

## TECHNICAL NOTE

# Identification of fungi found on desiccated human remains in an arid outdoor environment

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## Abstract

Three fungi not previously reported on desiccated human remains were identified on cadavers at the Forensic Investigation Research Station (FIRS) in Whitewater, Colorado. The location of the FIRS provides the unique opportunity to observe the stages of decomposition in a high desert environment. The two cadavers used in the study were in the late stages of decomposition (PMI of approximately 1520 and 1820 days) to the point of desiccation and had developed an extensive black crust on the skin that remained. Skin samples of the two cadavers were taken and plated onto potato dextrose agar to determine whether fungi were present on the desiccated tissues. Three different fungi consistently dominated cultures grown from numerous samples taken from each cadaver. Based on morphological observations, nuclear rDNA sequence data, and phylogenetic analyses, two fungi were identified to species (*Aureobasidium melanogenum* and *Didymella glomerata*) and one fungus was identified to the genus level (*Alternaria*). These results will contribute to the understanding of the role that fungi might play in late-stage decomposition and the extended postmortem period.

## KEYWORDS

18 SSU rDNA, 28S LSU rDNA, *Alternaria*, *Aureobasidium*, decomposition, *Didymella*, forensic mycology, fungi, high desert, ITS, molds, taphonomy

## Highlights

- Fungi are involved in long-term human decomposition in arid environments.
- Three dominant fungi identified thus far using morphological, molecular, and phylogenetic techniques.
- Sequence data and phylogenetic analyses placed the fungi into three different genera: *Aureobasidium*, *Alternaria*, and *Didymella*.
- The presence of these fungi so late in the decomposition processes suggests that they may be useful for predicting long-term postmortem intervals.

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## 1 | INTRODUCTION

The current research was undertaken at the Colorado Mesa University Forensic Investigation Research Station (FIRS) located in Whitewater, Colorado. The FIRS was established in 2012 as a taphonomic research facility and presents a unique location in which to observe the progression of human decomposition that includes the identification of indicators for long-term postmortem intervals (PMIs). The FIRS is situated in a high desert environment at 1457 meters above mean sea level where it receives less than 25 cm of rain and around 19 cm of snow annually. Due to the arid environment at the FIRS, cadavers are left to decompose and desiccate. The desiccated skin tissue often develops a black crust that thickens over time. This is typically associated with a postmortem interval of minimally 2 years (Figure 1). The questions arose whether the black crust might be composed of fungi, and if so, what species were present, what role they might play in the decomposition of desiccated tissue, and whether they could be used as indicators for long-term post-mortem interval (PMI) estimations. The purpose of the current study was to address the first question of whether fungi were present and to determine the identification of these fungi.

Fungi are known to play a role in the decomposition of human remains and have been explored as a forensic science tool (i.e., forensic mycology) [1–3]. Recently, one goal has been to determine whether the succession of fungal species can be used similarly to insect succession to determine the postmortem interval [4–7]. In addition, the presence of certain fungal species in the soil has been proposed as a method for identifying grave sites [8, 9].

While the area of forensic mycology is still expanding, most work has focused on the decomposition of non-human mammals, on the early decomposition stages, or on decomposition in high humidity environments that are generally conducive to fungal growth. As noted above, the FIRS presents a unique opportunity to explore the role that fungi play in the late stages of human decomposition (i.e., desiccation and skeletonization) in an arid outdoor environment. Particularly with desiccation, the extended postmortem period shows wide variation in decomposition trajectory. The more that is known about the variables that impact this trajectory, the closer the postmortem interval can be estimated and the more efficient taphonomists can be when it comes to determining what happened to the

individual at or about the time of death. Both are important goals in any medicolegal investigation.

## 2 | MATERIALS AND METHODS

### 2.1 | Fungal sample collection

The FIRS has a human body donation program, where donors or next-of-kin donate human remains for taphonomic study and for the osteological collection. Donors are placed in an outdoor, fenced area that eliminates large scavengers, and are left to decompose. The facility includes a HOBO weather station that takes hourly readings of temperature, precipitation, wind speed and direction, and solar radiation.

