

Molecular identification of fungi found on decomposed human bodies in forensic autopsy cases

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Abstract To investigate which fungi can be found during forensic autopsies, a PubMed literature review was done in regard to fungal growth on decomposed human bodies. Unfortunately, the existing data is limited and not all fungi were identified to the species level. We, therefore, collected skin samples with macroscopically visible fungal growth from 23 autopsy cases in Germany and identified the fungi to the species level by molecular methods. The identified species

included *Aspergillus fumigatus* and *Candida albicans*, which pose an allergenic risk, especially to persons with underlying lung diseases. Because safety standards are lacking, we recommend the use of respiratory protection during exhumations and forensic autopsies, when fungal growth is noted. With regard to the future, a database was set up which could possibly be used as a forensic tool to determine the time of death.

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Introduction

Invasive fungal infections are often opportunistic and occur mostly in immunocompromised patients [1]. But fungi can also lead to severe infections, even in the immunocompetent host, either because the fungus is a true pathogen (e.g., belongs to risk group 3) [2] or because there is particular exposure [3–5]. The fungi actually listed as risk group 3 organisms, *Blastomyces dermatitidis*, *Cladophialophora bantiana*, *Coccidioides immitis*, *Coccidioides posadasii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Penicillium marneffei*, and *Rhinocladiella mackenziei*, are uncommon in Germany. Nevertheless, inhalation of spores of non-risk group 3 organisms may lead to pulmonary infections and is a well-known cause of allergies and asthma [6], especially for persons subjected to repeated exposure or with underlying conditions, such as preexisting lung disease or diabetes mellitus.

In forensic autopsies, fungi are mainly found on decomposing human cadavers, such as mummies, bodies found in the environment, or bodies from exhumations [7–14]. Experience has also shown that strongly decomposed cadavers, in particular those that are highly mummified, often

evinced visible fungal growth only after they have been placed in cold storage.

During regular clinical autopsies, fungi should only be an issue if the deceased suffered from a fungal infection during life—an event that is not uncommon, as large studies on specific patient groups [15, 16] and on the general population [17–19] have shown. However, due to the lack of applicable safety standards, no special safety precautions are taken during forensic autopsies when fungal growth is noted.

But fungi are not only of interest because they are possible pathogens. Already in 1955, Dósa proposed to use fungi as a forensic tool to determine the time of death. On a limited number of fungi, he showed that these species only occur at certain post-mortem intervals [8].

The current study reviews the literature for fungi that can be found on mummies and on cadavers during autopsies. Furthermore, with regard to the possible use of fungi as a forensic tool, it is necessary to build up a database. We, therefore, identified fungi to the species level by molecular methods. We started with skin samples from the decomposed human bodies of 23 autopsy cases, collected in the Departments of Forensic Medicine at the Universities of Giessen and Hamburg, in Germany.

Materials and methods

Literature research

Literature research was done with PubMed, using the key words fungi, mummy, corpse, autopsy, and forensic autopsy. For the definition of risk groups, the Directive 2000/54/EC of the European Parliament was used (Table 1) [20].

Table 1 Risk group definition according to the directive 2000/54/EC of the European Parliament [20]

Risk group ^a	Definition
1	a biological agent that is most unlikely to cause human disease
2	a biological agent that may cause human disease and may be a hazard to laboratory workers, but is unlikely to spread in the community laboratory exposure rarely produces infection and effective prophylaxis or treatment is available
3	a biological agent that may cause severe human disease and presents a serious hazard to laboratory workers it may present a risk of spread in the community, but there is usually effective prophylaxis or treatment
4	a biological agent that causes severe human disease and is a serious hazard to laboratory workers it may present a high risk of spread in the community, and there is usually no effective prophylaxis or treatment

^a No fungi are currently classified as risk group 4

Classification of fungi in risk groups was done according to de Hoog et al. [21] and to the CBS (<http://www.cbs.knaw.nl>).

Cases included in the study

Overall, 23 cases were included in this study (Table 3). Cases were only included, if the skin exhibited macroscopically visible fungal growth.

Prior approval for this study was obtained from the Ethics Committee of the Medical Department of the Justus-Liebig-University in Giessen, Germany (AZ: 73/13).

