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Stability and Qualification of a Legacy Fungal Collection

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Background: Microbial culture collections are valuable repositories for qualified and diverse microorganisms, playing a pivotal role in research, education, innovation, as well as in our response to current and emerging public health and societal challenges. However, such precious holdings, when not integrated in professional biobank infrastructures, may be vulnerable to major risks such as staff retirement, changes in the institutional strategy, or natural disasters. The process of preserving and rescuing "historical" collections can be long and treacherous with a loss of a part of the collection. At the Biological Resource Center of Institut Pasteur, we undertook the challenge of rescuing the dormant legacy fungal collection.
***Materials and Methods*:** A total of 64 freeze-dried strains, including yeasts and filamentous fungi, were characterized by using a polyphasic approach combining morphological features and molecular data. We assessed the viability, purity, and authenticity of selected strains isolated from multiple sources and stored for more than 20 years.
Results: Our preliminary results show long-term stability of the selected strains and successful qualification in terms of purity and authentication. Moreover, based on the most recent taxonomic revisions, we updated and revised the nomenclature, where applicable.
Conclusion: Our findings demonstrated the potential value of reviving historical microbial collections for biobanking and research activities and reassure us about the collection's future reopening.

Keywords: culture collection, reviving, purity evaluation, authentication, fungi

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Introduction

The kingdom *Fungi* is a diverse group of eukaryotic organisms currently distributed into nine phyla based on morphological, physiological, and molecular features.[1] Nevertheless, they share characteristics, such as transitions between different morphotypes, and the presence of a cell wall composed mostly of chitin and β-glucans.[1]

Fungi are cosmopolitan organisms virtually found in all habitats.[1] They present different lifestyles and must interact with other dead or living organisms or their derivative compounds in order to develop and survive.[1] In humans, they provoke superficial noncritical infections to invasive life-threatening ones depending on their transmission mode, infectivity, and human patient's immune status.[2] Last year, the World Health Organization proposed a list of 19 fungal pathogens provoking invasive acute and subacute systemic infections that are difficult to treat and manage, on which it is essential to focus our development, research and education activities, and management strategies.[2] At the top of the list are found *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Candida auris*, and *Candida albicans*, which present a high mortality rate and can be resistant to antifungal drugs and are present worldwide.[2] Fungi are also producing natural compounds that have been used for a long time in industrial settings and will continue to be used in the next years to evolve toward a bio-based-oriented society.[3–5]

Microbial culture collections in biobanks are strategic tools for the conservation and valorization of biological resources. They preserve and provide access to fit-for-purpose sample collections, associated data, and knowledge-based and experimental services.[6] Culture collections and biobanks present fungal holdings accounting for approximately 850,000

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strains.[7] In total, 793 of these structures are in 77 countries and regions, of which more than half are in Europe and North America.[7] In France, 13 culture collections with fungal holdings are listed on the WDCM website https://ccinfo.wdcm.org. These collections have governmental and/or academic status and exhibit different specificities. However, some of them could be inactive or shut down, as is the case with the "Collection de Champignons et Actinomycetes Pathogènes" initially hosted at the Unité de Mycologie at Institut Pasteur (UMIP) in Paris.

The process of preserving and rescuing old collections is a daunting task. For instance, the microbial collection mounted by Dr. Kral in Prague at the end of the 19th century had several difficult periods. At Kral's death, the collection was neglected for a few years before it was adopted by Pr. Ernst Pribman in Vienna, who started a qualification process and found that many of the cultures were not viable. Finally, despite all efforts, the collection was permanently closed. Currently, only a few samples from this collection are included in the ATCC catalog.[8]

In France, the UMIP collection was housed in the Mycology Unit at Institut Pasteur until 2001. It included over 1500 different yeast and mold strains with ecological and/or clinical relevance and primarily belonging to the phyla *Ascomycota*, *Basiodiomycota*, and *Zoopagomycota*. All fungi were freeze-dried, cryopreserved at −80°C or in liquid nitrogen, and/or maintained on mineral oil. In 2013, the collection was declared closed. Over the years, UMIP fungal strains have been used in numerous studies. For instance, they were used to revise the taxonomical affiliation of *Lichtheimia corymbifera* complex isolates associated with human mycosis.[9] Another example of previous valorization for industrial purposes is reported by Lotfyet al.[10] The most recent study based on UMIP samples is that of Nyuykonge et al. who used UMIP81.77, UMIP582.60, and UMIP1137.76 to identify fungi associated with black-grain mycetoma in two Mexican patients and to develop diagnostic tools for strain identification at the species level.[11]

The revivification of dormant collections can increase the diversity of available microbial resources and provide a window into the past characteristics of extant species of medical or other interest. The purpose of this study was to assess the viability/culturability, purity, and authenticity of representative UMIP fungal strains to determine whether the UMIP collection can be reopened.

Materials and Methods

Fungal strains

We selected 33 yeasts and 31 filamentous fungi to assess their viability, purity, and identity. Strains had diverse isolation years, sources, and geographical locations and were representative of different genera. As listed in Table 1, yeast are represented by basidiomycetous (*Cryptococcus* and *Malassezia*) and ascomycetous (*Candida*, *Geotrichum*, *Kluyveromyces*, and *Pichia*) fungi. Molds encompass the following ascomycetous genera: *Acremonium*, *Fusarium*, *Trichoderma*, *Beauveria*, *Aspergillus*, *Paecilomyces*, *Penicillium*, *Talaromyces*, *Alternaria*, *Exophiala*, *Fonsecaea*, *Scopulariopsis*, *Phoma*, and *Trichophyton*.

