.7% of the 447 bats sampled), *Aureobasidium* spp. (17.2%), *Vishniacozyma* spp. (6.5%), *Filobasidium* spp. (3.4%), and *Holtermanniella* sp. (2.9%). Bats had a mean ± standard deviation (SD) of 0.9 ± 1.1 yeast OTUs and 41.4 ± 97.5 yeast colony forming units (CFU) per individual, not including singleton OTUs.

The Shannon diversity index and number of CFU (abundance) of yeast assemblages on bat wings varied among species, seasons, sampling locations, and whether bats were sampled at underground sites (Fig. 1; see Table S2 in the supplemental material). Shannon diversity decreased as the number of days samples were stored before processing increased, although this had no effect on yeast abundance (Table S2). Both Shannon diversity and yeast abundance were higher at underground sites compared to samples collected at other areas (e.g., forest sites, maternity roosts). Samples were collected at underground sites in all seasons, but winter samples were exclusively collected at underground sites. Shannon diversity and yeast abundance were highest in winter and lowest in summer. *Myotis velifer* and *Eptesicus fuscus* had the highest Shannon diversity and yeast abundance compared to the other bat species tested (Fig. 1; Table S2). *Corynorhinus townsendii* and *Myotis yumanensis* were more likely to have no yeast than the other tested bat species.

Skin fungal assemblage composition was significantly associated with bat species (pseudo-$F_{12,184} = 1.79$, $R^2 = 0.078$, $P = 0.001$), collection location (pseudo-$F_{11,184} = 2.58$, $R^2 = 0.103$, $P = 0.001$), season of collection (pseudo-$F_{3,184} = 2.35$, $R^2 = 0.026$, $P = 0.001$), and whether samples were collected at an underground site (pseudo-$F_{1,184} = 1.84$, $R^2 = 0.007$, $P = 0.008$), but not with the number of days swabs were stored before processing (pseudo-$F_{1,184} = 1.25$, $R^2 = 0.005$, $P = 0.187$).

Several isolates of *Aureobasidium* spp. showed weak inhibition of *P. destructans* growth under a variety of conditions (Table 1). Results varied depending on the medium type used, including results with the positive control voriconazole. *Holtermanniella takashimae* only inhibited *P. destructans* growth on Sabouraud dextrose (SD) medium (Table 1). Most of the tested yeast strains showed no inhibition of *P. destructans*. As demonstrated with