Isolation and purification of strains

Skin samples exhibiting visible fungal growth were taken and stored at −20 °C until the day of use. For some cases, several skin samples were taken. Samples were cultured on Sabouraud-chloramphenicol agar plates at 24 °C, for up to 7 days to obtain fungal growth. Small pieces of agar from the edge of the colonies were cultured on Sabouraud-chloramphenicol agar plates until pure cultures were obtained. All pure strains were stored as spore suspensions for filamentous fungi, or as heavy cell suspensions for yeasts, at −20 °C in 40 % glycerol, until used.

Culture

Strains were sub-cultured from frozen stocks on Sabouraud-chloramphenicol agar plates for up to 7 days at 24 °C. From the cultures, spore suspensions for filamentous fungi, or heavy cell suspensions for yeasts, were prepared in 0.9 % NaCl. Twenty milliliters of RPMI 1640 (Sigma-Aldrich, Seelze, Germany) with L-glutamine, but without sodium bicarbonate, buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (Sigma), was inoculated with these suspensions, and the fungi were grown for 48 to 72 h, at 24 °C, with agitation. For isolates exhibiting insufficient growth, 20 ml Sabouraud liquid broth was inoculated with the suspensions in 0.9 % NaCl, and also incubated at 24 °C, with agitation, until sufficient growth was obtained.

DNA extraction

Complete genomic DNA was extracted from mycelium grown in RPMI 1640, or in liquid Sabouraud broth, according to the hexacetyltrimethylammonium bromide (CTAB; Sigma) protocol described previously, with modifications [22]. Mycelium or yeast were homogenized in a 50-ml tube containing 1 ml CTAB extraction buffer (100 mM Tris-HCl [pH 8.4], 1.4 M NaCl, 25 mM EDTA, 2 % CTAB), three glass beads (0.5 cm) (Sigma), and 500 mg of 425- to 600-µm diameter glass beads (Sigma). The suspension was vigorously vortexed till homogenization was obtained.

Table 2 Results of literature research: summary of identified fungi potentially pathogenic for humans

Species	Risk group	References
<i>Acremonium potronii</i>	1	[8]
<i>Acremonium strictum</i>	1	[8]
<i>Alternaria</i> spp.	1	[7, 10]
<i>Ambrosiozyma platypodis</i>	1	[8]
<i>Aspergillus candidus</i>	1	[8]
<i>Aspergillus clavatus</i>	1	[8]
<i>Aspergillus flavus</i>	2	[8]
<i>Aspergillus fumigatus</i> ^a	2	[7, 8]
<i>Aspergillus nidulans</i>	1	[7]
<i>Aspergillus niger</i>	1	[8]
<i>Aspergillus repens</i>	1	[9]
<i>Aspergillus ruber</i>	1	[9]
<i>Aspergillus terreus</i>	1	[12]
<i>Aspergillus</i> sp.	≤2	[10]
<i>Beauveria</i> sp.	1	[10]
<i>Bipolaris</i> sp.	1	[10]
<i>Candida</i> sp. ^a	≤2	[10]
<i>Chaetomium globosum</i>	1	[8, 14]
<i>Chrysosporium</i> spp.	1	[7, 10]
<i>Cladophialophora</i> sp.	≤3	[10]
<i>Cladosporium</i> sp.	≤3 ^a	[11]
<i>Curvularia</i> spp.	1	[10]
<i>Dendryphion comosum</i>	1	[8]
<i>Fonsecaea</i> sp.	≤2	[10]
<i>Fumago</i> sp.	1	[8]
<i>Fusarium</i> sp.	1	[10, 11, 13]
<i>Geotrichum candidum</i>	1	[11]
<i>Geotrichum</i> sp.	1	[10, 13]
<i>Glenospora graphii</i>	2	[8]
<i>Glocladium</i> sp.	1	[9]
<i>Helminthosporium</i> sp.	1	[10]
<i>Hormodendrum</i> sp.	1	[11]
<i>Hypomyces aurantius</i>	1	[8]
<i>Hypomyces perniciosus</i>	1	[8]
<i>Lichtheimia corymbifera</i>	1	[8, 14]
<i>Monilia</i> sp.	≤2	[10]
<i>Mortierella</i> sp.	1	[11]
<i>Mucor</i> sp.	1	[10, 13]
<i>Mucor racemosus</i>	1	[8]
<i>Paecilomyces</i> sp.	1	[10]
<i>Penicillium chrysogenum</i>	1	[11]
<i>Penicillium</i> spp.	≤3	[7, 8, 10, 12]
<i>Phialophora</i> sp.	≤2	[10]
<i>Pythium</i> sp.	≤2	[13]
<i>Rhizopus</i> spp.	1	[7, 10]
<i>Rhodotorula mucilaginosa</i>	1	[8]
<i>Rhodotorula</i> sp.	1	[10]
<i>Saccharomyces</i> sp.	1	[8]