Strains' revival

Freeze-dried yeast samples were removed from the stocker refrigerator and opened with an 80% ethanol-precleaned lime. Except for *Malassezia* species, which were resuspended in sterile distilled water, all fungi received 1 mL of Sabouraud liquid broth supplemented with 0.05% chloramphenicol. Plates of Sabouraud Dextrose Agar (SDA) medium supplemented with chloramphenicol were inoculated with 500 µL of suspensions for all strains except *Malassezia* species, which were plated on Dixon medium (malt extract 36 g, desiccated oxbile 20 g, Tween 40 10 mL, glycerol 2 mL, peptone 6 g, agar 15 g, 1L osmotic water, and pH 6). At least two replicates were used for all strains, and the plates were incubated at 30°C until the appearance of colonies. Molds were revived using the same procedure, with the following difference: before being inoculated on SDA plates, all strains were left overnight in 1 mL Sabouraud broth. *Beauveria bassiana*, *Trichophyton rubrum*, *Acremonium strictum*, *Talaromyces flavus*, and *Trichophyton interdigitale* were incubated at 25°C, and all the other molds were incubated at 30°C until the appearance of colonies. If no growth was observed after 10 days, the strain was considered nonviable.

Purity evaluation

Microscopic examination was performed using aniline blue-lactic acid (aniline blue 50 mg and lactic acid 90%) wet mount preparations to ensure the absence of contamination. The purity of the *Candida* strains was also determined by streaking them onto chromogenic BBL CHROMagar *Candida* medium (Becton Dickinson France) and incubating the plates for 2 days at 35°C. The screening of single color colonies confirmed our purity check. In addition, for strains labeled as *Cryptococcus* species, we evaluated purity as well as melanin production (appearance of pigmented brown colonies) after subculturing the yeast cells on niger seed agar medium[12] for 48 hours to 7 days at 30°C.

Mold purity, on the other hand, was verified by observing monomorphic colonies on SDA plates. Briefly, molds were removed from the revival plate with a moistened Q-tip obtained by soaking in a 10 mL solution of 0.1% Tween®20. The fungus-infected Q-tip was returned to the soaking solution and squeezed against the tube walls. Finally, 10-fold dilutions were prepared, and 1 mL of these diluted suspensions were plated onto SDA plates and incubated at 25°C or 30°C depending on the optimal growth temperature of the different fungal groups.

Mycological identification

A polyphasic approach combining phenotype and molecular data was used to assess species identification. All strains were visualized both macroscopically and microscopically. For all *Cryptococcus* spp. labeled strains, direct wet India ink mounts were performed to determine the presence or absence of the polysaccharide capsule. For the rest of the isolates, squash wet mount preparations in aniline blue-lactic acid or slide cultures on adapted media were used to observe yeast features or mold conidiogenesis. The media for subculturing molds were SDA medium, 2% Malt Extract Agar (MEA), Potato Carrot Agar (PCA; 100 mL concentrated extract 10X [potatoes 200 g, carrots 200 g, osmotic water 1L], agar 20 g,

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TABLE 1. AUTHENTICATION OF THE 64 SELECTED UMIP YEAST (Y) AND MOLD (M) STRAINS.

