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Mr. Toad's wild fungi: Fungal isolate diversity on Colorado boreal toads and their capacity for pathogen inhibition

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ABSTRACT

The amphibian skin pathogen *Batrachochytrium dendrobatidis* (*Bd*) has caused an ongoing biodiversity crisis, including in the locally endangered Colorado boreal toad (*Anaxyrus boreas*). Although researchers have investigated the bacteria living on amphibian skin and how they interact with *Bd*, there is less information about fungal community members. This study describes (1) the diversity of culturable fungi from boreal toad skin, (2) which subset of these isolates is *Bd*-inhibitory, and (3) how *Bd* affects these isolates' growth and morphology. Most isolates were from the orders Capnodiales, Helotiales, and Pleosporales. Of 16 isolates tested for *Bd*-inhibition, two from the genus *Neobulgaria* and three from *Pseudeurotium* inhibited *Bd*. Fungal growth in co-culture with *Bd* varied with weak statistical support for *Neobulgaria* sp. (isolate BTF_36) and cf *Psychrophila* (isolate BTF_60) (p-values = 0.076 and 0.092, respectively). Fungal morphology remained unchanged in co-culture with *Bd*, however, these results could be attributed to low replication per isolate. Nonetheless, two fungal isolates' growth may have been affected by *Bd*, implying that fungal growth changes in *Bd* co-culture could be a variable worth measuring in the future (with higher replication). These findings add to the sparse but growing literature on amphibian-associated fungi and suggest further study may uncover the relevance of fungi to amphibian health and *Bd* infection.

1. Introduction

Host-associated fungi are little-understood, yet important to vertebrate health in myriad ways, from host development to disease progression, and often through multifaceted inter-domain microbial interactions (Cui et al., 2013; Hoarau et al., 2016; Limon et al., 2017; Oever and Netea, 2014). In addition, emerging fungal pathogens have taken an alarming toll on human, wildlife, and plant health worldwide in unprecedented ways (Farrer et al., 2011; Fisher et al., 2012). Along with more than five hundred amphibian species around the world, the Colorado boreal toads (*Anaxyrus boreas*) have been experiencing a steep decline throughout the southern Rocky Mountains over the past few decades, largely due to the emerging fungal skin pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Carey, 1993; Carey et al., 2006; Kilpatrick et al., 2010; Muths et al., 2003; Scheele et al., 2019). There is evidence that amphibian skin microbes interact with *Bd* in ways that affect disease outcomes, but most studies focus largely on bacteria (Bletz et al., 2013; Woodhams et al., 2014; Kueneman et al., 2016a, 2016b; McKenzie et al., 2018; Rebollar et al., 2016, 2020; Chen et al., 2022). The present study leverages culture-dependent techniques to discover the fungal diversity of boreal toad skin as well as test their interactions *in vitro* with *Bd*.

There are several reasons why investigating the fungal diversity of boreal toad skin is important. The Colorado boreal toad is a high-elevation species that has become locally endangered in Colorado due to *Bd*, which causes chytridiomycosis (Carey, 1993; Hammerson, 1999; Muths et al., 2003). Despite their dwindling numbers, boreal toads are long-lived (>20 years) and remain key players in subalpine and alpine systems over the course of their entire lifespans; they are Colorado's only alpine toad species, thus filling an important niche in pond food webs (Hammerson, 1999). Boreal toads, like all amphibians, undergo dynamic physiological changes over the course of their life cycle (i.e., egg, tadpole, metamorph, subadult, adult), which has been shown to greatly affect their skin microbiome and *Bd* infection progression (Hammerson, 1999; Kueneman et al., 2016a, 2016b). There are many potential inventions for mitigating chytridiomycosis caused by *Bd*, and one of these uses symbiotic toad skin microbes (Bletz et al., 2013;

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Kueneman et al., 2016a; McKenzie et al., 2018; Rebollar et al., 2016, 2020; Woodhams et al., 2014). Most of these investigations have focused on bacteria. The few amphibian mycobiome papers that exist are promising – fungi can combat *Bd* directly or indirectly by affecting other, *Bd*-inhibitory microbes, and might use metabolites or space/resource competition to do so (Kearns et al., 2017; Kueneman et al., 2016b; Medina et al., 2019). From these studies, it is clear that microbes across multiple domains interact with each other on amphibian skin. This may affect amphibian health in the context of disease, especially with regard to disease outcomes. To determine this, we need to investigate more members of these microbial communities in order to disentangle the role of various microbes on the skin surface of amphibians, including host-associated fungi. The first step towards this goal is to carry out descriptive studies that characterize the fungi on boreal toads.

Although *Bd* infections can be treated using broad-spectrum antifungal compounds, this treatment cannot be applied safely in the wild, can be toxic to amphibians, and may lead to the pathogen evolving drug resistance (Brannelly et al., 2012; Pessier and Mendelson, 2010). Probiotics, or beneficial microbes associated with the host, may provide a safer mitigation strategy for *Bd* infection. The goal of applying probiotics is not to add non-native microbes to an ecosystem, but to identify existing, native microbes from toad skin that can inhibit *Bd* infection or growth, and formulate a treatment to increase its abundance on toad skin. Ongoing research hypothesizes that these probiotics can act as a surrogate immune system for the toad by delaying the onset of the disease until the animal's innate immune response kicks in as it matures (McKenzie et al., 2018). Many bacterial and some fungal strains from healthy, wild amphibians have been found to have *Bd*-inhibiting abilities (Becker et al., 2011, 2021; Bletz et al., 2013; Kearns et al., 2017; Kueneman et al., 2016a; Loudon et al., 2014; Medina et al., 2019; Myers et al., 2012; Woodhams et al., 2015), and one bacterial strain is currently being field-tested in wild boreal toads with the help of Colorado Parks and Wildlife (unpublished work). Of the currently tested strains in boreal toads, few are fungi, despite new research that fungi can also effectively decrease *Bd* growth *in vitro* in other amphibian species (Kearns et al., 2017; Medina et al., 2019). Fungi could provide a different mechanism for *Bd*-inhibition that is not possible with only bacteria, giving wildlife ecologists one more tool in their conservation arsenal. Not only can fungi possibly inhibit the growth of *Bd,* but they can also be affected themselves by *Bd* or bacterial probiotics, which could influence host health outcomes.