Table 2 (continued)

Species	Risk group	References
<i>Scopulariopsis brevicaulis</i>	1	[8]
<i>Scopulariopsis</i> sp.	1	[10]
<i>Trichoderma</i> sp.	1	[10]
<i>Trichosporon</i> sp.	≤2	[10]
<i>Trichothecium</i> sp.	1	[10]
<i>Trichothecium roseum</i>	1	[8]

^a Fungi with possible allergenic potential

^b Some species described as *Cladosporium* have been reassigned to other species including group 3 pathogens such as *Cladophialophora bantiana*

Seven hundred microliters of the suspension was transferred into a 2-ml microcentrifuge tube. An equal volume of chloroform was added to the mixture, vortexed for 5 s, and spun for 10 min at 14,000×g. Five hundred microliters of the upper phase was transferred to a new 2-ml microcentrifuge tube, and DNA was precipitated by addition of an equal volume of isopropanol. DNA was pelleted at 14,000×g for 1 min. After the supernatant had been discarded, the pellet was washed with 100 % ethanol and re-suspended in 200 µl of TE buffer (10 mM Tris-HCl [pH 8.0]; 1 mM EDTA). Genomic DNA was stored at −20 °C.

PCR, primers, and sequencing

Ribosomal DNA, including the ITS1-5.8S-ITS2 region, was amplified with the fungal universal primers ITS1 (5'-TCCG TAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTT ATTGATATGC-3') [23]. For *Aspergillus* and *Penicillium* spp., part of the β-Tubulin gene was additionally amplified using the primers Bt1a (5'-TTCCCCCGTCTCCACTTCTT CATG-3') and Bt1b (5'-GACGAGATCGTTCATGTTGAAC TC-3') [24]. Amplification mixtures (50 µl) contained 2.5 µl of the extracted genomic DNA, 1.25 µl of 20 µM concentrations of each primer, 5 µl of 2.5 mM [each] dATP, dTTP, dGTP, dCTP (Roche Diagnostics GmbH, Mannheim, Germany), 5 µl of 25 mM MgCl₂, 3.125 U of AmpliTaq polymerase (Roche), and 5 µl of 10× PCR buffer (Roche). Amplification of the PCR products was done in a Applied Biosystems GeneAmp PCR System 9700 (Darmstadt, Germany) with the following cycling parameters: initial denaturizing step of 10 min at 94 °C, 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and elongation for 30 s at 72 °C, with a final extension for 10 min at 72 °C. PCR products were purified with the QIAquick® Purification Kit (QIAGEN, Hilden, Germany), and both strands were sequenced once with the BigDye terminator cycle sequencing ready reaction kit, version 1.1 (Applied Biosystems), with the primer set IT1 and IT4 or the primer set Bt1a and Bt1b. Sequences were

blasted in GenBank using nucleotide BLAST and compared to deposit sequences.

Light microscopy

As a control, the morphology of the first five fungal isolates that were isolated was examined by light microscopy using an Olympus BX51 microscope (Olympus Europa Holding, Hamburg, Germany) and compared to the sequencing results. One drop of spore suspension in saline was placed in the center of a Sabouraud chloramphenicol petri dish and incubated at 24 °C. Fungi were grown for a minimum of 4 days to achieve sufficient sporulation. A small piece from the edge of the colony was placed on a microslide, and one drop of methylene blue solution (Carl Roth, Karlsruhe, Germany) was added. Microscopy was performed at different magnifications ($\times 100$, $\times 200$, $\times 400$, $\times 1000$). Photographs were acquired with a DP72 (Olympus) camera and the program Olympus cell^D (Olympus).