Initial samples were taken in March 2018 from a male cadaver that had been decomposing in the outdoor environment for 4 years (PMI of 1520 days) and was totally desiccated with little skeletal material showing (Figure 1). The individual was the first individual placed at the FIRS (January 17, 2014) and therefore represented the longest PMI available for this study. The cadaver had developed an obvious, extensive black crust on the skin 2 years after placement. Initial samples of this crust were obtained by swabbing the surface of the skin on the abdomen and legs with a cotton-tipped applicator or by cutting or scraping small samples of the same skin using a scalpel. Samples were plated directly onto potato dextrose agar (PDA) plates in the field, then taken immediately to the laboratory to grow at room temperature. Initial growth was noted within 48–72 h and distinct colonies/sporulation could be distinguished within 4 days of incubation. Of note was the fact that there appeared to be a limited number of different fungi that dominated the culture plates, regardless of how samples were taken. To determine if these results could be replicated, subsequent skin samples were taken from the same male cadaver in May 2018, April 2019, and April 2020. In addition, in 2018 and 2019, skin samples were taken from a female cadaver with a date of death and placement only 4 months later than the initial donor (May 19, 2014; approximate PMI of 1820 days). To minimize potential contamination from outside air, all skin samples used for morphological and molecular identification discussed in this article were taken using a scalpel or forceps and placed into clean Petri



**FIGURE 1** Typical condition of skin and bones used to obtain samples analyzed in this article. This cadaver had been decomposing for 4 years at the time these photos were taken.

dishes or sample tubes for transport to the laboratory. Separate, clean tools were used to take samples from the two cadavers and from different areas of each cadaver. Once in the laboratory, the samples were plated directly onto PDA plates by embedding small chunks of skin into the media or by crumbling the skin samples over the media. In each case, three fungi dominated the cultures, as shown in Figure 2.

## 2.2 | Morphological identification of fungi

Once fungal colonies began to grow, subcultures were taken from each of the three dominant fungal colonies to obtain pure cultures to be used for more detailed morphological identification. Pure cultures were grown on PDA and observed under an Olympus CX31 compound microscope. Preliminary identification of each fungus was based on colony growth habit, color, and morphology as well as spore or fruiting body morphology (Figures 3, 4, 5).

## 2.3 | Fungal DNA extraction, sequencing, sequence identification, and phylogenetic analyses

Samples for DNA extraction were taken from 3-week-old pure cultures of each fungus by scraping a sterile pipette tip across the surface of the mature, spore-producing portion of each culture. Qiagen

DNeasy Plant Pro kit (Qiagen, Germantown, MD, U.S.A.) was used for DNA extraction. The fungal material amounts ranged between 20 and 30mg and were placed into separate 2-mL tissue disruption tubes and submerged in 500 $\mu$ L of Solution CD1. Homogenization was done on a Vortex-Genie® 2 with a Vortex Adaptor. DNA extractions were performed according to the protocol provided by the manufacturer. The presence and quality of DNA were confirmed for each sample by agarose gel electrophoresis.