UMIP no.	*Phylum*	*Order*	*Original identification (Y/M)*	*New identification[◇] or nomenclature update[†] or further investigation needed*	*ID marker*	*Identity matches*	*Match with collection sequence strain*
1215.80	Ascomycota	*Saccharomycetales*	*Candida albicans* (Y)	—	ITS	885/887 [100%]	CBS 562^{T}
1407.82	Ascomycota	*Saccharomycetales*	*Candida albicans* (Y)	—	ITS	827/827 [100%]	CBS 562^{T}
803.63	Ascomycota	*Saccharomycetales*	*Candida brumptii* (Y)	[†]*Diutina catenulata*	ITS	414/414 [100%]	CBS 565^{T}
813.63	Ascomycota	*Saccharomycetales*	*Candida intermedia* (Y)	—	ITS	614/614 [100%]	CBS 7153
838.63	Ascomycota	*Saccharomycetales*	*Candida zeylanoides* (Y)	—	ITS	595/595 [100%]	CBS 6519^{T}
1926.90	Basidiomycota	*Filobasidiales*	*Cryptococcus albidus var. kuetzingii* (Y)	[†]*Naganishia albida*	ITS	621/621 [100%]	CBS 1926
2093.92	Basidiomycota	*Tremellales*	*Cryptococcus feraegula* (Y)	[◇]*Sirobasidium magnum*	ITS	485/485 [100%]	CBS 6803
1972.91	Basidiomycota	*Filobasidiales*	*Cryptococcus laurentii* (Y)	[◇]*Papiliotrema flavescens*	ITS	681/681 [100%]	CBS 9956
1973.91	Basidiomycota	*Filobasidiales*	*Cryptococcus laurentii* (Y)	[◇]*Papiliotrema flavescens*	ITS	713/713 [100%]	CBS 9956
1940.90	Basidiomycota	*Filobasidiales*	*Cryptococcus neoformans var. gattii* (Y)	**Cryptococcus gattii* complex	ITS	633/633 [100%]	CBS 883
1939.90	Basidiomycota	*Filobasidiales*	*Cryptococcus neoformans var. gattii* (Y)	**Cryptococcus gattii* complex	ITS	437/437 [100%]	CBS 10575
1938.90	Basidiomycota	*Filobasidiales*	*Cryptococcus neoformans var. gattii* (Y)	**Cryptococcus gattii*complex	ITS	633/633 [100%]	CBS 883
1556.84	Ascomycota	*Saccharomycetales*	*Geotrichum capitatum* (Y)	[†]*Magnusiomyces capitatus*	ITS	379/379 [100%]	CBS 162.80
1640.86	Ascomycota	*Saccharomycetales*	*Geotrichum capitatum* (Y)	[†]*Magnusiomyces capitatus*	ITS	379/379 [100%]	CBS 162.80
2059.92	Ascomycota	*Saccharomycetales*	*Geotrichum capitatum* (Y)	[†]*Magnusiomyces capitatus*	ITS	465/465 [100%]	CBS 623.96
820.63	Ascomycota	*Saccharomycetales*	*Pichia anomala* (Y)	[†]*Wickerhamomyces anomalus*	ITS	671/671 [100%]	CBS 250^{T}
2149.93	Ascomycota	*Saccharomycetales*	*Candida glabrata* (Y)	[†]*Nakaseomyces glabratus*	ITS	878/878 [100%]	CBS 6633
810.63	Ascomycota	*Saccharomycetales*	*Candida glabrata* (Y)	[†]*Nakaseomyces glabratus*	ITS	784/784 [100%]	CBS 14388
2423.96	Ascomycota	*Saccharomycetales*	*Candida krusei* (Y)	[†]*Pichia kudriavzevii*	ITS	827/827 [100%]	CBS 5147^{T}
2148.93	Ascomycota	*Saccharomycetales*	*Candida tropicalis* (Y)	—	ITS	537/537 [100%]	CBS 94^{T}
205.52	Ascomycota	*Saccharomycetales*	*Candida parapsilosis* (Y)	—	ITS	854/854 [100%]	CBS 2197^{T}
2234.94	Ascomycota	*Saccharomycetales*	*Kluyveromyces marxianus* (Y)	—	ITS	697/698 [99.8%]	CBS 1554
2386.96	Basidiomycota	*Malasseziales*	*Malassezia furfur* (Y)	—	ITS	781/781 [100%]	CBS 7019^{NT}
2063.92	Basidiomycota	*Malasseziales*	*Malassezia furfur* (Y)	—	ITS	761/761 [100%]	CBS 7984
2062.92	Basidiomycota	*Malasseziales*	*Malassezia furfur* (Y)	—	ITS	822/822 [100%]	CBS 9369
2321.95	Basidiomycota	*Malasseziales*	*Malassezia pachydermatis* (Y)	—	ITS	787/787 [100%]	CBS 6541
2064.92	Basidiomycota	*Malasseziales*	*Malassezia pachydermatis* (Y)	[†]*Malassezia* cf. *pachydermatis*	ITS	645/669 [96.7%]	CBS 10527
2065.92	Basidiomycota	*Malasseziales*	*Malassezia pachydermatis* (Y)	—	ITS	760/760 [100%]	CBS 10528
2066.92	Basidiomycota	*Malasseziales*	*Malassezia pachydermatis* (Y)	—	ITS	766/766 [100%]	CBS 10531
2067.92	Basidiomycota	*Malasseziales*	*Malassezia pachydermatis* (Y)	—	ITS	782/783 [99.9%]	CBS 1892
2194.93	Basidiomycota	*Malasseziales*	*Malassezia pachydermatis* (Y)	[†]*Malassezia* cf. *pachydermatis*	ITS	646/669 [96.6%]	CBS 10527
2334.95	Ascomycota	*Hypocreales*	*Acremonium strictum* (M)	[◇]*Acremonium egyptiacum*	ITS	533/533 [100%]	CBS 114785
1958.90	Ascomycota	*Pleosporales*	*Alternaria alternata* (M)	—	ITS	584/584 [100%]	CBS 479.90
296.55	Ascomycota	*Eurotiales*	*Aspergillus clavatus* (M)	—	β-tubulin	432/433 [99.7%]	CBS 513.65
1144.76	Ascomycota	*Eurotiales*	*Aspergillus clavatus* (M)	—	β-tubulin	495/497 [99.6%]	NRRL 1
597.69	Ascomycota	*Eurotiales*	*Aspergillus flavus* (M)	—	Calmodulin	639/642 [99.5%]	CBS 569.65^{NT}

(*continued*)

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TABLE 1. (CONTINUED)