Results

The results of the literature review are summarized in Table 2. For fungi not identified to the species level, the highest possible risk group for members of the given genera was assumed. The fungi mostly belonged to risk groups 1 and 2, although some fungi may possibly even belonged to risk group 3.

An overview of the included cases is given in Table 3. Twenty-six percent of the corpses were female. The mean age of the 21 cases with known age was 65 years, with a range of 25 to 98 years. Most of the corpses were found in their apartments; two corpses were from exhumations; and one each was found in a forest, a river, or a school toilet. In one case, the corpse had been stored in the cold room of a hospital for 2 months before the autopsy was done. The cause of death was not determined in all cases. In Hamburg, autopsies for corpses found in apartments were only performed if a suspicion of homicide was noted during the inspection of the corpse. Putrefaction was found in all 23 cases, while eight

Table 3 Overview of cases included in the study

Case ^a	Age	Sex	Post-mortem interval	Ambient condition	Cause of death	Height [cm]	Weight [kg]	State of decomposition
1	58	Male	Unknown	Apartment	Sudden cardiac death	178	59	Putrefaction
2	50	Male	1–2 weeks	Apartment	Hypothermia	184	60	Putrefaction, mummification
3	69	Female	5 weeks	Individual grave	Intangible	173	66	Putrefaction
4	98	Female	10 weeks	Individual grave	Pneumonia	147	45	Putrefaction, adipocere, mummification
5	54	Female	2 weeks	Hospital cold room	Rhinocerebral mucormycosis	160	56	Putrefaction
6	69	Male	Unknown	Forest	Intangible	178	Unknown	Putrefaction, mummification
7	25	Male	4–6 days	School toilet	opiate intoxication	191	66	Putrefaction
8	52	Male	Unknown	Apartment	Shot in the head	185	Unknown	Putrefaction
9	64	Female	Unknown	Apartment	Myocard ishaemia	Unknown	Unknown	Putrefaction, mummification
10	37	Male	Unknown	River	Drowning	184	98	Putrefaction
11	66	Male	Unknown	Apartment	No autopsy	174	77	Putrefaction
12	65	Male	Unknown	Apartment	No autopsy	176	34	Putrefaction, mummification
13	73	Female	Unknown	Apartment	No autopsy	154	59	Putrefaction, mummification
14	75	Male	Unknown	Apartment	No autopsy	183	78	Putrefaction, mummification
15	78	Male	Unknown	Apartment	No autopsy	163	61	Putrefaction
16	Unknown	Male	Unknown	Unknown	No autopsy	180	89	Putrefaction
17	64	Male	Unknown	Unknown	No autopsy	178	88	Putrefaction
18	68	Male	Unknown	Apartment	No autopsy	181	705	Putrefaction, mummification, skeletonization
19	52	Male	Unknown	Apartment	No autopsy	173	80	Putrefaction
20	77	Male	Unknown	Apartment	No autopsy	179	48	Putrefaction
21	49	Male	Unknown	Unknown	No autopsy	187	91	Putrefaction
22	84	Male	Unknown	Apartment	Hypothermia	157	38	Putrefaction
23	85	Female	2 month	Hospital cold room	Intangible	163	63	Putrefaction

^a Samples 1–9 were collected at the Institute of Forensic Medicine in Giessen, and samples 10–23 were collected at the Institute of Forensic Medicine in Hamburg

cadavers showed mummification; skeletonization and adipocere were only found in one case each.

The sequencing results are given in Table 4. None of the collected samples was negative for fungal growth after 7 days of incubation. Overall, 24 different species could be purified and identified. Four species belonged to risk group 2: They were identified as *Aspergillus flavus*, *A. fumigatus*, *C. albicans*, and *Candida tropicalis*. The other species belonged to risk group 1 and included known human pathogens, such as *Candida glabrata*, *Lichtheimia corymbifera*, *Mucor circinelloides*, *Rhizopus microsporus*, and *Scopulariopsis brevicaulis*.

Figure 1 shows the morphology of four of the five fungal isolates used as controls. The isolates were identified as *A. fumigatus* (case 6) (A and B), *R. microsporus* (case 5) (C and D), *M. circinelloides* (case 3) (E and F), and *L. corymbifera* (case 1) (G and H). Isolate 5, *M. circinelloides* (case 4), is not shown. Picture A shows the septated hyphae and characteristic conidial heads (Picture B) of *A. fumigatus*.