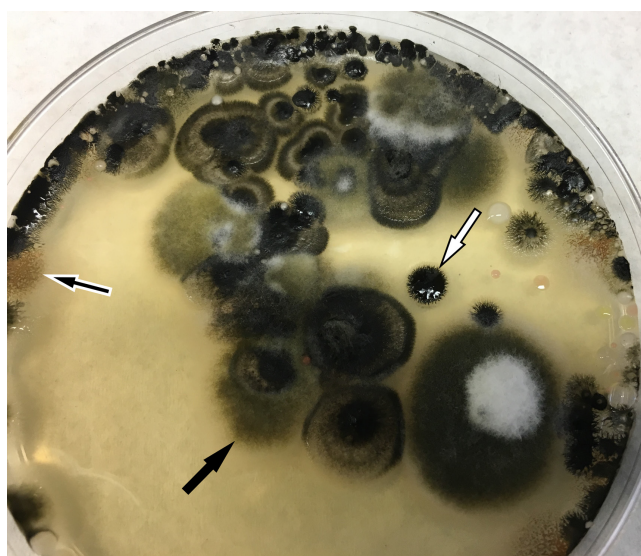
PCR amplification of the 28S LSU rDNA, the 18S SSU rDNA, and the ITS regions from each sample was achieved using three primer pairs. The LSU was amplified using the primer pair LR6/LROR (10; [https://sites.duke.edu/vilgalyslab/rdna\\_primers\\_for\\_fungi/](https://sites.duke.edu/vilgalyslab/rdna_primers_for_fungi/)) and the primer pair NS1/NS4 [11] was used to amplify the SSU region. The third set of primers ITS1F and LR3 [10, 11], was used to amplify the ITS1 and ITS2 regions. The polymerase chain reactions contained NEB Taq polymerase (1.25 units), 1X ThermoPol Buffer (New England Biolabs), 0.2 $\mu$ M of each primer, 0.2mM dNTPs, 1 $\mu$ L of genomic DNA, and water to bring the final volume to 30 $\mu$ L. Initial denaturation was set at 94°C for 5 min, followed by 40 cycles of denaturation (95°C for 45 s), annealing (52°C for 60 s), and extension (72°C for 90 s). The final extension was set for 5 min at 72°C. The amplified DNA was run on a 0.8% agarose gel (1X TAE) and extracted using a QIAquick Gel Extraction Kit (Qiagen Corp, Valencia, CA). The resulting DNA fragments were sequenced by Genewiz, Inc. (South Plainfield, New Jersey) using the LROR, LR3, LR3R, and LR6 primers for LSU rDNA; NS1, NS2, N3, and NS4 primers for the SSU rDNA and ITS1f and ITS4 primers for the ITS region [ [10, 11], [https://sites.duke.edu/vilgalyslab/rdna\\_primers\\_for\\_fungi/](https://sites.duke.edu/vilgalyslab/rdna_primers_for_fungi/)]. Sequencher® (version 5.0.1 DNA sequence software, Gene Codes Corporation, Ann Arbor, MI USA) was used to assemble the regions.

The assembled LSU, SSU, and ITS fungal sequences were compared using BLASTn on GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). This initial comparison was followed up with a pairwise alignment of the ITS sequences using MycoBank (previously CBS-KNAW) where the sequences were compared to multiple fungal sequence databases simultaneously ([https://www.mycobank.org/page/Pairwise\\_alignment](https://www.mycobank.org/page/Pairwise_alignment)). In addition, the ITS sequences from each fungus were compared, using BLASTn, to specific sequences published in taxonomic studies for each genus. The sequences obtained in this study were deposited in GenBank and assigned the following accession numbers ON055253 (*Aureobasidium melanogenum*), ON140589 (*Alternaria* spp.), and ON140590 (*Didymella glomerata*).