UMIP no.	*Phylum*	*Order*	*Original identification (Y/M)*	*New identification◇ or nomenclature update† or further investigation needed*	*ID marker*	*Identity matches*	*Match with collection sequence strain*
864.64	Ascomycota	*Eurotiales*	*Aspergillus fumigatus* (M)	—	β-tubulin	413/413 [100%]	CBS 133.61
1041.72	Ascomycota	*Eurotiales*	*Aspergillus ochraceus* (M)	◇*Aspergillus westerdijkiae*	Calmodulin	503/503 [100%]	NRRL 3174^{hT}
1142.76	Ascomycota	*Eurotiales*	*Aspergillus parasiticus* (M)	—	Calmodulin	702/705 [99.6%]	CBS100926
1298.82	Ascomycota	*Eurotiales*	*Aspergillus repens* (M)	†*Aspergillus pseudoglaucus*	Calmodulin	576/578 [99.6%]	NRRL 40^{NT}
1017.70	Ascomycota	*Eurotiales*	*Aspergillus tamarii* (M)	—	Calmodulin	538/542 [99.3%]	CBS 104.13^{NT}
1043.72	Ascomycota	*Eurotiales*	*Aspergillus terreus* (M)	◇*Aspergillus citrinoterreus*	Calmodulin	625/633 [98.7%]	CBS 138921^{HT}
219.52	Ascomycota	*Eurotiales*	*Aspergillus versicolor* (M)	◇*Aspergillus amoenus*	Calmodulin	635/638 [99.5%]	NRRL 4838^{HT}
689.63	Ascomycota	*Eurotiales*	*Aspergillus versicolor* (M)	◇*Aspergillus sydowii*	Calmodulin	560/561 [99.8%]	CBS 593.65^{NT}
2298.95	Ascomycota	*Hypocreales*	*Beauveria bassiana* (M)	—	ITS	568/568 [100%]	CBS 132.36
1394.82	Ascomycota	*Hypocreales*	*Beauveria bassiana* (M)	—	ITS	568/568 [100%]	CBS 132.36
1802.88	Ascomycota	*Chaetothyriales*	*Exophiala dermatitidis* (M)	—	ITS	435/435 [100%]	CBS 270.35^{T}
505.60	Ascomycota	*Chaetothyriales*	*Fonsecaea pedrosoi* (M)	◇*Fonsecaea monophora*	ITS	559/572 [98%]	CBS 269.37^{T}
1412.82	Ascomycota	*Hypocreales*	*Fusarium gramniearum* (M)	—	EF 1-α	674/674 [100%]	NRRL 31615
853.64	Ascomycota	*Hypocreales*	*Paecilomyces viridis* (M)	†*Metarhizium viride*	ITS	619/619 [100%]	CBS 659.71
1668.86	Ascomycota	*Eurotiales*	*Penicillium expansum* (M)	—	β-tubulin	446/450 [99.1%]	MUCL 50
1613.8	Ascomycota	*Eurotiales*	*Penicillium purpurogenum* (M)	◇*Talaromyces stollii*	β-tubulin	401/401 [100%]	CBS 100372
2237.94	Ascomycota	*Pleosporales*	*Phoma cava* (M)	◇*Neocucurbitaria cava*	β-tubulin	346/346 [100%]	CBS 115979
691.63	Ascomycota	*Microascales*	*Scopulariopsis brevicaulis* (M)	—	β-tubulin	486/487 [99.2%]	CBS 127812^{T}
2381.96	Ascomycota	*Eurotiales*	*Talaromyces flavus* (M)	◇*Talaromyces muroii*	β-tubulin	402/402 [100%]	CBS 756.96^{T}
2430.96	Ascomycota	*Eurotiales*	*Talaromyces trachyspermus* (M)	—	β-tubulin	336/336 [100%]	CBS 116556
2406.96	Ascomycota	*Hypocreales*	*Trichoderma koningii* (M)	—	β-tubulin	533/533 [100%]	CBS 457.96^{NT}
2403.96	Ascomycota	*Hypocreales*	*Trichoderma longibrachiatum* (M)	—	β-tubulin	522/522 [100%]	ATCC 18648^{T}
102.77	Ascomycota	*Onygenales*	*Trichophyton mentagrophytes* var. *interdigitale* (M)	†*Trichophyton interdigitale*	ITS	608/608 [100%]	CBS 428.63
2375.96	Ascomycota	*Onygenales*	*Trichophyton raubitschekii* (M)	**Trichophyton rubrum* complex	β-tubulin	412/413 [99.8%]	CBS 100084
1464.83	Ascomycota	*Onygenales*	*Trichophyton rubrum* (M)	**Trichophyton rubrum* complex	β-tubulin	387/389 [99.5%]	CBS 392.58
2376.96	Ascomycota	*Onygenales*	*Trichophyton rubrum* (M)	**Trichophyton rubrum* complex	β-tubulin	380/382 [99.5%]	CBS 392.58

Yeasts and molds Are separated by a dividing line; UMIP, Unité de Mycologie at Institut Pasteur

1L osmotic water) and/or Oatmeal Agar (OA; oatmeal 30 g, agar 15 g, and osmotic water 1L) depending on their capacity to sporulate. The presence of distinctive sporulating structures, when present, was studied.

The molecular characterization for yeasts and molds was performed by extracting total genomic DNA[13] and by sequencing the recommended fungal barcode comprising the ITS1-5.8S-ITS2 region of the ribosomal deoxyribonucleic acid (rDNA). The D1-D2 domain of the 26/28S large subunit ribosomal DNA was also amplified[13] as were small fragments of protein coding genes such as β-tubulin (benA, tub-2),[14] calmodulin (caM),[15] and elongation factor-1α (EF-1α). For the latter, the amplification was done with primers EF-1 and EF-2[16] with the following PCR mixture in a 50 μL final volume: 3 μL of the extracted genomic DNA, 1X PCR buffer (Applied Biosystems), 3 mM MgCl2 (Applied Biosystems), 0.25 μM of each primer, 0.25 mM of each deoxynucleoside triphosphate (Sigma), and 1.25U of AmpliTaq DNA polymerase (Applied Biosystems). The PCR conditions were predenaturation at 94°C for 5 minutes; 35 cycles at 95°C for 30 seconds, 58°C for 1 minutes, and 72°C for 1 minutes; and a final incubation at 72°C for 7 minutes.

The PCR products were then sequenced at Eurofins sequencing facility by using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XL sequencing machines (Applied Biosystems). For every chromatogram, a manual quality checking was performed. Consensus sequences were obtained by using the Sequencher® version 5.4.6 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA) and subjected to pairwise alignments against curated fungal reference databases available at the online Institut PasteurFungiBank (https://fungibank.pasteur.fr/) and MycoBank database (http://www.mycobank.org/). In addition, a BLAST search against known sequences in NCBI Genbank (http://www.ncbi.nlm.nih.gov) was performed. Sequence identities with a cut-off of ≥98% were considered significant. Figure 1 depicts our technical approach.