Table 4 Identified fungi in the present study

Fungus	Risk group	Case ^a
<i>Aspergillus flavus</i>	2	7
<i>Aspergillus fumigatus</i> ^b	2	3, 6, 20
<i>Aspergillus pseudoglaucus</i>	1	6, 13
<i>Candida albicans</i> ^b	2	1
<i>Candida glabrata</i>	1	2
<i>Candida tropicalis</i>	2	17
<i>Debaryomyces hansenii</i>	1	6
<i>Helicostylum elegans</i>	1	18
<i>Lichtheimia corymbifera</i>	1	1
<i>Mucor circinelloides</i>	1	3, 4
<i>Mucor flavus</i>	1	18, 23
<i>Mucor plumbeus</i>	1	9, 10, 11, 21
<i>Mucor saturninus</i>	1	15, 22
<i>Mucor racemosus</i>	1	8
<i>Penicillium brevicompactum</i>	1	17, 23
<i>Penicillium chrysogenum</i>	1	11, 20
<i>Penicillium crustosum</i>	1	7, 14
<i>Penicillium expansum</i>	1	14, 23
<i>Penicillium polonicum</i>	1	8
<i>Pseudogymnoascus pannorum</i>	1	16
<i>Rhizopus microspores</i>	1	5
<i>Rhizopus stolonifer</i>	1	9
<i>Scopulariopsis brevicaulis</i>	1	12, 19
<i>Yarrowia lipolytica</i>	1	14

^a Samples 1–9 were collected at the Institute of Forensic Medicine in Giessen, and samples 10–23 were collected at the Institute of Forensic Medicine in Hamburg

^b Fungi with possible allergenic potential

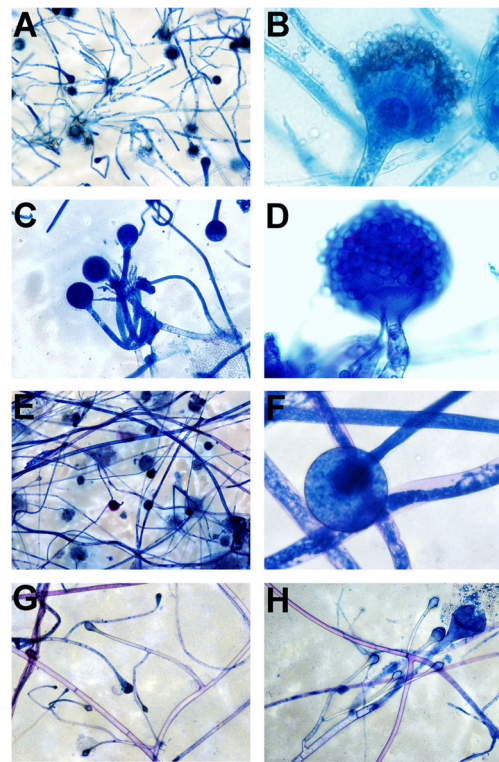


Fig. 1 Morphological characteristics of *A. fumigatus* (case 6) (a, b), *R. microsporus* (case 5), *M. circinelloides* (case 3) (e, f), and *L. corymbifera* (case 1) (g, h). Magnifications: $\times 100$ (e), $\times 200$ (a, c, g), $\times 400$ (f), $\times 1000$ (b, d, h). Methylene blue

Picture C shows the non-septate hyphae with rhizoids and a typical sporangium of *R. microsporus* in Picture D. Picture E shows the non-septate hyphae and a sporangium of *M. circinelloides* in Picture F. Picture G shows the branched sporangiophores and a sporangium marked with apophysis, typical for *L. corymbifera*, in Picture G.

Discussion

The aim of the study was to identify macroscopically visible fungal species from cadavers at different stages of decomposition. As soon as populations of filamentous fungi are visible, a high level of risk should be assumed for the examiner because these fungi layers can produce spores, and the threat of an airborne infection can, thus, not be excluded.