To expand on possible probiotic targets and better characterize amphibian-associated skin fungi, we used culture-dependent techniques to determine: (1) the diversity of culturable fungal isolates from boreal toad skin, (2) if certain fungi or groups of fungi inhibit *Bd* growth, and (3) if the colony morphology or growth of fungal isolates change when grown with *Bd*?

2. Methods

2.1. Field collection and isolation

During the summer of 2019, we obtained skin swabs from a subalpine boreal toad site in Chaffee County, Colorado with an approved IACUC protocol (#2629) and an active Colorado Parks and Wildlife scientific collection permit (19HP0998). Since past research has shown the importance of life stage to skin mycobiome composition and diversity (Alexiev et al., 2021; Kueneman et al., 2016b), we sampled at least 3 individuals from each life stage (tadpole, metamorph, subadult, and adult). Individual boreal toads were captured by hand while wearing sterile nitrile gloves and swabbed 10 times using double-headed rayon-tipped swabs (BD BBL, East Rutherford, New Jersey) (n = 16 animals swabbed total). To sample boreal toad eggs, we swabbed a length of ten eggs from 4 egg masses. One of each of the swab heads was then rubbed and rolled thoroughly across the surface of either a Sabourad Dextrose agar (SDA) plate or a Potato Dextrose agar (PDA) plate (both made with chloramphenicol [50 mg used per 1 L of media] to inhibit bacterial growth). Plates were then stored at ambient temperatures (approximately 25 °C). Media recipes and sampling protocols can be found on FigShare (McKenzieLabBoulder et al., 2022a).

Plates were checked every 24 h for the growth of fungal colonies. Each visually unique fungal colony was sampled and transferred to clean SDA or PDA plates (without chloramphenicol) by extracting a plug from the original plate's agar until an isolated and pure culture was obtained. This method likely under-samples fungal isolates that are morphologically convergent, however, we did not have access to high-throughput culturing mechanisms that would allow collection of such a large and varied sample of isolates. Thus, we worked with what we could realistically collect and isolate at the time. The resulting plates were stored at 4 °C once a pure culture was isolated, for subsequent DNA extraction and production of glycerol stocks. Agar plugs from these plates were transferred to glycerol stocks and kept at −80 °C for long-term storage (McKenzieLabBoulder et al., 2022a).

2.2. Identification of fungi via Sanger sequencing

Pure fungal isolates were grown on agar plates (SDA or PDA, respectively) overlain with sterilized cellophane to allow an easy removal of fungal tissue from the plate following previously described methods (Cassago et al., 2002). Cultures were grown at ambient temperature on top of the cellophane for subsequent DNA extraction. Plates were incubated for varying durations – as little as 24 h and up to 3 weeks, at ambient temperature until enough tissue necessary for the subsequent DNA extraction was able to be harvested. Once adequately grown, we used a sterile spatula in a biosafety cabinet to scrape fungal colony tissue from the surface of the cellophane into a sterile 1.5 mL tube. We lyophilized the tissue in these tubes for 24 h at ambient temperature using a vacuum lyophilizer (FreeZone Plus 6 Liter Cascade Console Freeze Dry Systems, Labconco, Kansas City, MO). Using sterile technique in a biosafety hood, we crushed the tissue with a pestle until powdered. This resulting tissue was used for DNA extraction (DNeasy Plant Mini Kit, Qiagen, Hilden, Germany).

We PCR amplified part of the ITS region using ITS1-F and ITS4 primers (10 μM concentration) (Martin and Rygiewicz, 2005), which was longer than the high-throughput sequencing project previously completed. Unpurified PCR products were then sent to GeneWiz LLC (South Plainfield, NJ) for Sanger sequencing. All protocols for DNA extraction, PCR, and sample preparation can be found on FigShare (McKenzieLabBoulder et al., 2022a). Sanger sequences are also on FigShare (McKenzieLabBoulder et al., 2022b).

We Sanger sequenced 120 axenically cultured fungal isolates, which encompassed 38 genetically unique genera. UGENE was used to trim both ends of the reads (to a minimum quality value of 30) (Okonechnikov et al., 2012). Reads were each aligned to their respective forward or reverse sequence with MUSCLE August 3, 1551 (Edgar, 2004). Overhanging sequences were manually trimmed and consensus sequences were generated using UGENE. Sequences were run through the BLASTN algorithm against the NCBI nucleotide (nt) databases to identify taxa with high sequence similarity. Taxonomy was assigned to our sequences by selecting the BLAST hit(s) with the greatest percent identity (Table S1).

Using the above method, one isolate (BTF_60), which subsequently became important to downstream analyses, was only identified via its order, Helotiales. This isolate is part of the class Leotiomycetes, a group with a largely unresolved phylogeny (Johnston et al., 2019; Quandt and Haelewaters, 2021) and for which many ITS marker gene sequences available in Genbank are derived from environmental sequencing studies where identification below the ordinal level is uncommon for Helotiales (Nilsson et al., 2019a). In an effort to refine the identity of this particular isolate, we placed the BTF_60 ITS sequence in a recently published pan-Helotiales ITS alignment (Lebeuf et al., 2021). Muscle (v3.8.1551) was used for sequence alignment, and RaxML (v8.2.12) was

Table 1
The counts of total isolates grown from different toad life stages, as well as the media used to grow and isolate them.

	Isolates grown from eggs	Isolates grown from tadpoles	Isolates grown from metamorphs	Isolates grown from subadults	Isolates grown from adults	Totals
Isolates grown on PDA	46	4	3	6	8	67
Isolates grown on SDA	29	2	5	8	9	53
Totals	75	6	8	14	17	120

used with the following settings: f a, the GTRCAT model, with 200 rapid bootstraps. Based on the large phylogeny, a subset of sequences closely related to the BTF_60 isolate were separately aligned and subjected to phylogenetic reconstruction.