The ITS sequencing was used for the authentication and characterization of the majority of UMIP strains. For those strains where ITS barcode markers do not provide species-level discrimination, we amplified additional loci: elongation factor-1α for *Fusarium*; β-tubulin for *Aspergillus* section *Fumigati*, *Talaromyces* spp. or *Trichophyton* spp.; calmodulin for *Aspergillus* spp.

To assign the most up-to-date nomenclature, a taxonomic monitoring based on the Index Fungorum database (https://www.indexfungorum.org), literature updates, specialized works, and databases was performed.

Results

We evaluated the revival status, purity, and authenticity of the selected 33 yeast and 31 mold UMIP strains. Table 1 and Figures 2, 3, and 4 display the results.

Yeasts

From the 33 yeast selected, only 2 failed to grow: UMIP 1118.75 (labeled as *C. albicans*) and UMIP 2079.92 (labeled as *Cryptococcus ater*).

All yeast strains were confirmed to be pure cultures on chromogenic agar, or Dixon medium (for *Malassezia* species). The colonies of all strains were either mucous, glossy, or dry, with white to cream colors. Except for *Geotrichum* strains that exhibited holoarthric conidiogenesis (arthroconidia formation), the rest of yeast strains showed typical budding. Seven strains categorized under the genus *Cryptococcus* were all pure. Only the three strains labeled as *C. neoformans* var. *gattii* grew as melanized colonies after 4 days of incubation on Niger seed agar. UMIP1926.90 and UMIP 2093.92 showed nonmelanized (white/cream color) colonies (Fig. 2). Partial melanization was observed for UMIP1972.91 and 1973.91 strains (Fig. 2). The presence of a polysaccharide capsule was confirmed for all originally labeled *Cryptococcus* strains except for UMIP 2093.92 in the tested conditions (Fig. 2).

Barcode ITS sequencing (Table 1) unambiguously validated all examined strains except UMIP1972.91 and UMIP1973.91 (labeled as *Cryptococcus laurentii*) for which we identified *Papiliotrema flavescens* (100% identity type CBS 942; 440/440 bp), and UMIP2093.92 (labeled as *Cryptococcus feraegula*) for which we identified *Sirobasidium magnum* (100% identity type CBS 6803; 513/513 bp) (Fig. 3).

Several strains were renamed as a result of recent nomenclature updates: *Candida brumptii* (UMIP803.63) as *Diutinacatenulata*; *Candida glabrata* (UMIP2149.93, 810.63) as *Nakaseomyces glabratus; Candida krusei* (UMIP 2423.96) as *Pichia kudriavzevii; Cryptococcus albidus* var. *kuetzingii* (UMIP1926.90) as *Naganishia albida; Geotrichum capitatum* (UMIP1556.84, 1640.86, 2059.92) *as Magnusiomyces capitatum*, and *Pichia anomala* (UMIP820.63) as *Wickerhamomyces anomalus* (Table 1 and Fig. 3).

Finally, further investigation is needed to reach the species level for UMIP 2064.92 and 2194.93 strains labeled as *Malassezia pachydermatis* and UMIP 1940.90, 1939.90, and 1938.90 labeled as *Cryptococcus neoformans var. gattii* (Table 1 and Fig. 3).

Molds

The verification of purity was successful for all the 31 molds examined (Table 1 and Fig. 3). The authentication, with no subsequent name modification, was performed by ITS sequence analysis for the following strains: *Alternaria alternata* (UMIP1958.90); *Trichoderma koningii* (UMIP2406.96); *Trichoderma longibrachiatum* (UMIP2403.96); and *B. bassiana* (UMIP1394.82 and 2298.95).

The strains of *A. fumigatus* (UMIP864.64), *Penicillium expansum* (UMIP1668.86), *T. flavus* (UMIP2381.96), *Talaromyces trachyspermus* (UMIP2430.96), *Exophiala dermatitidis* (UMIP1802.88), *T. rubrum* (UMIP1464.83, 2376.96), *T. interdigitale* (UMIP102.77), and *Scopulariopsis brevicaulis* (UMIP691.63) were authenticated based on the β-tubulin gene sequence data (Table 1 and Fig. 3).

Furthermore, the identity of *Aspergillus flavus* (UMIP597.69), *A. parasiticus* (UMIP1142.76), *A. tamarii* (UMIP1017.70), and *A. clavatus* (UMIP296.55 and 1144.76) was performed by calmodulin sequence analysis. The strain of *Fusarium graminearum* (UMIP1412.82) was successfully validated based on the partial sequencing of the elongation factor-1α gene (Table 1 and Fig. 3). In contrast, *Aspergillus ochraceous* (UMIP1041.72) and *A. terreus* (1043.72) were, respectively, reidentified as