In the first part of the study, we reviewed the literature. In the second part, we analyzed visible fungal layers (Fig. 2) by microscopy and molecular biology, to determine the genus and, in particular, the species level to assess the associated hazard to staff and visitors during autopsies in such cases. It was not our goal to identify all potentially dangerous fungi that might grow on a cadaver or those present inside the cadaver, such as in the respiratory tract or organs [25]. Such

Fig. 2 Macroscopically visible fungal layers on an exhumed body



investigations could be approached in various ways in future studies.

Compared to the literature [7–14], we confirmed that fungi potentially pathogenic to humans (Tables 2 and 4) can be found on decomposed human bodies. Unfortunately, not all fungi mentioned in the literature were identified to the species level. This must be considered problematic, as even within a genus, the species have different risk potentials. For example, in the genus *Aspergillus*, *A. fumigatus* is a risk group 2 pathogen with high allergenic potential, whereas some other *Aspergillus* spp. are counted as harmless. But even if only risk group 1 were noted for some fungi, these species can still pose a high allergenic risk (e.g., *Alternaria* spp).

In the literature, findings of *Cladophialophora* sp. are reported [10]. This genus includes species belonging to risk group 3, which can cause severe human disease and present a serious hazard to workers. It remains unclear if these species (*C. bantiana*, or other group 3 fungi) can really be found on human corpses. This lack of clarity calls for further analysis.

Our investigations to identify fungi growing on decomposed bodies to the species level by molecular biology led to deeper insights about the different species of fungi growing on human corpses at diverse states of putrefaction. We were able to illustrate the necessity of knowing which fungi can be found on decomposed human bodies and of implementing safety precautions for staff and visitors at forensic autopsies. For a total of 23 cases, skin samples exhibiting visible fungal growth were analyzed. Especially three cases were of particular interest, in which species were found that can pose an allergenic risk (e.g., *A. fumigatus* and *C. albicans*) [6].

Although risk group 3 fungi are uncommon in Germany, they may play a role in forensic autopsies in other countries. To detect possible risk group 3 fungi on human corpses, the study design has to be changed slightly. Growth of some of these fungi, e.g., *H. capsulatum* and *C. immitis*, lasts several weeks, and the cultivation procedure for these fungi is recommended to be performed between 4 to 6 weeks [26–28].

Additionally, selective agar media can be used to suppress growth of commensal fungi, e.g., by using cycloheximide or by adding ammonium hydroxide to the surface of the agar to increase the pH, as the coexistence of slowly growing risk group 3 fungi cannot be otherwise excluded, and they may be overgrown by the commensal flora, especially of saprophytic fungi. Furthermore, additional incubation at 37 °C should be performed, which can afford mold-to-yeast conversion of risk group 3 fungi.

Further studies on fungal growth on decomposed human bodies are warranted.

Current safety precautions mainly aim at protecting against bacterial and viral infections. The required protection equipment for forensic autopsies differs between countries and institutions but should always include double-layer gloves with an interposed layer of cut-proof synthetic mesh, an impermeable gown, and a face mask to act as physical barriers to airborne infection [29]. Because applicable safety standards are missing, no specific safety precautions are taken during autopsies of bodies exhibiting extensive fungal growth. The frequently held assumption that the older a dead body is, the less danger there is for the examiner, does not hold for filamentous fungi because of their ability to produce spores. Based on the results of this study in particular, we recommend the use of respiratory protection during exhumations and forensic autopsies when fungal growth is noted. In addition to double-layer gloves, personal protection equipment should at least include N-95 respirators and eye protection. As an alternative, full face masks with P-100 filters can be used when very high concentrations of spores are expected [30]. Especially immunocompromised persons and persons with underlying diseases such as preexisting lung diseases or diabetes mellitus should not be present at forensic autopsies when fungal spores are expected.

A database was set up with regard to future studies and the possible use of fungi as a forensic tool. In this database, colonizing fungal species are cross-linked to the ambient conditions and the post-mortem interval. Unfortunately, in

our study, cases with known post-mortem interval were rare and the post-mortem intervals were short. It would be desirable to have post-mortem intervals that span several years, for example 1, 2, 4, 6, 8, and 10 years to validate the assumption, as proposed by Dósa, that the colonizing species change with time and correlate to the post-mortem interval [8]. A larger number of cases with known post-mortem intervals are still needed for this purpose.

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