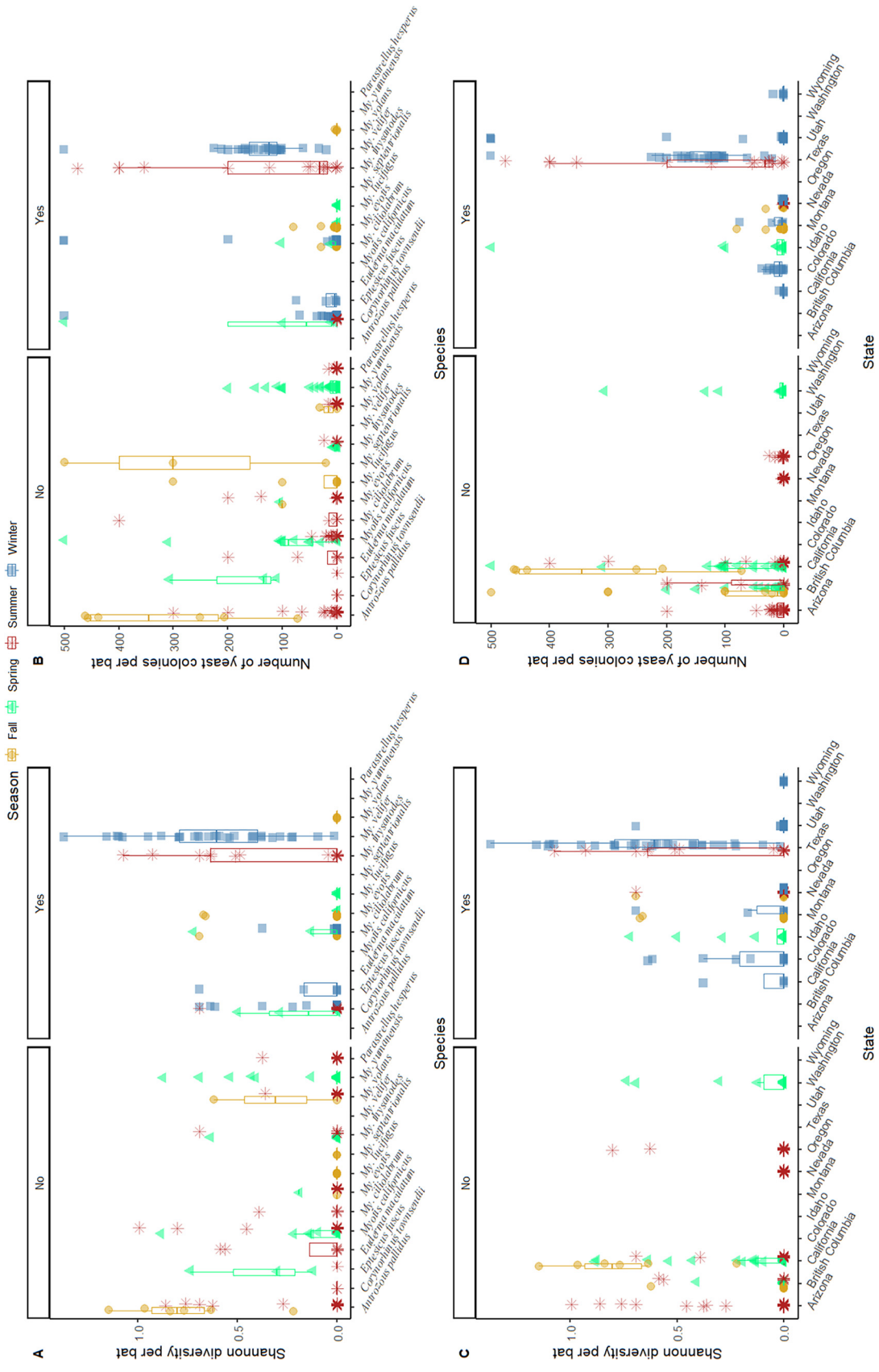

FIG 1 The Shannon diversity index (A and C) and number of yeast CFU (B and D) for each bat by species (A and B) and location of sample collection (C and D), colored by season of collection, are shown for western North America. Whether a sample was collected at an underground site (cave or mine) is indicated by "yes" and "no." We defined winter as December through February, spring as April through May, summer as June through August, and fall as September through October.

TABLE 1 Mean diameter of the zone of inhibition of *Pseudogymnoascus destructans* in the presence of the indicated yeast strains after 2 weeks of incubation at 7°C under various conditions *in vitro*

	Mean diam of zone of inhibition (mm) on medium[a]:						
				YM			
Yeast strain or condition	SD	BHI	BHIB	pH 4.5	pH 5.0	pH 5.0 with 6% NaCl[b]	pH 7.0
Aureobasidium pullulans 46370-1185-2SD	2[c]	0	0	3[c]	0	0	0
Aureobasidium sp. 46370-1064-1LNA	2[c]	0	0	0	0	0	0
A. pullulans 45699-972-4aSD	2, 12[c]	12[c]	7[c]	2, 16[c]	7[c]	0	3[c]
A. pullulans 46379-835-2LNA	0	0	0	0	0	5[c]	0
Holtermanniella takashimae 45701-673-1SD	4[c]	0	0	0	0	0	0
Voriconazole	6[c]	25	12[c]	10	19	CI[d]	17
PBST	0	0	0	0	0	0	0

[a]Shown are the mean diameters of the zone of inhibition ($n = 3$ replicates for each condition except positive controls) of *P. destructans* in the presence of the indicated yeast strains after 2 weeks of incubation at 7°C under the following conditions *in vitro*: Sabouraud dextrose medium (SD), brain heart infusion medium (BHI), brain heart infusion agar with 10% sheep blood (BHIB), and yeast morphology medium (YM). Voriconazole and phosphate-buffered saline with 0.5% Tween 20 (PBST) were used as positive and negative controls, respectively. *Filobasidium magnum* (46370-1167-0LNA), *Filobasidium* sp. (46379-1438-2LNA), *Vishniacozyma* sp. cluster 9 (46388-1843-1LNA), *Blastobotrys buckinghamii* (45699-84-1LNA), *Vishniacozyma victoriae* cluster 11 (45701-666-2SD), *Debaryomyces hansenii* (45701-670-3SD, 45701-677-1SD), *Debaryomyces* sp. 1 (45702-288-1SD), *Debaryomyces* sp. 3 (45698-832-4SD), *Trichosporon otae* (44797-05-2SD), and *Cutaneotrichosporon moniliiforme* (44797-11-1LNA) showed no inhibition under any conditions and thus do not appear in the table.

[b]Plates checked at 7 weeks due to slow growth of *P. destructans* on this medium.

[c]Inhibition weak, with some growth in the "inhibited" area.

[d]CI, complete inhibition.

Aureobasidium spp., the ability of yeast to inhibit *P. destructans* growth *in vitro* is strain specific. Although strains of *Cutaneotrichosporon moniliiforme* isolated from eastern bats inhibited *P. destructans in vitro* (11), strains of *Cu. moniliiforme* isolated from western bats showed no inhibition.

The four yeast taxa that were enriched on WNS-resistant species of bats in eastern North America were not as abundant on western bats: *Cu. moniliiforme* was isolated from 17.3% of eastern bats and 2% of western bats, *Debaryomyces* sp. 1 from 14.3% of eastern and 6.3% of western bats, *Debaryomyces* sp. 3 from 12.3% of eastern and 8.7% of western bats, and *Debaryomyces hansenii* from 29.1% of eastern bats and 12.5% of western bats. Based on models of the presence of three of the four yeast taxa enriched on resistant bat species in eastern North America, the only western bat species predicted to be WNS resistant was *Myotis velifer* (Fig. 2 and Table 2). The remaining 12 species sampled were predicted to be WNS susceptible, based on all criteria examined, although some species (e.g., *E. fuscus*) approached the percent cutoff for WNS resistance in some models (Fig. 2).

DISCUSSION

Based on models of the presence of three yeast taxa enriched on resistant bat species in eastern North America, the only western bat species predicted to be WNS resistant was *Myotis velifer* (Fig. 2 and Table 2). However, unlike the other species, the *My. velifer* samples all originated from the same site, which may bias results. Furthermore, the species was considered WNS susceptible based upon the overall yeast abundance model, making it difficult to predict WNS-associated impacts on *My. velifer* populations based on mycobiome traits. The remaining 12 species sampled were predicted to be WNS susceptible, based on all criteria examined, although some species (e.g., *E. fuscus*) approached the percent cutoff for WNS resistance in some models (Fig. 2).