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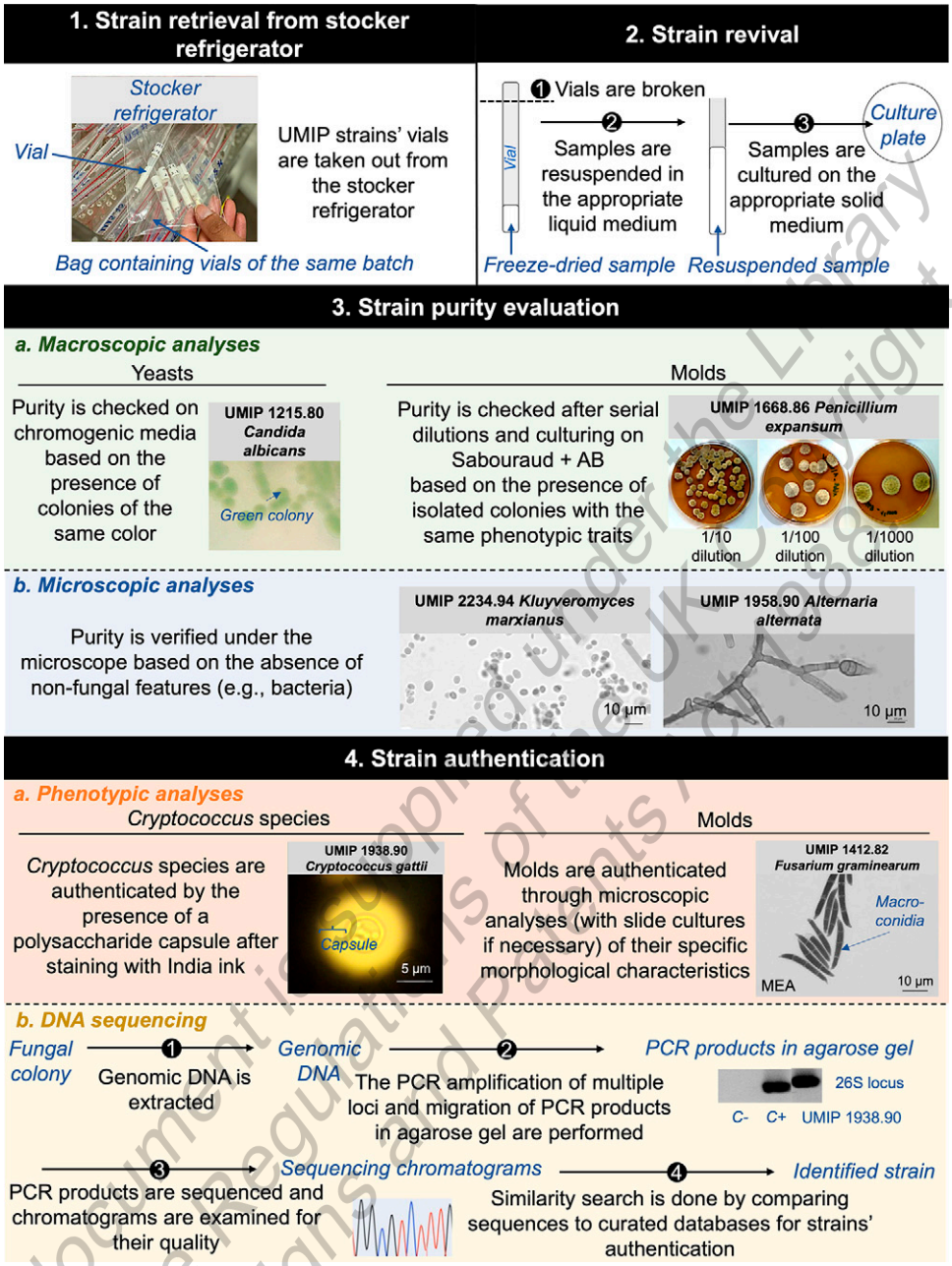

FIG. 1. Workflow used to evaluate the viability, purity, and identity of selected UMIP yeast and mold strains. UMIP, Unité de Mycologie at Institut Pasteur.

A. westerdijkiae and *A. citrinoterreus* after calmodulin sequence analysis, as were two *A. versicolor* strains, *Aspergillus amoenus* (UMIP219.52) and *A. sydowii* (UMIP 689.63) (Table 1 and Fig. 3). Furthermore, barcode ITS sequencing confirmed the new denomination of *A. strictum* (UMIP2334.95) as *A. egyptiacum* and *Fonsecaeapedrosoi* (UMIP505.60) as *F. monophora* (Table 1 and Fig. 3). Finally, based on the β-tubulin sequences, *Penicillium purpurogenum* (UMIP1613.85) and *T. flavus* (2381.96) were renamed *Talaromyces stollii* and *T. muroii*, respectively (Table 1 and Fig. 3).

We followed nomenclature updates to rename *Aspergillus repens* (calmoduline sequence data-UMIP1298.82) as *A. pseudoglaucus*; *Paecilomyces viridis* (ITS sequence data-UMIP 853.64) as *Metarhizium viride*; *Phoma cava* (β-tubulin-UMIP2237.94) as *Neocucurbitaria cava*, *Trichophyton mentagrophytes* var. *interdigitale* (β-tubulin-UMIP102.77) as *T. interdigitale,* and *Trichophyton raubitschekii* (β-tubulin-UMIP 2375.96) as *T. rubrum* (Table 1 and Fig. 3).

Finally, both UMIP1464.83 and 2376.96 labeled as *T. rubrum* were classified in the *T. rubrum* complex, and in-depth phylogenetic studies are needed to determine their species name.

We also visualized all strains' macro- and microscopic features, which aligned with our molecular analysis. Figure 4 depicts colony growth of *A. amoenus* (UMIP 219.52), *Aspergillus westerdijkiae* (UMIP 1041.72), and *Acremonium egyptiacum* (UMIP 2334.95) on SDA media for 14 days at 37°C. Additionally, Figure 4 shows