2.3. Fungi versus Bd lawn plate assay

We selected representatives from 16 unique fungal isolates, which was a tractable number to work with given laboratory resources. We used previously published ITS marker gene sequences from a past research study of the boreal toad skin mycobiome (sequences are deposited at the European Nucleotide Archive (ENA) in association with the project accession PRJEB41738), which identified fungal OTU's that were associated with the host (at each distinct life stage) as opposed to its habitat (Alexiev et al., 2021). Although we cultured and sequenced 120 distinct isolates, we did not have the capacity to test each against *Bd* at the time, so we used our previous study results to choose a tractable number to test. Therefore, we then chose cultured isolates to test against *Bd* in co-culture in the present study based on groups that were observed to be host-associated or consistently present across most boreal toad life stages in the previous study (Alexiev et al., 2021). Once we had Sanger sequences of the fungal isolates, we created a custom BLAST database of the Sanger sequences from fungal isolates in this study (amplified ITS1F-ITS4 with average read length of 583 base pairs), which are longer than the high-throughput sequences (amplified ITS1F-ITS2 with average read length of 158 base pairs), and matched the ITS high-throughput sequence data from the 2021 study to these (a histogram of percent identity matches is shown in Suppl Fig. 1).

We grew fungi and *Bd* on 1% tryptone agar plates for 8 days, to examine how fungal and *Bd* growth might be affected when co-cultured together versus alone. We had three types of plates, all in duplicate, per fungal isolate and using the same vial of *Bd* (standardized via hemocytometer count) per set: (1) experimental co-culture plates with a *Bd* lawn and fungal isolate agar plug in the center, (2) control plates with only *Bd* lawn, and (3) control plates with only the fungal isolate agar plug. We grew pure cultures of each fungal isolate in duplicate, originating from the same single plate in cold storage at −4 °C, so that plates (1) and (3) used agar plugs from each set of duplicate pure cultures and are therefore paired. *Bd* culture was continuously maintained in liquid culture flasks at ambient temperature (McKenzieLabBoulder et al., 2022a). To prepare the plates, we created a *Bd* zoospore lawn using plate washes (i.e., 5 mL of 1% tryptone broth is left on a *Bd* agar plate for 30 min then pipetted off) measuring on average 1.3 × 10^6 zoospores/mL (concentrations were standardized using a hemocytometer count). Once the

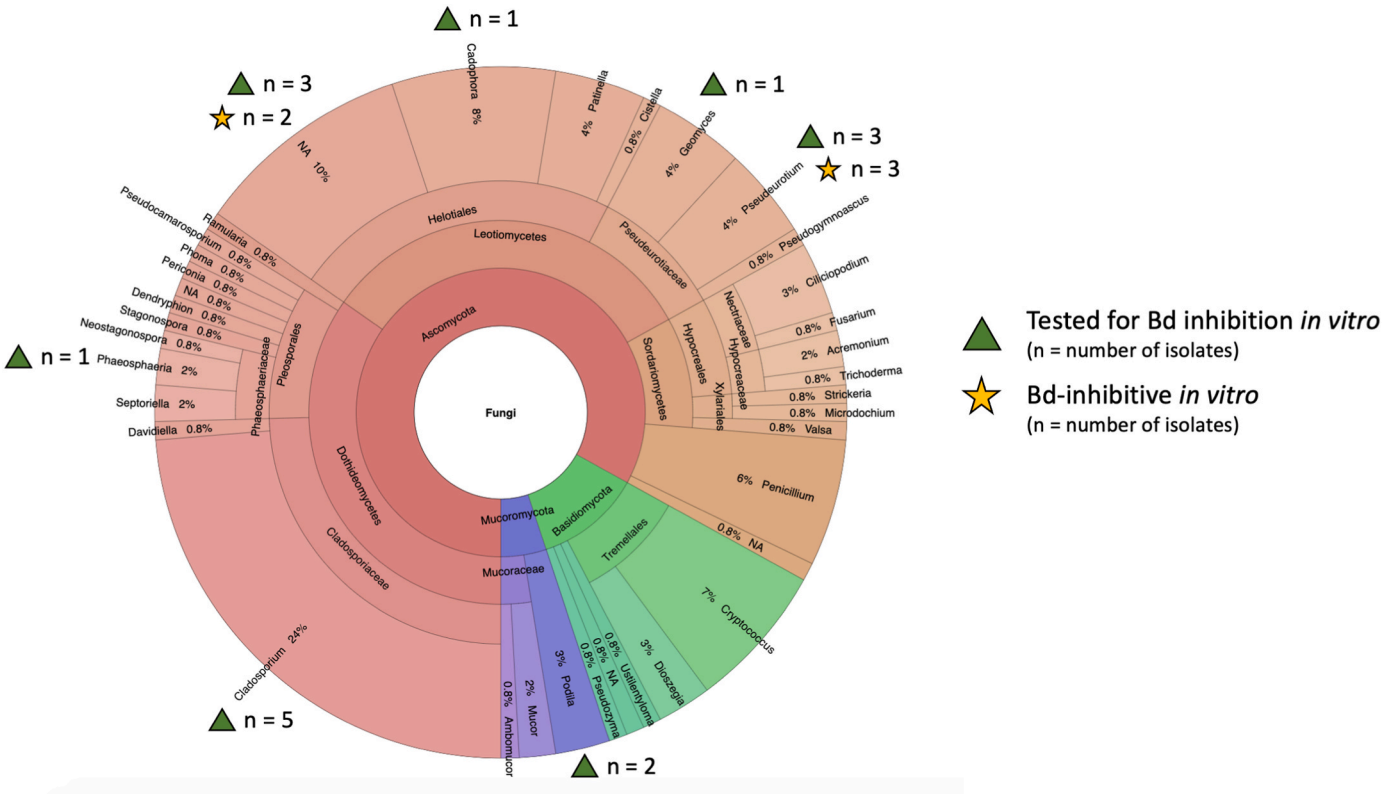

Fig. 1. Krona graph of the percentage of the Sanger sequence data (derived from isolates) that matched with certain fungal phylogenetic groups. Green triangles indicate groups that were tested for *Bd*-inhibition *in vitro* in co-culture petri plate assays, with the number of isolates tested. Of these, yellow stars then indicate groups and number of isolates that were inhibitive to *Bd*.