Aside from *E. fuscus*, predictions of susceptibility for western populations of species with continental distributions (e.g., *Myotis lucifugus*) were consistent with the known susceptibilities of their eastern counterparts. However, it is important to note that the mycobiome characteristics we used in our predictions do not distinguish WNS-susceptible from WNS-impervious bat species in eastern North America, as both have low yeast diversity and abundance on their skin. This means that a bat classified as "susceptible" by our model may be either susceptible or impervious. Western populations of *Co. townsendii* were classified as "susceptible" by our model, but they are likely to be

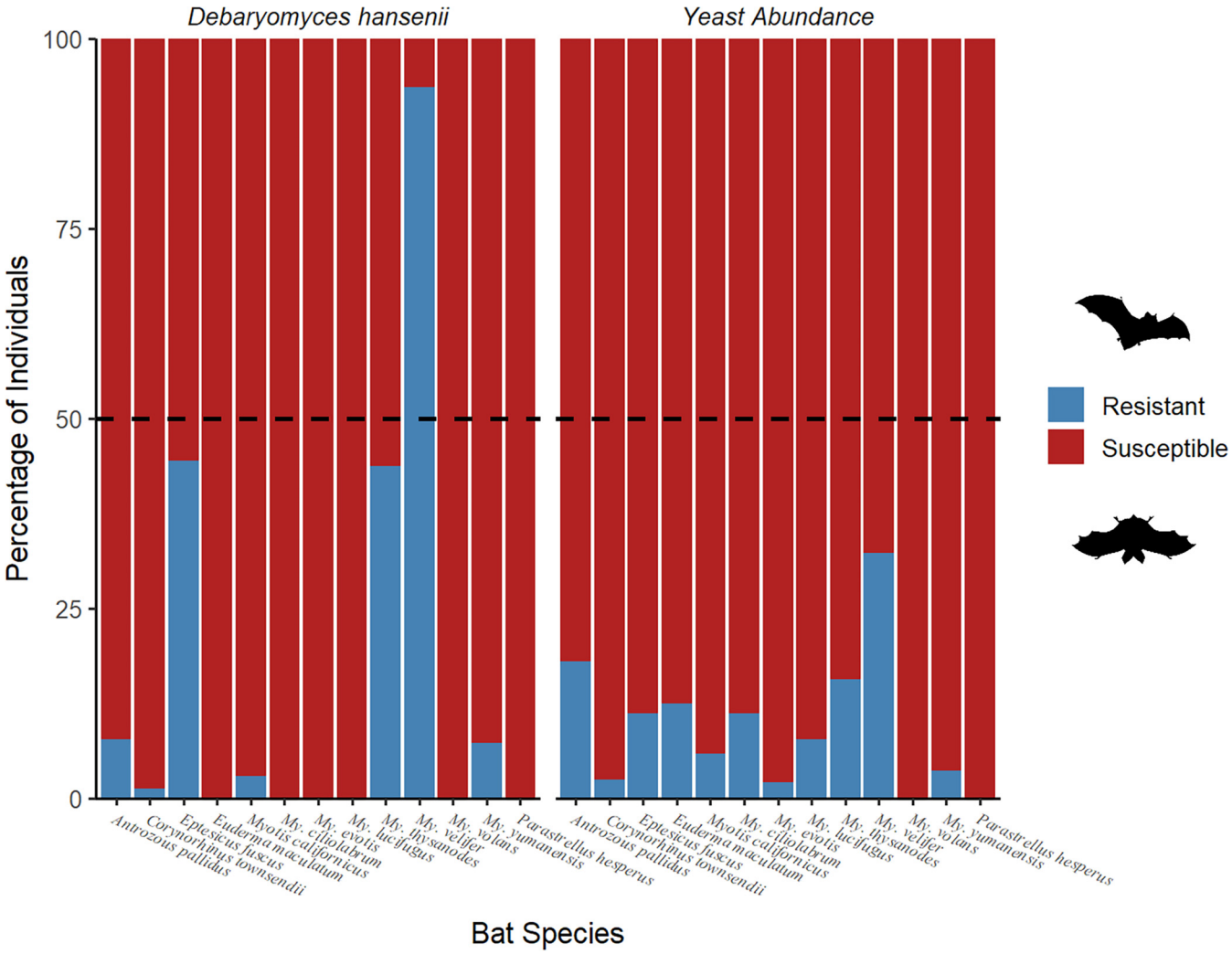

FIG 2 The percentage of individuals of each bat species sampled in western North America predicted to be resistant or susceptible to white-nose syndrome based on the presence of *Debaryomyces hansenii* and overall yeast abundance (counts of CFU per bat) cultured from wing swabs. A species with >50% of individuals predicted as resistant (portion of blue bar above 50%) was considered resistant. Bat images used in this figure were free-for-use through a Creative Commons license (Attribution-NonCommercial 4.0 License [http://getdrawings.com/bat-silhouette-images]; Natasha Sinegina, Creative Commons Attribution-Share Alike 4.0 License [http://www.supercoloring.com/silhouettes/bat]).

WNS impervious based on observations from eastern North America (12). Haase et al. (13) predicted that *My. velifer*, *E. fuscus*, and *Co. townsendii* would have a higher probability of survival when infected with *P. destructans* compared to other bat species in the western United States, such as *My. lucifugus* and *Myotis evotis*, based on models with body mass and hibernacula microclimate. Aside from bat species that do not hibernate for long periods, currently the only known WNS-impervious bat species are in the genus *Corynorhinus*. *Corynorhinus* is phylogenetically divergent from *Myotis*, among which there are no known impervious taxa. Thus, our predictions may be more accurate for species of *Myotis* and *Parastrellus* (the latter of which is a sister genus to *Perimyotis*, an eastern North American taxon that is highly susceptible to WNS [4, 8]). Several western species included in our model do not fall into the WNS-impervious category because individuals of these species have been confirmed to develop WNS. For example, *My. velifer*, *My. evotis*, *Myotis thysanodes*, *Myotis volans*, and *My. yumanensis* have recently been confirmed to develop WNS, although population declines have not yet been observed (7), indicating these species are not WNS impervious. There is, however, more uncertainty in predictions for some other western bat species for which no close relatives occur in eastern North America (e.g., *Euderma maculatum*, *Antrozous pallidus*), and these species may be WNS impervious independent of their mycobiomes.