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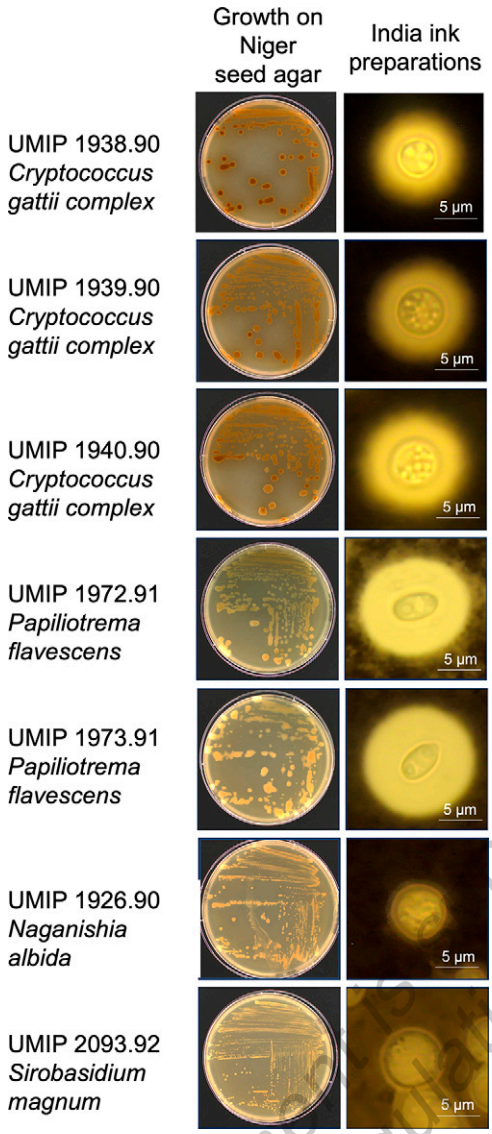

FIG. 2. Macro- and microscopic features for *Cryptococcus* labeled species. Growth on Niger seed agar medium reports differential melanin production by the *Cryptococcus* labeled species after four days of incubation at 30°C. India ink mounts show the polysaccharide capsule, when present.

microscopic reproductive features of *A. alternata* (UMIP 1958.90) strain with longitudinally and transversally septate pear-shaped dictyospores, of *F. graminearum* (UMIP 1412.82) strain with transversally septate macroconidia characteristic for the genus *Fusarium*, and of *Aspergillus parasiticus* (UMIP 1142.76) with biseriate conidial head with finely rough conidia.

We authenticated 31 yeast strains, with 45% (14/31) retaining their original assigned identity, 29% (9/31) having a nomenclature updated identity, 10% (3/31) having a different taxon name than the originally assigned, and 16% (5/31) needing further investigation to determine their name modification. In the same manner for molds, we validated 31 strains, with 52% (16/31) retaining their original assigned identity, 16% (5/31) having a nomenclature updated identity, 26% (8/31) having a different taxon name than the originally assigned, and 6% (2/31) having a name change but requiring further investigation to determine the nature of this modification.

Discussion

Our study showed that most of the analyzed fungal strains of the legacy UMIP collection were viable (31/33 of yeasts and 31/31 of molds) and pure (31/31 of yeasts and 31/31 of molds).

Importantly, our study included strains that were isolated from diverse substrates and that are relevant in either medical or environmental communities of practice. For instance, the yeast UMIP 2423.96T (authenticated as *Pichia kudriavzevii*) was isolated from the sputum of a patient, and UMIP 1973.91 (*P. flavescens*) was isolated from maize. The same goes for molds as we studied UMIP 597.69 (*A. flavus*) isolated from a human with endocarditis and UMIP 1412.82 (*F. graminearum*) from wheat.

The freeze-drying method is often used for the preservation of microorganisms, as samples can be stored and shipped at ambient temperature. However, this method may induce risks for the cell viability and stability. Its efficiency depends on the nature of the cryoprotectant, the type of organism, the growth conditions before freeze-drying, the parameters applied during the freeze-drying process, and the conditions of long-term storage.[17,18,19] In our study, we showed that fungi were successfully revived, except UMIP 1118.75 (labeled as *C. albicans*) and UMIP 2079.92 (labeled as *C. ater*), based on two vials from different batches. One or a combination of the critical freeze-drying parameters mentioned above can explain such a failure. However, we suggest that the cellular properties of UMIP 1118.75 and UMIP 2079.92 are not a root cause, as other strains of *Candida* and *Cryptococcus* were successfully revived. The exact culprit cannot be identified as we do not have information about the exact protocols that had been applied to prepare and freeze-dry the samples. Through our study, we also witnessed that a vial of UMIP 1407.82 (labeled as *C. albicans*) with a beige/brownish unusual color did not revive; however, the retrieval of another vial with the typical white color led to the successful growth of the strain (Table 1 and Fig. 4). The latter information supports that viability issues encountered through our study were most probably unrelated to intrinsic properties of the microorganism.

The study by J. Bosmans qualifying the revival state of approximately 5000 vials of freeze-dried microbial samples, including yeasts and filamentous fungi, indicated that freeze-drying was generally a better preservation method than periodic transfer, conservation under mineral or paraffin oil, soil agar, sterile water, and deep freeze.[20] Interestingly, the author showed that yeasts have lower stability than filamentous fungi after 10 years, as 25% did not resuscitate. He also highlighted that a particular group of fungi, the *Entomophtorales*, did not support lyophilization in the tested conditions, indicating that the freeze-dried method can be optimized for this group of fungi and/or that alternative preservation methods must be used. Palacio et al. also reported that the basidiomycetes *Agaricus blazei*, *Ganaderma lucidum*, *Grifola frondosa*, and *Pleurotus pulmorius* were best preserved in distilled water at 24°C and in sawdust and rice bran with 10% glycerol at −80°C than