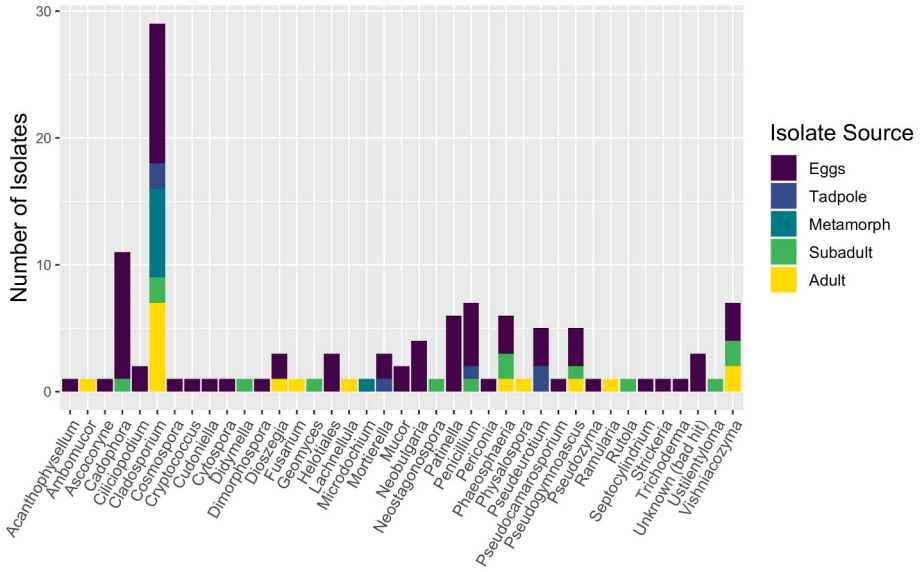

Fig. 2. The number of fungal isolates per fungal taxon and their source (i.e., life stage of the toad). Each isolate represents a distinct BLAST match to the UNITE database.

lawn dried, we excised a 1 cm diameter circle from the edge (newest growth) of a pure fungal colony and pressed it lightly face down in the center of the *Bd* lawn, so that fungal tissue was touching the *Bd* lawn. Plate assay preparation is described in the protocol on FigShare (McKenzieLabBoulder et al., 2022a).

Over the course of 8 days, we recorded fungal morphology and color, measured fungal colony size (diameter in mm), and measured the *Bd* inhibition zone (diameter in mm) if present. We used Fiji software (version 2.1.0/1.53c) to measure the size change (mm) using photos taken on days 0, 1, 3, and 8. Protocols can be found on FigShare (McKenzieLabBoulder et al., 2022a).

2.4. Statistical analysis and visualizations

We created a Krona graph with the fungal Sanger sequences using https://github.com/marbl/Krona and added metadata on *Bd* inhibition to visualize the abundances of different phylogenetic groups of fungi we cultured. All other analyses and visualizations were created using R (version 4.0.5 (2021-03-31)) and ggplot2 (R Core Team, 2019; Wickham, 2016). For each sequence, we identified the host's stage of development (i.e., eggs, tadpoles, metamorphs, etc.) and how the sequence identities compared to ITS high-throughput sequence whole community data from Alexiev et al., 2021. We compared our findings to data demonstrating whether ITS-identified OTUs were host- or habitat-associated (Alexiev et al., 2021) and then plotted how many of these were Sanger-sequenced and had consistent phylogenetic identification.

We compared the average inhibition zones of *Bd*-inhibitive fungal colonies (2 replicates per isolate) using a one-way ANOVA with Tukey post-hoc analysis (zone of inhibition diameter [mm] ~ plate type [*Bd* or control]). We used the Fiji-generated growth over 8 days (colony area in mm^2) of fungi to compare control and treatment plates via T-test (colony area [mm^2] ~ plate type [*Bd* or control]). All analyses scripts and data inputs and outputs can be found on GitHub: https://github.com/aalexiev/FungalIsolatesRepo.

3. Results and discussion

3.1. Are certain phylogenetic groups more readily culturable off boreal toads?

Of the 120 unique fungi isolated from boreal toad skin, 38 fungal genera were represented. The isolate counts and their respective origin are shown in Table 1 and details on the results of matching the BLASTn database to the Sanger sequences can be found in Supplementary Table 1. The majority of isolates (83%) were from the phylum Ascomycota (Fig. 1), which is a demonstrated pattern among other amphibian-associated fungi (Kearns et al., 2017; Medina et al., 2019). We found representatives primarily from the classes Leotiomycetes (32% of all isolates) and Dothideomycetes (35% of all isolates) (Fig. 1). Twenty-three percent of fungal isolates were from the genus *Cladosporium*, which mostly originated from eggs, adults, and metamorphs (Figs. 1 and 2). Helotiales (23% of all isolates) and Pleosporales (9% of all isolates) were the most highly culturable orders from boreal toads (Fig. 1). Most isolates were cultured from eggs (Fig. 2). This initial sequence-based identification helped us establish a few fungal groups that were highly culturable from boreal toad skin across all life stages and could be of interest for future investigation into whether and how they interact with the host amphibian and its resident skin microbiota. For example, *Pseudogymnoascus* and *Geomyces* both include psychrophiles, which is consistent with the fact that boreal toads live in a cold, high-elevation pond and often overwinter in hibernacula beneath the snow (Hammerson, 1999), implying some level of niche specialization in skin microbiota for this particular host habitat. Leotiomycetes were another highly represented fungal group in this isolate collection, and were dominated by Helotiales (of which 10% of the isolates were only identified down to order). Leotiomycetes and subsequently Helotiales are understudied, and the phylogeny of these groups remains difficult to resolve (Gernandt et al., 2001; Johnston et al., 2019; Quandt and Haelewaters, 2021; Wang et al., 2006; Zhang and Wang, 2015). Animals that harbor host-associated microbiota, like amphibians may be a source of isolates to help resolve such phylogenetic uncertainties, and the lack of research into these systems to date could explain why their phylogeny is currently poorly resolved.