TABLE 2 Model predictions of which bat species in western North America will be resistant to white-nose syndrome based on different mycobiome characteristics

Bat species (*n*) and parameter	Susceptibility or resistance based on[a]:				
	Yeast abundance	*Cutaneotrichosporon moniliiforme*	*Debaryomyces* sp. 3	*Debaryomyces* sp. 1	*Debaryomyces hansenii*
Matthews correlation coefficient	0.80	0.48	0.05	0.31	0.85
Antrozous pallidus (39)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Corynorhinus townsendii (83)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Eptesicus fuscus (9)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Euderma maculatum (8)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Myotis californicus (34)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Myotis ciliolabrum (36)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Myotis evotis (47)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Myotis lucifugus (13)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Myotis thysanodes (32)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Myotis velifer (31)	Susceptible	Susceptible	Resistant	Resistant	Resistant
Myotis volans (43)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Myotis yumanensis (55)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Parastrellus hesperus (17)	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible

[a]Shown are the results of our model predictions of which bat species in western North America will be resistant to white-nose syndrome (WNS) based on different mycobiome characteristics, including yeast abundance cultured from skin swabs (counts of CFU) and the presence of four yeast taxa that were differentially abundant on WNS-resistant bat species in eastern North America (as determined by Vanderwolf et al. [11]). A Matthews correlation coefficient of +1 represents perfect prediction, 0 no better than random prediction, and −1 indicates total disagreement between prediction and observation for each model. "*n*" indicates sample size.

If our model predictions are accurate, more western bat species in North America could be WNS susceptible than eastern bat species. However, there are several caveats. First, the mycobiome is but one of several factors that influence host susceptibility to WNS. Our models intentionally focused on the mycobiome because of its association with host susceptibility in our previous study (11), but we fully acknowledge the multifactorial nature of host susceptibility to fungal pathogens. Second, the traits used in our predictive models likely vary temporally and spatially beyond the parameter ranges that we measured and modeled. For instance, the bacterial microbiomes of several western bat species differed between individuals sampled in underground (i.e., caves and mines) versus surface sites (14). Many bats sampled for our project were captured from the surface and during the nonhibernation season (i.e., when the species would not be at risk of developing WNS) due to the difficulty of locating hibernacula for most western bat species, and we found that Shannon diversity and yeast abundance and composition differed between bats sampled at underground sites compared to surface sites.

Yeast assemblages on bat skin are believed to represent primarily commensal rather than transient inhabitants compared to filamentous fungi (11), and thus are more likely to be permanent residents of the skin. Although multiple yeast taxa were shared between bats in both eastern and western North America, several differed in abundance or were unique to the west. Of the five yeast taxa associated with WNS-resistant eastern bat species (11), one taxon was detected on a single bat, and the other four taxa were generally uncommon on western bats. Whether yeast taxa unique to the west are associated with WNS resistance is unknown, although our inhibition assays uncovered few yeasts that inhibit *P. destructans* growth *in vitro*. Potentially other microbes on the skin, such as bacteria, perform this role (15).

Several yeast strains isolated from bats in western North America inhibited *P. destructans* growth *in vitro*. *Aureobasidium* spp. were more common on bats in western North America than in the east, and several isolates of *Aureobasidium* showed weak inhibition of *P. destructans* growth under a variety of conditions (Table 1). *Holtermanniella takashimae* only inhibited *P. destructans* growth on Sabouraud dextrose medium (Table 1). As demonstrated with *Aureobasidium* spp., the ability of yeast to inhibit *P. destructans* growth *in vitro* is strain specific. *Cutaneotrichosporon moniliiforme* isolated from bats in the east inhibited *P. destructans* growth on two types of media (11), but *Cu. moniliiforme* isolated from bats in western North America did not inhibit *P. destructans* growth on any of the same types

of media tested (Table 1). Similar to results of inhibition assays performed with yeasts isolated from bats in eastern North America, few yeasts isolated from bats in western North America inhibited *P. destructans* growth *in vitro*. It is unknown which yeasts could inhibit *P. destructans* on bat wings, given that *in vivo* conditions would differ from *in vitro* conditions and the chemical environment on bat wings is largely unknown. Finally, it is plausible that yeast assemblage characteristics common to resistant bat species do not directly modulate disease susceptibility but are rather a result of other behavioral or physiological processes that also influence resistance (11).

Yeast assemblage traits used in the predictive models could also exhibit variation due to geography and associated host genetics. Eastern and western populations of North American bat species with continent-wide distributions are genetically distinct, often exhibiting higher genetic diversity in the west (16, 17). As such, susceptibility to WNS could be different between eastern and western populations and even within western populations. There was greater intraspecific variation in yeast community traits observed in some western bat species compared to eastern species. For example, 7.7% of individuals from western populations of *My. lucifugus*, a WNS-susceptible species, were classified as resistant by the yeast abundance model, whereas 0% of individuals from eastern populations had yeast abundance traits consistent with WNS resistance. The disparity in yeast abundance profiles between susceptible and resistant species of bats in eastern North America creates uncertainty between predicted and observed disease susceptibilities.