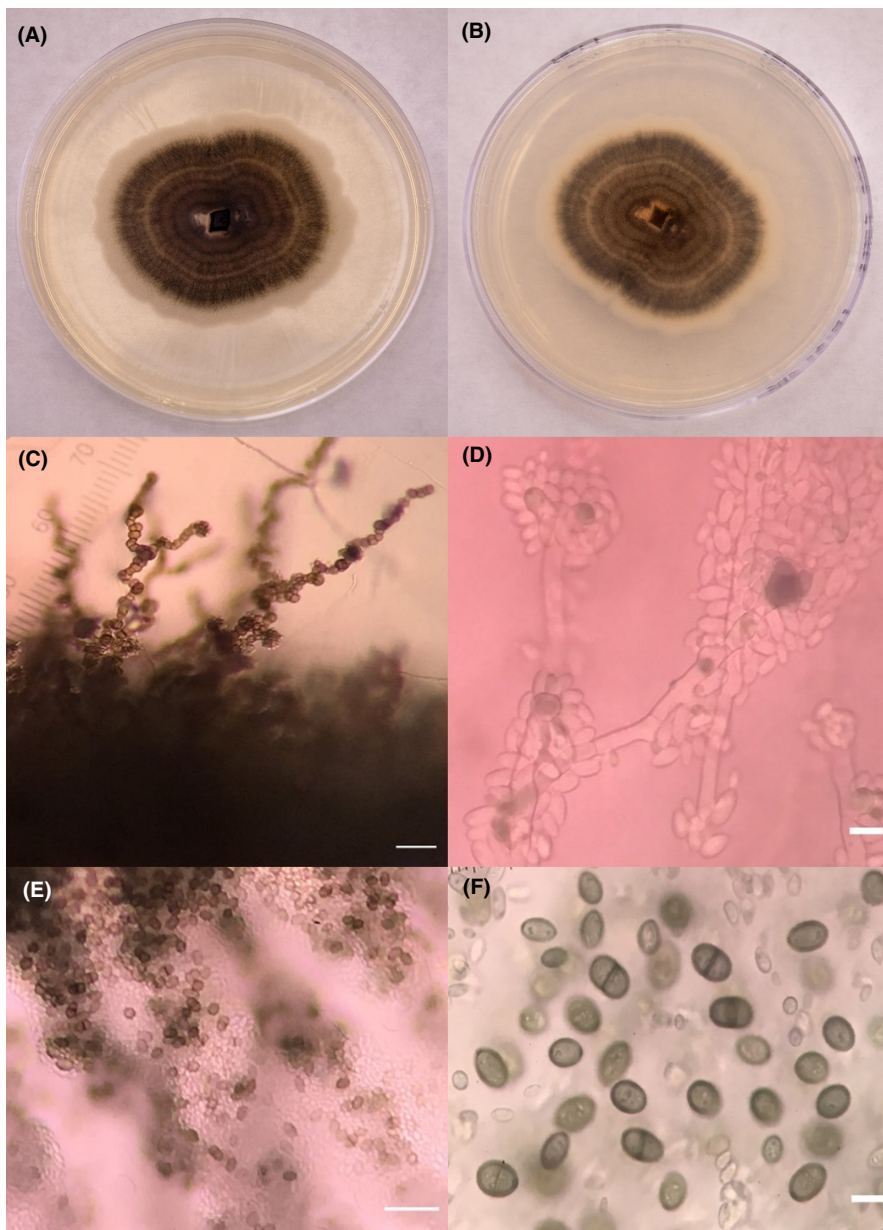
Phylogenetic analyses of the ITS sequences were performed using MEGA X [12]. DNA sequences produced in this study and published sequences of suspected and related species were analyzed using the maximum likelihood (500 bootstrap replications) and neighborhood joining methods.

## 3 | RESULTS AND DISCUSSION

The samples obtained from both of the cadavers used for this study, regardless of date or tissue type, consistently yielded three distinct



**FIGURE 2** Typical mixed colony growth from a single sample (4 days old on PDA). Of note are the numerous shiny, black colonies (white arrow outlined in black), the many olive green colonies (black arrow), and the diffuse brown colonies (black arrow outlined in white). These three fungi were consistently present in every sample taken from both cadavers used for this study. This particular culture was from an abdominal skin sample taken in the field then transported to the laboratory and plated by crushing the sample over a PDA plate.