Helotiales fungi in general seem to potentially have a connection to the host that is worth further investigation (Alexiev et al., 2021). This is an interesting fungal group because many are plant-associated and their

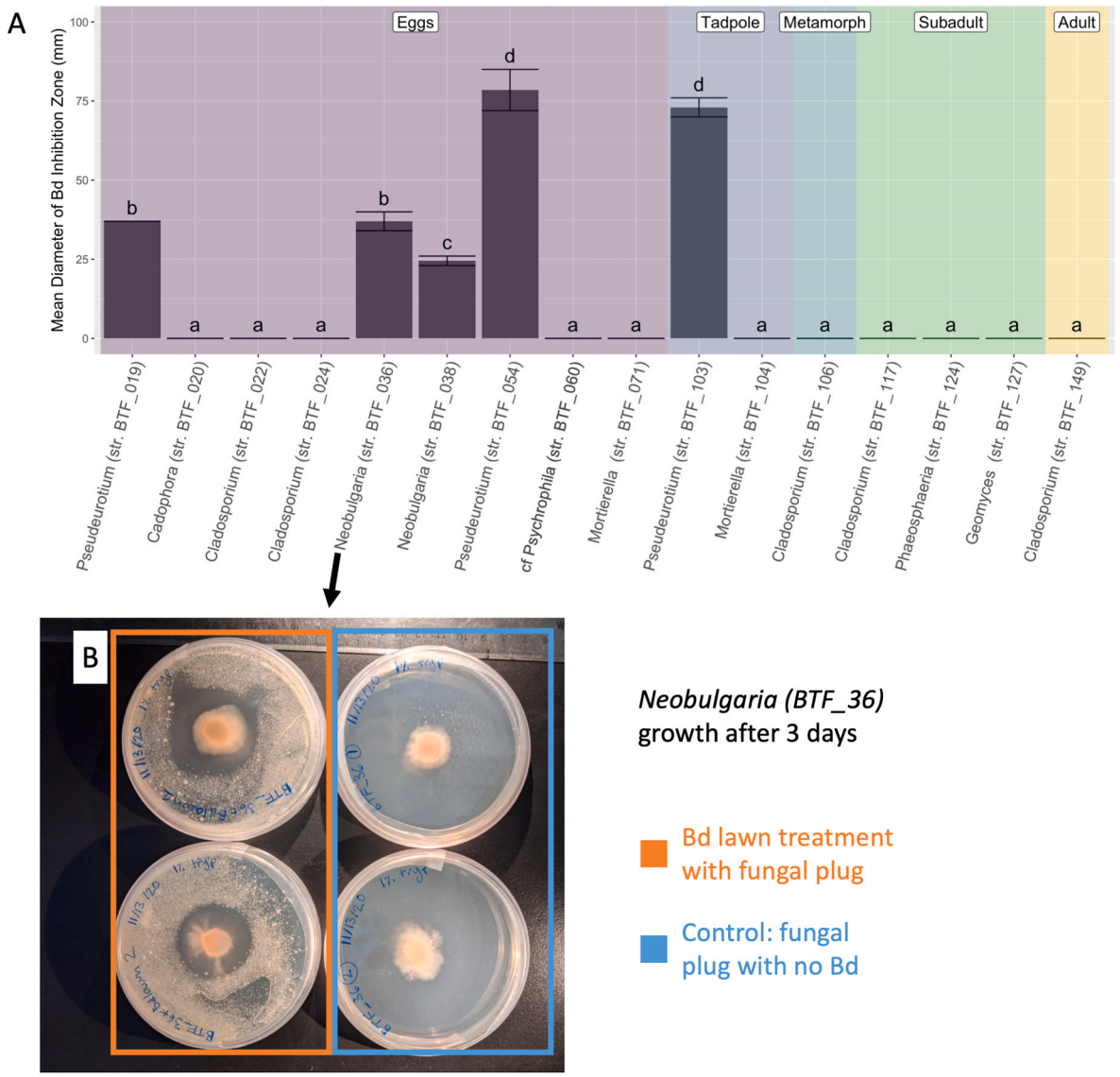

Fig. 3. *Bd* inhibition assay results. (A) Shows the degree of *Bd* inhibition, measured as the diameter of *Bd* inhibition zone (mean mm taken across duplicates), for each of 16 tested fungal isolates. The letters above each bar attribute significance groupings based on an ANOVA with Tukey post-hoc analysis across all isolates. The graph is colored and labeled based on the life stage the isolate originated from when initially swabbed. (B) Shows a photo of *Neobulgaria* sp. (BTF_36) on *Bd* lawn plates from the assay to serve as an example of a zone of inhibition from a fungus that successfully inhibits *Bd* after three days of growth.

phylogeny is not well-resolved (Gernandt et al., 2001; Johnston et al., 2010, 2019; Quijada et al., 2017; Wang et al., 2006; Zhang and Wang, 2015). Helotiales sequences and isolates have only recently been associated with amphibians (Alexiev et al., 2021; Medina et al., 2019), and Helotiales fungi were generally very abundant on boreal toad skin (Figs. 1 and 2), representing a new potential source of fungal isolates of the Helotiales group. For example, *Ascocoryne*, a saprobe of plant material, is one of many groups of isolates from Helotiales that we cultured, and multiple genetically distinct *Ascocoryne* isolates were cultured readily from boreal toad skin (Fig. 2). If Helotiales fungi are truly host-associated and perhaps play some currently unknown role on toad skin, it's possible that *Bd* could influence the growth and/or activity of Helotiales strains. This also supports the concern that using broad-spectrum antifungal drugs to combat *Bd* could just as easily affect symbiotic fungal microbes on amphibian skin and in turn have an unintended negative effect even if the pathogen is cleared in the short term.