Wildlife diseases such as WNS pose significant management challenges due to the difficulty of treating free-ranging populations, especially once a disease has become established. Thus, management strategies focusing on prevention or early intervention hold the most promise for controlling wildlife diseases before they impact new populations or emerge in new locations. Such proactive strategies, however, require intimate knowledge of a host's susceptibility to a pathogen. The microbiome is increasingly recognized as an important moderator of disease (18, 19) but has rarely been used to predict host susceptibility. We have attempted to forecast disease impacts on novel host species using traits of the mycobiome. We emphasize that our predictions will likely change as new data on bat skin microbiomes are generated, such as the role of bacteria and viruses. More data will help assess the utility of skin microbiomes in forecasting disease susceptibility. However, despite uncertainties, our results provide information that may assist wildlife managers and strategic decision-makers. Specifically, our model predicts that WNS will likely pose a continent-wide conservation threat to *Myotis* spp., and the disease may have more severe ecological impacts in western North America due the greater number of potentially susceptible host species. Continued monitoring of western bat populations will serve to document population and susceptibility trends, determine the accuracy of the mycobiome in predicting WNS resistance, and identify additional predictors to assess the vulnerability of naive bat populations to WNS.

MATERIALS AND METHODS

Sampling. We collected swab samples from a total of 36 sites in 11 U.S. states and one Canadian province in western North America from 2015 to 2018. In all, we sampled 450 individual bats, representing 14 species (Table 3). We sampled only those western North American bat species known to hibernate (8, 9), because bats that do not hibernate are not known to be susceptible to WNS. Unlike areas of eastern North America where multiple species of bats form large hibernation aggregations (>100 individuals) in caves and mines during winter, hibernation locales for many western bat species are unknown or contain small numbers of bats (9, 20). Due to the difficulties of locating many western bat species during the hibernation period, we collected samples year-round, only some of which were at underground sites (caves or mines). Other sampling sites included maternity colonies (bat boxes, bat condos, buildings) and forest landscapes. We conducted sampling under U.S. Geological Survey—National Wildlife Health Center Institutional Animal Care and Use Committee Protocols EP140212 and EP081124-A2.

Fungal culture and identification. We collected and processed swabs of bat wings (each bat swabbed once) following the methods of Vanderwolf et al. (11), with some modifications. First, swabs were stored from 2 to 30 days (mean ± SD, 9.5 ± 7.8 days) before processing due to time variation in shipping samples to the laboratory. Second, we cultured fungi only on Sabouraud dextrose (SD) agar

TABLE 3 Bat species, sample sizes, and locations sampled in western North America[a]

Bat species	WNS susceptibility	Sample size (*n*)	State/province-no. of sites
Antrozous pallidus	?	39	AZ-1, CA-1, OR-1
Corynorhinus townsendii	Impervious in East	83	CA-2, CO-1, ID-1, MT-1, NV-1, UT-1, WY-2
Eptesicus fuscus	Resistant in East	9	AZ-1, MT-1, WA-1
Euderma maculatum	?	8	BC-1, NV-1
Myotis californicus	?	34	AZ-1, CA-3, OR-2
Myotis ciliolabrum	?	36	AZ-1, BC-1, CA-2, ID-1, NV-2, UT-1, WY-2
Myotis evotis	?*	47	BC-2, CA-2, ID-1, MT-1, NV-5, OR-2
Myotis lucifugus	Susceptible in East	13	BC-2, ID-1
Myotis septentrionalis	Susceptible in East	3	BC-1
Myotis thysanodes	?*	32	AZ-1, CA-3, OR-1, TX-1
Myotis velifer	?*	31	TX-1
Myotis volans	?*	43	AZ-1, BC-2, CA-1, MT-1, NV-4, OR-3
Myotis yumanensis	?*	55	BC-1, CA-4, WA-2
Parastrellus hesperus	?	17	AZ-1, NV-1

[a]The names and coordinates of the collection sites have been withheld due to the sensitive nature of bat hibernacula. "East" refers to eastern North America. AZ, Arizona; CA, California; CO, Colorado; BC, British Columbia; ID, Idaho; MT, Montana; NV, Nevada; OR, Oregon; TX, Texas; UT, Utah; WA, Washington; and WY, Wyoming. "?" indicates unknown susceptibility to white-nose syndrome (WNS), and "*" indicates a species in which WNS has been confirmed, indicating the species is either susceptible or resistant (but not impervious) to the disease.

plates with chloramphenicol and gentamicin and Leeming and Notman agar; we did not use dermatophyte test medium because in our previous study, this medium contributed little to the overall assessment of fungal abundance and diversity on bat skin (11). Finally, we selected only fungi with a yeast morphotype for identification because the abundance of yeasts (CFU) was the only component of the mycobiome strongly associated with WNS susceptibility (11).

Briefly, we streaked each swab five times, discretely, across the two medium types and incubated plates in darkness at 7°C (to approximate typical conditions in hibernacula) for 2 months. We checked plates weekly and isolated morphologically unique fungal colonies in pure culture. We counted the number of colonies of each morphotype on each plate weekly for the 2-month incubation period, or until confluent growth precluded accurate counts, to determine the number of CFU. We identified pure cultures by analyzing the full-length internal transcribed spacer (ITS) region of the fungal rRNA gene as previously described (11). Briefly, after cells were lysed, we performed a PCR targeting the ITS using universal fungal primers ITS1-F and ITS4 (cycling conditions of 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 3 min, with a final extension for 10 min at 72°C) (21). All PCRs were conducted using GoTaq Flexi DNA polymerase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions, with 0.5 ml template (extracted from fungal isolates using PrepMan Ultra reagent [Life Technologies Corporation, Carlsbad, CA, USA]) per 25-ml reaction. Sanger sequencing of amplicons was performed at the University of Wisconsin—Madison Biotechnology Center.