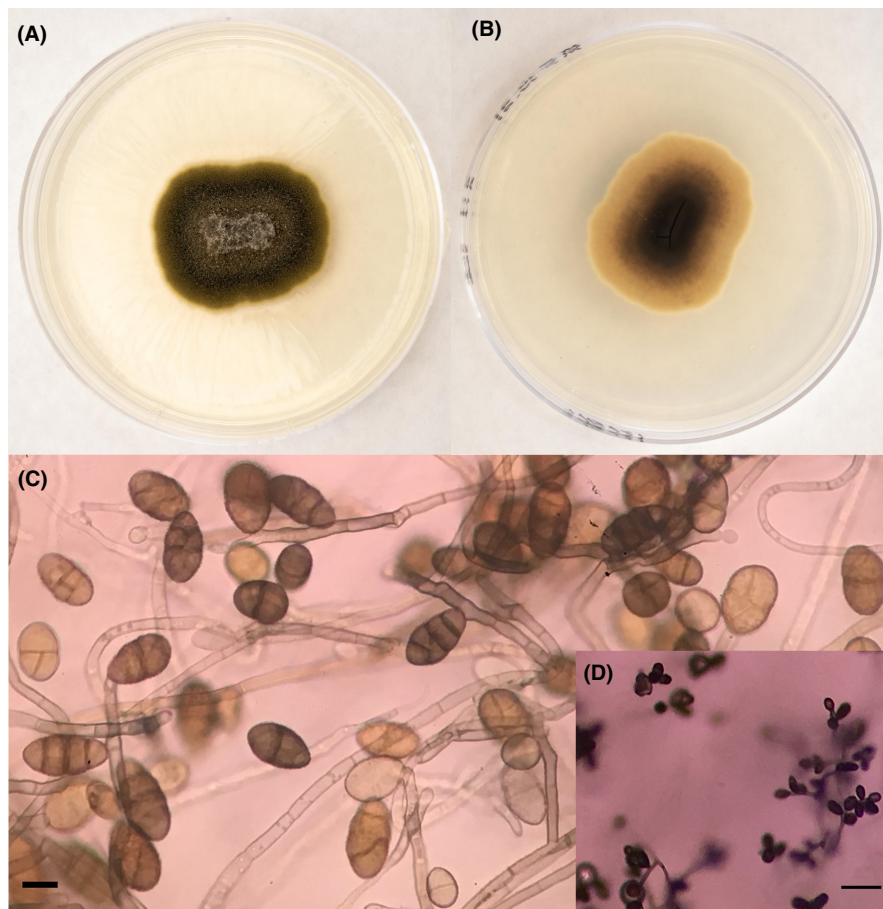
**FIGURE 3** (A, B) Pure culture of *Aureobasidium melanogenum* (11 days old on PDA), the black fungus typically seen in mixed cultures (A. front, B. back). (C) Aerial hyphae that emerge from the center of the colony. (D) Black leading edge of the colony. (E) Pigmented conidia from the black leading edge. (F) Hyphae and hyaline conidia at the white/clear leading edge of the colony. Scale bars: C, E = 50  $\mu\text{m}$ , D, F = 10  $\mu\text{m}$ . Photo credit for A, B Bronson Henriques.

fungi when cultured on potato dextrose agar growth media. The morphology of each fungus was easily distinguishable from the others, with one developing into shiny, black colonies, a second that produced olive green colonies, and a third that could be identified based on its diffuse brown growth (Figure 2). The morphological observations gave us a starting point for identification but required further verification using DNA sequences. Generally speaking, 28S LSU rDNA, the 18S SSU rDNA sequences evolve slowly and are useful for identification to higher taxonomic levels (e.g., phyla, class, order, family, and genus), whereas the ITS regions evolve more quickly making them suitable for identifying some fungi to species [13].

Based on the authors' initial morphological observations, the shiny, black colonies were tentatively identified as belonging to the genus *Aureobasidium* (Figure 3) [14, 15]. This assumption was supported by the LSU and SSU sequence results and further confirmed by the ITS sequences, which showed 100% sequence homology

(61% query coverage, E value 0.0) with at least one published strain of *Aureobasidium melanogenum* (FJ150887) [15] as well as with numerous sequences published on GenBank (accession numbers KY294710, KY294709, KY294708, KY659501, KY294711 all with 97% query coverage and E values of 0.0). The pairwise comparison on MycoBank yielded similar results. Additionally, neighborhood joining and maximum likelihood analysis of the ITS sequences also supported this species designation. Finally, comparisons of the morphology of the FIRS cultures to those published in recent taxonomic evaluations of the genus *Aureobasidium* solidified the identity as *A. melanogenum* (syn. *A. pullulans* var. *melanogenum*), a ubiquitous black yeast species [14–16]. The morphological features of note that separate this fungus from other *Aureobasidium* species include the dark, black coloration caused by the production of large amounts of melanin, the dark aerial hyphae that emerge from the older portions of the colonies (Figure 3A), and the shape of the dark conidia

**FIGURE 4** (A, B) Pure culture of *Alternaria* spp. (7 days old on PDA), the olive green fungus typically seen in mixed cultures (A. front, B. back). (C) Conidia and hyphae. (D) View from top of culture showing conidial attachment to conidiophores. Scale bars: C = 10  $\mu$ m, D = 50  $\mu$ m. Photo credit for A, B Bronson Henriques.