Many of the cultured fungi were highly represented in sequence databases, which confirms a potential bias in that culturable taxa are more represented in sequence-based databases. Ninety-six percent of the OTUs found in the high-throughput sequencing project (Alexiev et al., 2021) were recovered in culture during the present study. This does not include fungal colonies or OTUs where DNA extraction or PCR amplification did not work, which is a shared bias in both culture-independent and -dependent methods used here. Additionally, many fungal cultures did not yield enough genetic material for Sanger sequencing, or their sequences matched poorly to existing databases used for identification. These unrepresented fungi could be important to the host, however, we could not evaluate them with the experimental methods described here. This reinforces the need for innovative new fungal descriptive techniques. Single-cell whole-genome sequencing and sequencing of multiple different marker genes could help expand on known fungal groups, but remains expensive and technically challenging (Gawad et al., 2016; Macaulay and Voet, 2014; Nilsson et al., 2019b). High-throughput culturing has been used to isolate host-associated bacteria and could be useful to isolate host-associated or symbiotic fungi as well (Highlander, 2012).

Table 2
Strains and their descriptive statistics with regards to the zones of inhibition in Bd co-culture plate assays.

Strain name	Mean zone of inhibition when grown on *Bd* lawn (mm)	Standard Error (mm)
***Pseudeurotium* (str. BTF_019)**	37.0	0.0
***Cadophora* (str. BTF_020)**	0.0	0.0
***Cladosporium* (str. BTF_022)**	0.0	0.0
***Cladosporium* (str. BTF_024)**	0.0	0.0
***Neobulgaria* (str. BTF_036)**	37.0	3.0
***Neobulgaria* (str. BTF_038)**	24.5	1.5
***Pseudeurotium* (str. BTF_054)**	78.5	6.5
cf *Psychrophilla* (str. BTF_060) [previously Helotiales (str. BTF_60)]	0.0	0.0
***Mortierella* (str. BTF_071)**	0.0	0.0
***Pseudeurotium* (str. BTF_103)**	73.0	3.0
***Mortierella* (str. BTF_104)**	0.0	0.0
***Cladosporium* (str. BTF_106)**	0.0	0.0
***Cladosporium* (str. BTF_117)**	0.0	0.0
***Phaeosphaeria* (str. BTF_124)**	0.0	0.0
***Geomyces* (str. BTF_127)**	0.0	0.0
***Cladosporium* (str. BTF_149)**	0.0	0.0

3.2. Do some fungi inhibit Bd? Are some groups of fungi more inhibitive than others?

Thirty-one percent (5 out of 16) of the fungi we challenged against *Bd in vitro* were *Bd*-inhibitive. Two *Neobulgaria* and three *Pseudeurotium* isolates were significantly *Bd*-inhibitive, with two *Pseudeortium* isolates being the most inhibitive (Fig. 3, Table 2). Generally, *Pseudeurotium* isolates were more inhibitive than *Neobulgaria* isolates (62.8 mm average zone of inhibition versus 30.75 mm average zone of inhibition, respectively). Furthermore, this is the first time many isolates that are consistently found in association with boreal toads have been characterized in a vertebrate host. *Neobulgaria* isolates are often found on plants (Johnston et al., 2010; Roll-Hansen and Roll-Hansen, 1979) and *Pseudeurotium* is typically found abundantly in soil (Idid et al., 2014; Santos et al., 2020), but neither have been studied in amphibians until recently (Alexiev et al., 2021; Kearns et al., 2017; Medina et al., 2019). Studies have demonstrated an association between *Pseudeurotium* and ocean invertebrates like sponges, corals and sea fans (Kumla et al., 2014). As in past studies (Alexiev et al., 2021; Kearns et al., 2017; Medina et al., 2019), we found that most of the fungi we tested have not been previously studied as host-associated organisms, despite being repeatedly found living on amphibians. Both *Neobulgaria* and *Pseudeurotium* include cold-tolerant fungal species (Untereiner et al., 2019; Wang et al., 2015), which makes sense considering boreal toads live at high elevation and overwinter in burrows beneath the snow (Hammerson, 1999). This gives us reason to explore a new possible niche for study and targeted isolation for many fungi.

The *Bd*-inhibitory isolates we recovered largely originated from the earlier life stages of the host (i.e., eggs and tadpoles). Previous data demonstrated that earlier life stages tend to have a more unique fungal mycobiome relative to the environment compared to later developmental life stages. Eggs in particular contain a greater relative abundance of key host-associated indicator taxa, which match what we recovered in our culturing efforts, however egg-associated fungi are also found across all life stages (Alexiev et al., 2021). Although these isolates were largely cultured from eggs and likely came from environmental microbes landing on eggs, they also appeared in other life stages. Tadpoles emerge from eggs, coming into direct contact with the jelly encasing that these fungi live on, and show overlap in the fungal sequences recovered from both life stages as well as those from later life stages (Alexiev et al., 2021) (Suppl Table S2). Therefore it is possible they are seeded by microbes in this process, which is also consistent with data about the bacterial community (Prest et al., 2018). *Bd* is also thought to persist at low abundance in water until it comes upon a host