Sequences were collapsed into representative operational taxonomic units (OTUs) using USEARCH (22) with a 97% similarity threshold (23). We applied a 99% similarity threshold for the genus *Debaromyces* because of minimal genetic variation exhibited in the ITS region among *Debaromyces* species (24). We assigned taxonomy to sequences in R, using the assigntaxonomy function (DADA2 package) (25) with UNITE (26, 27). Some sequences were not identified to the genus using UNITE, and we compared these to NCBI's GenBank database using BLAST (28). We manually generated a community matrix of annotated OTUs and their CFU for each bat.

Inhibition assays. We screened yeasts found on bats for *P. destructans*-antagonistic properties by spore germination and growth inhibition/competition assays using methods identical to those of Vanderwolf et al. (11). We tested OTUs that were present on ≥5 bats each. Briefly, we used different types of media, pH, and salt conditions, including SD agar (pH 5.6), brain heart infusion (BHI [pH 7.4]), BHI with 10% sheep blood (pH 7.4), yeast morphology (YM) medium with the pH adjusted to either 4.5, 5.0, or 7.0 with 0.1 M citrate-phosphate buffer, and YM supplemented with 6% (wt/vol) NaCl at pH 5.0. We harvested *P. destructans* conidia, spread 150 μl of *P. destructans* conidial suspension containing 2 million conidia onto agar medium, placed six presterilized Whatman no. 1 filter paper discs equidistant from one another on each plate, and inoculated each disc with 8-μl suspensions, each containing 500,000 cells of different yeast strains. We tested each yeast strain in triplicate, incubating all plates in the dark at 7°C. Negative controls were discs treated with sterile phosphate-buffered saline only, and positive controls were discs containing voriconazole (30 μg [Sensi-disc; Becton Dickinson]). We checked plates daily for the first week to assess inhibition of *P. destructans* germination near the discs and then weekly for 2 months or until *P. destructans* growth had covered the entire agar surface. We measured zones of inhibition around the discs to the nearest mm after 2 weeks of incubation.

Statistical analyses. Singletons (fungal OTUs that were isolated from one bat) were removed prior to analysis. *Myotis septentrionalis* samples were removed before analysis because we collected samples from only three individuals. We performed all analyses in R (29). We calculated the Shannon diversity index of fungi on each bat using the diversity function in the vegan package (30). To assess which factors influence skin fungal assemblage structure, we constructed a Gaussian zero-inflated model with Shannon index as the response variable and bat species (13-level factor), state (12-level factor), the number of days swabs were stored, whether samples were collected at underground sites (caves or mines

[binary factor]), and season (4-level factor) as explanatory variables (package glmmTMB) (31). We defined "winter" as December through February, "spring" as April through May, "summer" as June through August, and "fall" as September through October. No samples were collected in March or November. A separate model with yeast CFU (abundance) as the response variable and identical explanatory variables was also constructed. We used the function AICtab (package bbmle) (32) to compare model Akaike information criteria (AIC) values. We determined the best data transformation with the transformTukey function (package rcompanion) (33) for each response variable. Lambda values were 0.4 and 0.2 for Shannon diversity and yeast abundance, respectively. We tested multicollinearity among the variables using the corvif function (34).

To determine if wing fungal assemblage composition varied among WNS susceptibility groups, bat species, and sites, we implemented a nonparametric permutational multivariate analysis of variance (PERMANOVA) on abundance-based (CFU) Bray-Curtis dissimilarity coefficients using the function ADONIS (Vegan). As Bray-Curtis dissimilarity values cannot be calculated for samples that have no composition, we first removed individual bats with no cultured fungi and bats from which the only fungus cultured was the single representative of that OTU within our data set ($n = 17$ for *Antrozous pallidus*, $n = 57$ for *Corynorhinus townsendii*, $n = 1$ for *E. fuscus*, $n = 6$ for *Euderma maculatum*, $n = 14$ for *Myotis californicus*, $n = 16$ for *My. ciliolabrum*, $n = 33$ for *My. evotis*, $n = 11$ for *My. lucifugus*, $n = 10$ for *My. thysanodes*, $n = 34$ for *My. volans*, $n = 22$ for *My. yumanensis*, and $n = 12$ for *Parastrellus hesperus*). We ran PERMANOVA for 1,000 iterations, and report R^2 values when the variable enters the model last.