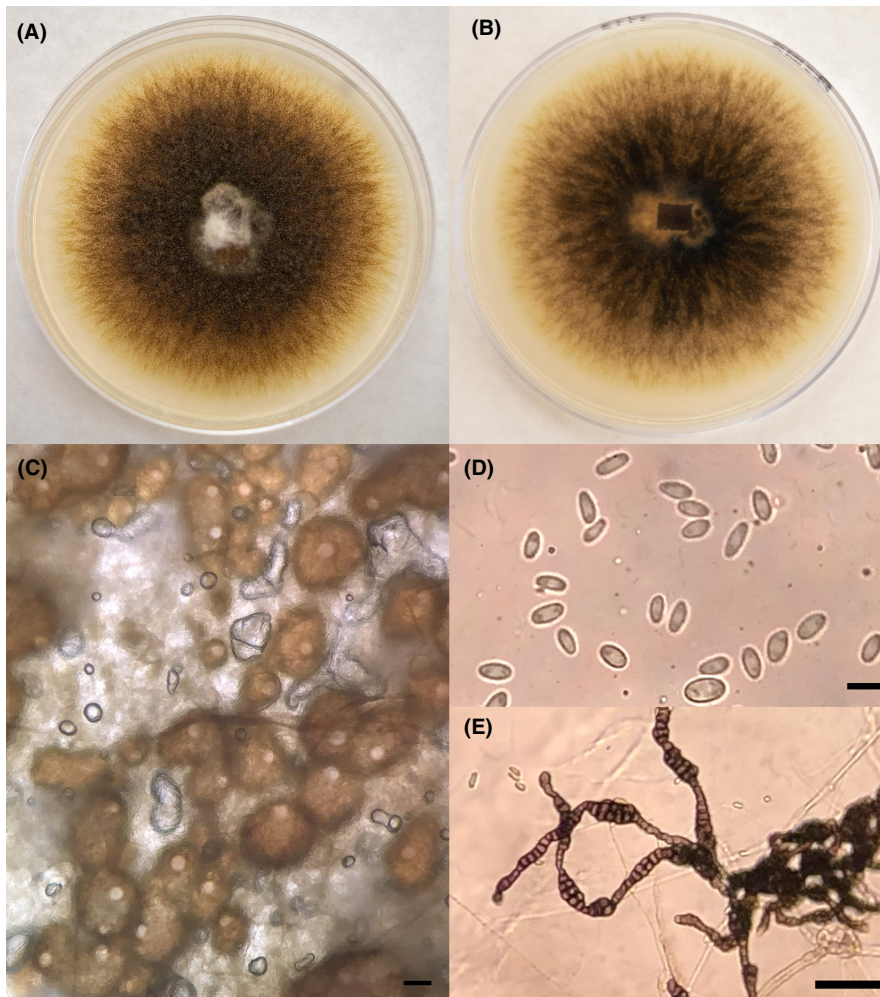


(Figure 3D and E). Although not previously reported as a fungus involved in human decomposition, *Aureobasidium melanogenum* is known to be an opportunistic human pathogen [16]. It is also interesting to note that the ITS sequence of our isolate is 98.87% homologous (67% query coverage, E value 0.0) to an *A. melanogenum* isolated from the soils of the Taklamakan desert in Northwest China [17], suggesting that perhaps, this fungus could inhabit the desert soils around FIRS.

The morphological features of the olive green colonies suggested that this fungus was a species of *Ulocladium* or *Alternaria* (Figure 4), which are considered to be allied genera [18]. The LSU and SSU sequence results suggested *Alternaria* sp. as did our initial BLASTn of the ITS sequence results. However, the “obovoid” shape of the conidia and the fact that the conidia were not produced in chains (Figure 4C and D) suggested that the fungus could be a species of *Ulocladium* [18, 19]. Further comparisons of the ITS sequences against GenBank sequences published in phylogenetic studies of *Ulocladium* and *Alternaria* by Runa et al. [19] and Woudenberg et al. [18], respectively, yielded no less than 97% homology with multiple species of *Ulocladium*, while a pairwise alignment of our ITS sequence using the MycoBank fungal sequence database suggested 100% sequence homology with *Alternaria terricola* (syn. *Ulocladium tuberculatum*) and *Alternaria atra* (syn. *Ulocladium atrum*) (query coverage/E value of 71.81%/0.0 and 74.19%/0.0, respectively). Both of