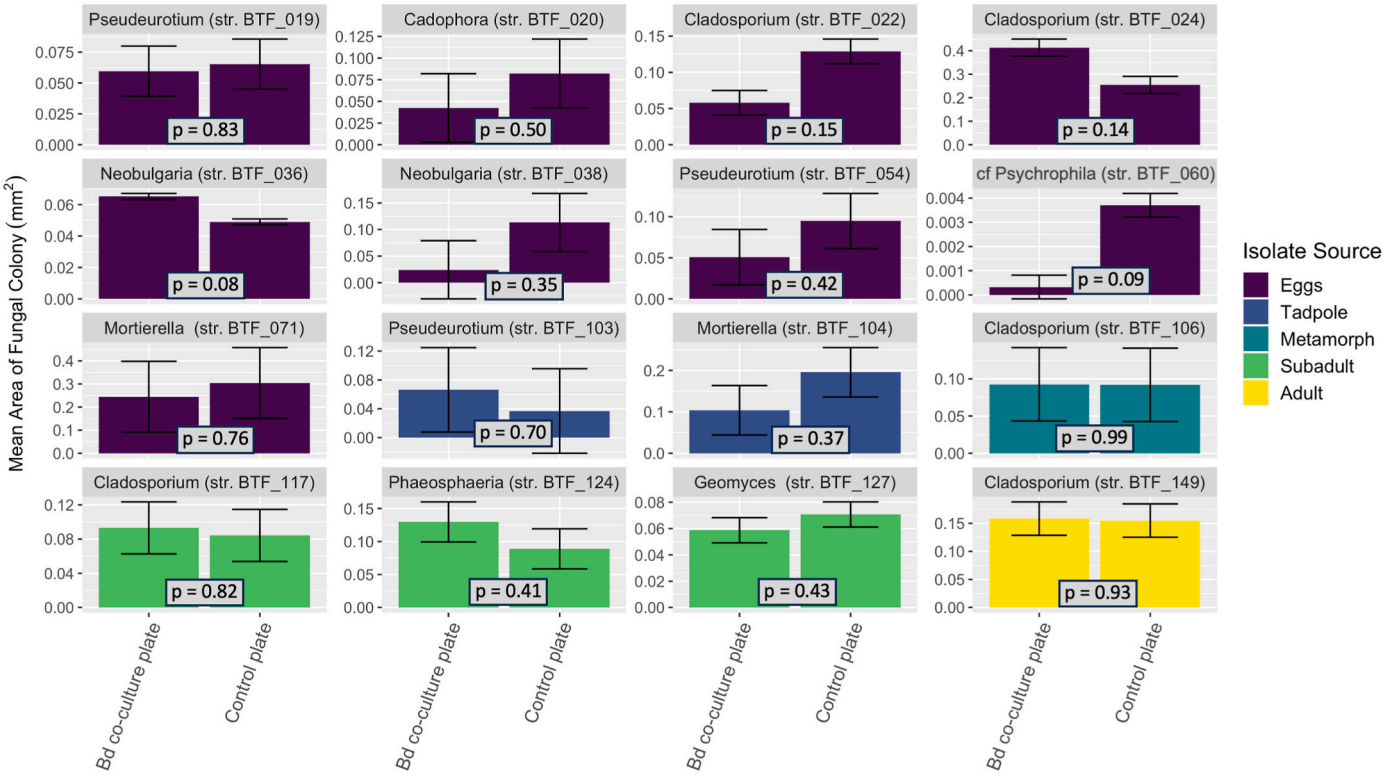

Fig. 4. Growth over 8 days per fungal isolate when grown on *Bd* co-culture plates (the same plates described in Fig. 3), colored by the life stage the isolate was originally sourced from. The names above each graph include the strain name we attributed to the isolate throughout the study. Growth was measured using the area of the fungal colony (mm^2), and each replicate is shown as 1 bar. Darker bars indicate the treatment (*Bd* lawn) and lighter bars indicate the control plates.

Table 3
Strains and their descriptive statistics with regard to fungal isolate growth when grown with and without Bd in co-culture.

Strain name	p-value from T-test	Mean of *Bd* co-culture plates (mm)	Standard Deviation of *Bd* co-culture plates (mm)	Mean of control (no *Bd*) plates (mm)	Standard Deviation of control (no *Bd*) plates (mm)	Standard error (mm)
***Pseudeurotium* (str. BTF_019)**	0.83	0.00	0.02	−0.01	0.00	0.02
***Cadophora* (str. BTF_020)**	0.50	0.00	0.02	0.03	0.01	0.04
***Cladosporium* (str. BTF_022)**	0.15	−0.02	0.00	0.03	0.00	0.02
***Cladosporium* (str. BTF_024)**	0.14	0.02	0.01	0.04	0.01	0.04
***Neobulgaria* (str. BTF_036)**	0.08	−0.02	0.00	−0.01	0.02	0.00
***Neobulgaria* (str. BTF_038)**	0.35	0.06	0.00	0.12	0.05	0.05
***Pseudeurotium* (str. BTF_054)**	0.42	0.00	0.01	0.00	0.00	0.03
cf *Psychrophilla* (str. BTF_060) [previously Helotiales (str. BTF_60)]	0.09	0.00	0.00	0.00	0.00	0.00
***Mortierella* (str. BTF_071)**	0.76	0.40	0.20	0.38	0.12	0.15
***Pseudeurotium* (str. BTF_103)**	0.70	0.03	0.01	0.04	0.04	0.06
***Mortierella* (str. BTF_104)**	0.37	0.13	0.05	0.15	0.07	0.06
***Cladosporium* (str. BTF_106)**	0.99	0.07	0.00	0.07	0.05	0.05
***Cladosporium* (str. BTF_117)**	0.82	0.04	0.00	0.05	0.03	0.03
***Phaeosphaeria* (str. BTF_124)**	0.41	0.02	0.00	0.04	0.00	0.03
***Geomyces* (str. BTF_127)**	0.43	0.23	0.07	0.12	0.08	0.01
***Cladosporium* (str. BTF_149)**	0.93	0.01	0.00	0.00	0.00	0.03