To predict WNS susceptibility in western bat species, we constructed a logistic regression model. We built our model using previously published data on the abundance of yeasts (CFU per bat) within skin mycobiomes of bat species of known WNS susceptibility sampled in eastern North America (11). We filtered the data set as previously described (11) and excluded the yeast *Malassezia vespertilionis* because it was commonly found on both WNS-susceptible and WNS-resistant bat species in the eastern United States (11) and thus is not a good predictor of WNS susceptibility status. We removed WNS-impervious bat species (*Corynorhinus townsendii* and *Corynorhinus rafinesquii*) from the eastern North American data set prior to constructing the model because WNS-susceptible and WNS-impervious bat species had similar yeast abundances on their skin (11) and were therefore uninformative for model purposes. We fitted WNS susceptibility status (binomial: resistant or susceptible) as our response variable and yeast abundance as the sole predictor variable. We assessed model performance with 5-fold cross validation using the function cross_validate in the cvms package (35). We report the Matthews correlation coefficient because it is considered the best metric for establishing the quality of a binary classifier (36). The eastern data set had unequal numbers of the two susceptibility groups, but the Matthews correlation coefficient can be used even if classes are of very different sizes (37). A coefficient of $+1$ represents perfect prediction, 0 indicates no better than random prediction, and -1 indicates total disagreement between prediction and observation. We used the "predict" function of the stats package (29) to predict the susceptibility of individuals of unknown susceptibility in the western North America data set. If $>50\%$ of individuals of a given western bat species were predicted as WNS resistant, we considered the species WNS resistant: otherwise, the species was considered WNS susceptible. In addition to yeast abundance, we constructed additional models that used the presence or absence of four yeast taxa that were enriched on WNS-resistant species of bats in eastern North America as binary predictor variables of WNS susceptibility for western bat species in separate binomial models constructed as described above. The four yeast taxa were *Cutaneotrichosporon moniliiforme*, *Debaryomyces* sp. 1, *Debaryomyces* sp. 3, and *Debaryomyces hansenii*.

Data availability. Metadata associated with this project is available at https://www.sciencebase.gov/catalog/item/5cf6c524e4b0d63728b9b463. The Fungal ITS sequences determined by Sanger sequencing and submitted to GenBank as described above are available under GenBank accession no. MK782157 to MK782494.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