these species have been part of an extensive phylogenetic analysis of the genus *Alternaria* and have been placed in section *Ulocladioides* within the *Alternaria* clade by Woudenberg et al. [18]. Neighborhood joining and maximum likelihood analysis of the ITS sequences from this study and Woudenberg et al. [18] supported the pairwise alignments where the species described here was placed in the same clade with the *Alternaria terricola* and *A. atra*. Sequencing of other loci such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RNA polymerase's second largest subunit (RPB2), and translation elongation factor 1-alpha (TEF-1) could be done in the future to improve the resolution of species identification. Although we did not resolve the identity of this particular fungus to species, it is interesting to note that *Alternaria* species have been identified as cutaneous opportunistic pathogens of humans [20, 21] as well as a dominant genus in the biological soil crusts of the Colorado Plateau [22, 23], an area that includes Whitewater, Colorado.

Initial morphological observations of the third fungus that produced the brown colonies led us to suspect that it was a species of *Phoma* (Figure 5); the LSU/SSU sequence results supported this initial hypothesis. Further sequencing work with the ITS primers initially suggested that it was a species in the Family *Didymellaceae*, of which *Phoma* is a member [24]. A deeper dive into the literature on the *Didymellaceae* revealed that some members of the family produce chains of alternaroid chlamydospores, a characteristic noted



**FIGURE 5** (A, B) Pure culture of *Didymella glomerata* (7 days old on PDA), the brown fungus typically seen in mixed cultures (A. front, B. back). (C) Flask-shaped pycnidia with ostioles visible. (D) Hyaline, aseptate conidia. (E) Chains of multicellular, alternaroid chlamydsopores from a 3-year-old culture that had been stored at 10°C. Scale bars: C = 100µm, D = 10 µm, E = 50µm. Photo credit for A, B Bronson Henriques.

in some of the older cultures in our collection (Figure 5E) [25]. Furthermore, the morphology of the chlamydsopores in the cultures matched the descriptions for *Didymella glomerata* (syn. *Phoma glomerata*, syn. *Peyronellaea glomerata*) [25–27]. Finally, a more directed comparison of the sequence to those published in taxonomic studies that included *Didymella glomerata* showed the FIRS sequence to be 99.79% homologous with at least two published sequences (FJ427013 and FJ427004, 60% query coverage and E value of 0.0 for both) [24, 28] and 100% homologous with at least one published sequence (MH864401; 69% query cover, E value 0.0) [29] and numerous *D. glomerata* sequences submitted to GenBank and MycoBank. Finally, the phylogenetic trees produced by both neighborhood joining and maximum likelihood analyses suggest that the FIRS ITS sequence belongs to the same clade as *Didymella glomerata*. Given that this fungus is ubiquitous and can be found in the soil, on plants (as a pathogen), and on animals (as an opportunistic pathogen), its presence on the human remains in our study is not surprising [24–26].

#### 4 | CONCLUSION

Using a combination of morphological observations, ribosomal sequence data, and phylogenetic analyses, we were able to identify

three fungi that appear to be involved in the late-stage decomposition of desiccated human remains. It is important to note that no one single method is sufficient when working to identify fungi and that in some cases (e.g., identification of *Alternaria*) the identification to species can be complicated by the fact that fungal taxonomy is in a constant state of flux. The three fungi identified here are all common environmental fungi, but to the authors' knowledge, have not previously been reported on human remains in the late stages of decomposition. The fact that three dominant fungi were consistently isolated, as described above, suggests a low fungal diversity during the later stages of decomposition where remains desiccate. This observation is in keeping with the results of other authors. For example, Forger et al. [5] found that fungal diversity decreased in the latter stages (dry/skeletal) of porcine decomposition. It is interesting to note that all three of the fungi described here can be found in soil/biological soil crusts [17, 22–26]. In fact, the soil has been suggested as a potential source for microbes involved in the decomposition of mammals in outdoor environments [6]. An analysis of the soil fungal community present at the FIRS would be an interesting follow-up study. In addition, further work is needed to understand more about when and how these fungi could be involved in the breakdown of human remains and whether they are useful markers for determining long-term PMI.

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