to infect and may use water as a mode of infection, waiting until animals mature before mounting an infection (Bosch et al., 2023; Johnson and Speare, 2003; Kirshtein et al., 2007). Further, Boreal toad skin bacterial communities go through cycles of succession, wherein hatching from eggs and metamorphosis are developmental stages that begin as blank slates for microbial assembly (Prest et al., 2018). Early-stage tadpoles and newly metamorphosed toads have skin bacterial communities dominated by fast-growing bacteria that are strong competitors, similar to many early-stage successional communities in nature (Prest et al., 2018). This could also be the case for amphibian skin fungi as well, especially considering the wide variety of growth rates seen in the fungal isolates we cultivated. As noted earlier, some grew as quickly as 24 h and others up to 3 weeks. We did not investigate successional dynamics in fungal skin communities, but this would be an interesting topic for further research. In addition, bacterial richness has been linked in the past to *Bd*'s ability to invade the skin microbiome (Chen et al., 2022; Piovia-Scott et al., 2017). Fungi have not been investigated as part of this concept; however, more fungal taxonomic diversity or biomass on the skin could provide *Bd* with more competitors, thus making it more difficult for the pathogen to colonize the host. A previous marker gene sequencing study found that the early life stages are composed of potentially anti-fungal bacterial taxa (Kueneman et al., 2016b), which could be competing with fungi for resources and space. Our present study found that many of the *Bd*-inhibitive cultured fungal isolates came from these early life stages (i.e., eggs and tadpoles). As such, we hypothesize that the toad's early-life community could be fairly antimicrobial in its function due to increased competition as microbes scramble to colonize young animals' skin. An additional benefit to this might be direct *Bd*-inhibition and/or less opportunity for the pathogen to colonize the host.

Finding such a wealth of *Bd*-inhibitive fungal isolates naturally occurring in the toad's mycobiome could be useful for application as probiotics, either as single- or multi-strain assemblages, and possibly in combination with existing probiotic bacteria. These interactions could boost or inhibit the ability for probiotics to more consistently establish and persist on host skin. Developing these probiotics would not only require extensive *in vitro* culture tests though, but also animal safety testing, as some microbes can be opportunistic pathogens or simply elicit an immune response at higher abundances in hosts (McKenzie et al., 2018). Generally, we know little about host-associated fungi in wildlife like amphibians, especially compared to bacteria, but it is clear from the work described here that they are worth investigating further.

3.3. Did morphology or growth of fungi change when cultured with bd?

We hypothesized that perhaps *Bd* could affect the toad skin fungal community in terms of morphology or growth. Colony morphology (color, shape, texture) did not change for any isolates when grown with *Bd*. Growth over 8 days did not significantly vary between treatments, but approached marginal significance (see p-values in Fig. 4 and Table 3). Although it is possible that with more replicates, some isolates would show a significant pattern, we could not obtain a higher replicate of plates given laboratory resources at the time. Therefore, there is a high level of variation between some duplicates, which is captured in the standard error bars of Fig. 4. Despite this high variation, we see a couple of isolates approach marginal significance that would be worth testing in higher replication in the future: the growth of *Neobulgaria* sp. (isolate BTF_36) and cf *Psychrophila* (isolate BTF_60) had the lowest p-values (p-values = 0.08 and 0.09, respectively) (Fig. 4, Table 3). Techniques that help with high-throughput testing of cultured fungi would be a valuable contribution to the field in the future.

In addition to the skin damage caused by chytridiomycosis, it is possible that *Bd* alters the toad fungal community members in other ways that affect host health. *Bd* is also not the only threat to amphibian health. Amphibian eggs in water can be overtaken by saprobic fungi since they are sessile in highly-microbially-diverse sediments, which affects survivorship of the animal (Densmore and Green, 2007; Pessier and Mendelson, 2010). The authors have seen eggs rotting away in ponds on field sampling trips in the past at the sample sites described here. Although we did not test these other pathogens like we did *Bd*, given that we observed antifungal-producing isolates (largely from the Helotiales group) mostly from eggs, it makes sense for eggs to harbor isolates with competitive strategies given the habitat. We hope to further investigate these suppositions in the future as they provide interesting and exciting questions regarding how amphibians survive their habitat in the context of fungal communities in, on, and around them.

4. Conclusion

About 31% of the culturable subset of fungal isolates tested in this study were inhibitory against *Bd*, indicating that fungal symbiont diversity is an overlooked but important aspect of microbial interactions in amphibian systems. Increased high-throughput approaches could offer more insight into the interactions among fungi in host-associated microbial systems. It is possible that fungi could have some competitive advantages over oft-used bacteria as probiotics and could even be tested in multi-strain probiotics combined with bacteria to reduce *Bd*. Knowing

which fungal species readily establish on amphibian skin could help determine which taxa offer more promise as potential probiotics. Much of this work hinges on future developments in high-throughput culturing and decreasing the cost of single-cell genome sequencing or sequencing multiple marker genes in a large dataset.

In addition, antifungal drugs or applied probiotics could alter resident symbiotic fungi that may be in the process of inhibiting *Bd*. This side effect is possible because the mechanisms by which these therapies work broadly targets shared fungal characteristics, like slowing fungal growth. Host safety would also need to be tested extensively before the use of any fungal probiotic, especially since many microbes are opportunistic. The future of amphibian work should focus on further investigating fungi and possibly developing multi-strain probiotics, with fungi included.

It is also important to think of how *Bd* affects the fungal community. Possibly the abundances of species in the innate amphibian mycobiome might change how effective an added probiotic is or how severely *Bd* affects host skin. Perhaps *Bd* has previously unknown effects on the mycobiome beyond its direct infection of skin cells, which could alter the health of survivors of the infection or semi-susceptible species. Future investigations into how fungi and bacteria interact on hosts are pivotal to understanding what probiotics we can add to amphibian skin, how to apply them, and how *Bd* interacts with these groups.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Valerie J. McKenzie reports financial support was provided by Morris Animal Foundation. Alexandra Alexiev reports financial support was provided by Indian Peaks Wilderness Association. Alexandra Alexiev reports financial support was provided by Colorado Mountain Club Foundation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2023.101297.

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