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REFERENCES

1. Blehert DS, Hicks AC, Behr M, Meteyer CU, Berlowski-Zier BM, Buckles EL, Coleman JTH, Darling SR, Gargas A, Niver R, Okoniewski JC, Rudd RJ, Stone WB. 2009. Bat white-nose syndrome: an emerging fungal pathogen? Science 323:227. https://doi.org/10.1126/science.1163874.
2. U.S. Fish and Wildlife Service. 2012. North American bat death toll exceeds 5.5 million from white-nose syndrome. https://www.fws.gov/news/ShowNews.cfm?ID=0DA47708-D93C-21F4-3AA3E6EEB217DAA0.
3. Lorch JM, Meteyer CU, Behr MJ, Boyles JG, Cryan PM, Hicks AC, Ballmann AE, Coleman JTH, Redell DN, Reeder DM, Blehert DS. 2011. Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. Nature 480:376–378. https://doi.org/10.1038/nature10590.
4. Turner G, Reeder D, Coleman J. 2011. A five-year assessment of mortality and geographic spread of white-nose syndrome in North American bats and a look to the future. Bat Res News 52:13–27.
5. Frick WF, Puechmaille SJ, Hoyt JR, Nickel BA, Langwig KE, Foster JT, Barlow KE, Bartonička T, Feller D, Haarsma AJ, Herzog C, Horáček I, van der Kooij J, Mulkens B, Petrov B, Reynolds R, Rodrigues L, Stihler CW, Turner GG, Kilpatrick AM. 2015. Disease alters macroecological patterns of North American bats. Glob Ecol Biogeogr 24:741–749. https://doi.org/10.1111/geb.12290.
6. Lorch JM, Palmer JM, Lindner DL, Ballmann AE, George KG, Griffin K, Knowles S, Huckabee JR, Haman KH, Anderson CD, Becker PA, Buchanan JB, Foster JT, Blehert DS. 2016. First detection of bat white-nose syndrome in western North America. mSphere 1:e00148-16. https://doi.org/10.1128/mSphere.00148-16.
7. White-nose Syndrome Response Team. 2020. Bats affected by WNS. https://www.whitenosesyndrome.org/static-page/bats-affected-by-wns. Accessed 5 December 2020.
8. Adams RA. 2003. Bats of the Rocky Mountain West: natural history, ecology, and conservation. University Press of Colorado, Boulder, CO.
9. O'Shea TJ, Cryan PM, Bogan MA. 2018. United States bat species of concern: a synthesis. Proc Calif Acad Sci Ser 4 65:1–279.
10. Frick W, Cheng T, Langwig K, Hoyt J, Janicki A, Parise K, Foster J, Kilpatrick A. 2017. Pathogen dynamics during invasion and establishment of white-nose syndrome explain mechanisms of host persistence. Ecology 98:624–631. https://doi.org/10.1002/ecy.1706.
11. Vanderwolf KJ, Campbell LJ, Goldberg TL, Blehert DS, Lorch JM. 2021. Skin fungal assemblages of bats vary based on susceptibility to white-nose syndrome. ISME J 15:909–920. https://doi.org/10.1038/s41396-020-00821-w.
12. Bernard RF, Foster JT, Willcox EV, Parise KL, McCracken GF. 2015. Molecular detection of the causative agent of white-nose syndrome on Rafinesque's big-eared bats (Corynorhinus rafinesquii) and two species of migratory bats in the southeastern USA. J Wildl Dis 51:519–522. https://doi.org/10.7589/2014-08-202.
13. Haase CG, Fuller NW, Dzal YA, Hranac CR, Hayman DTS, Lausen CL, Silas KA, Olson SH, Plowright RK. 2021. Body mass and hibernation microclimate may predict bat susceptibility to white-nose syndrome. Ecol Evol 11:506–515. https://doi.org/10.1002/ece3.7070.
14. Winter AS, Hathaway JJM, Kimble JC, Buecher DC, Valdez EW, Porras-Alfaro A, Young JM, Read KJH, Northup DE. 2017. Skin and fur bacterial diversity and community structure on American southwestern bats: effects of habitat, geography and bat traits. PeerJ 5:e3944. https://doi.org/10.7717/peerj.3944.
15. Hoyt J, Cheng T, Langwig K, Hee M, Frick W, Kilpatrick A. 2015. Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. PLoS One 10:e0121329. https://doi.org/10.1371/journal.pone.0121329.
16. Neubaum MA, Douglas MR, Douglas ME, O'Shea TJ. 2007. Molecular ecology of the big brown bat (*Eptesicus fuscus*): genetic and natural history variation in a hybrid zone. J Mammal 88:1230–1238. https://doi.org/10.1644/06-MAMM-A-228R1.1.
17. Vonhof MJ, Amelon SK, Currie RR, McCracken GF. 2016. Genetic structure of winter populations of the endangered Indiana bat (*Myotis sodalis*) prior to the white nose syndrome epidemic: implications for the risk of disease spread. Conserv Genet 17:1025–1040. https://doi.org/10.1007/s10592-016-0841-6.
18. Kong HH, Morris A. 2017. The emerging importance and challenges of the human mycobiome. Virulence 8:310–312. https://doi.org/10.1080/21505594.2017.1279780.
19. Kearns PJ, Fischer S, Fernández-Beaskoetxea S, Gabor CR, Bosch J, Bowen JL, Tlusty MF, Woodhams DC. 2017. Fight fungi with fungi: antifungal properties of the amphibian mycobiome. Front Microbiol 8:2494. https://doi.org/10.3389/fmicb.2017.02494.
20. Weller TJ, Rodhouse TJ, Neubaum DJ, Ormsbee PC, Dixon RD, Popp DL, Williams JA, Osborn SD, Rogers BW, Beard LO, Mcintire AM, Hersey KA, Tobin A, Bjornlie NL, Foote J, Bachen DA, Maxell BA, Morrison ML, Thomas SC, Oliver GV, Navo KW. 2018. A review of bat hibernacula across the western United States: implications for white-nose syndrome surveillance and management. PLoS One 13:e0205647. https://doi.org/10.1371/journal.pone.0205647.
21. Lorch JM, Minnis AM, Meteyer CU, Redell JA, White JP, Kaarakka HM, Muller LK, Lindner DL, Verant ML, Shearn-Bochsler V, Blehert DS. 2015. The fungus *Trichophyton redellii* sp. nov. causes skin infections that resemble white-nose syndrome of hibernating bats. J Wildl Dis 51:36–47. https://doi.org/10.7589/2014-05-134.
22. Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461. https://doi.org/10.1093/bioinformatics/btq461.
23. O'Brien HE, Parrent JL, Jackson JA, Moncalvo J-M, Vilgalys R. 2005. Fungal community analysis by large-scale sequencing of environmental samples. Appl Environ Microbiol 71:5544–5550. https://doi.org/10.1128/AEM.71.9.5544-5550.2005.
24. Martorell P, Fernández-Espinar MT, Querol A. 2005. Sequence-based identification of species belonging to the genus *Debaryomyces*. FEMS Yeast Res 5:1157–1165. https://doi.org/10.1016/j.femsyr.2005.05.002.
25. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583. https://doi.org/10.1038/nmeth.3869.
26. Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Douglas B, Drenkhan T, Eberhardt U, Dueñas M, Grebenc T, Griffith GW, Hartmann M, Kirk PM, Kohout P, Larsson E, Lindahl BD, Lücking R, Martín MP, Matheny PB, Nguyen NH, Niskanen T, Oja J, Peay KG, Peintner U, Peterson M, Põldmaa K, Saag L, Saar I, Schüßler A, Scott JA, Senés C, Smith ME, Suija A, Taylor DL, Telleria MT, Weiss M, Larsson KH. 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 22:5271–5277. https://doi.org/10.1111/mec.12481.
27. UNITE Community. 2017. UNITE general FASTA release. https://unite.ut.ee/repository.php.
28. Altschul S, Gish W, Miller W, Myers E, Lipman D. 1990. Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.
29. R Core Team. 2020. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
30. Oksanen J, Blanchet F, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin P, O'Hara R, Simpson G, Solymos P, Stevens M, Eduard Szoecs E, Wagner H. 2018. Vegan: community ecology package. R package version 2.5-2. https://cran.r-project.org/web/packages/vegan/.
31. Brooks ME, Kristensen K, van Benthem KJ, Magnusson A, Berg CW, Nielsen A, Skaug HJ, Maechler M, Bolker BM. 2017. Modeling zero-inflated count data with glmmTMB. bioRxiv. 132753. https://www.biorxiv.org/content/10.1101/132753v1.full.pdf.

32. Bolker B, R Development Core Team. 2017. bbmle: tools for general maximum likelihood estimation. R package version 1020. https://rdrr.io/cran/bbmle/.
33. Mangiafico S. 2019. rcompanion: functions to support extension education program evaluation. R package version 2010. https://cran.r-project.org/web/packages/rcompanion/index.html.
34. Zuur A, Ieno E, Walker N, Saveliev A, Smith G. 2009. Mixed effects models and extensions in ecology with R. Springer, New York, NY.
35. Olsen L, Zachariae B. 2020. cvms: cross-validation for model selection. R package version 120. https://cran.r-project.org/web/packages/cvms/.
36. Chicco D, Jurman G. 2020. The advantages of the Matthews correlation coefficient (MCC) over F1 score and accuracy in binary classification evaluation. BMC Genomics 21:6–13. https://doi.org/10.1186/s12864-019-6413-7.
37. Boughorbel S, Jarray F, El-Anbari M. 2017. Optimal classifier for imbalanced data using Matthews correlation coefficient metric. PLoS One 12: e0177678-17. https://doi.org/10.1371/journal.pone.0177678.