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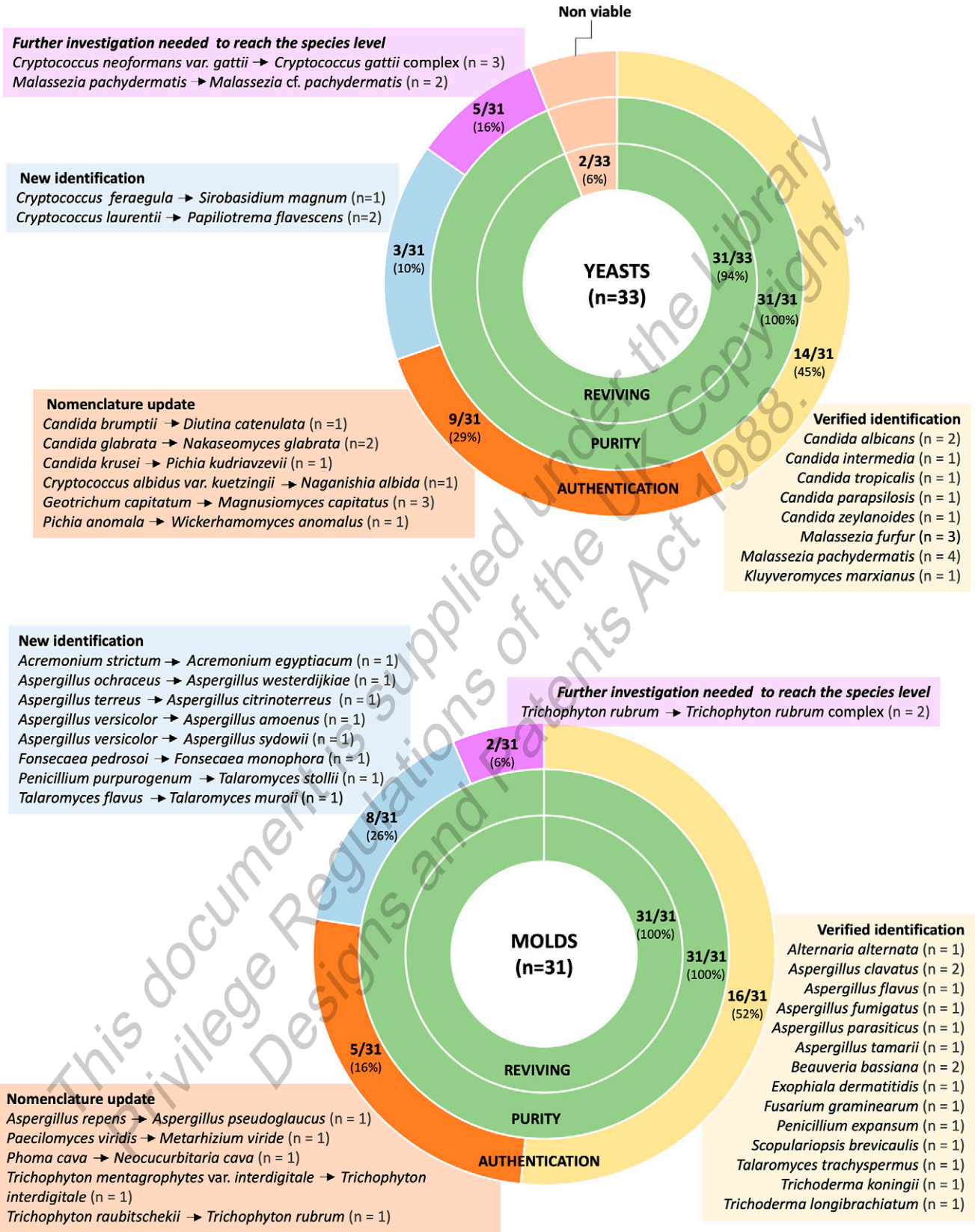

FIG. 3. Success rate of the reviving, purity, and authentication of the UMIP yeast and mold strains. UMIP, Unité de Mycologie at Institut Pasteur.

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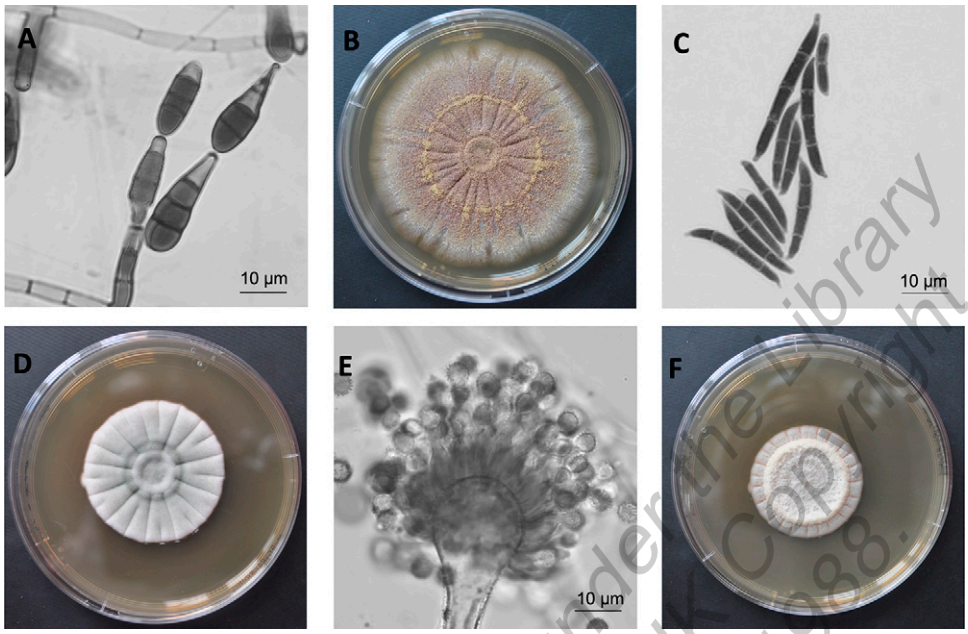

FIG. 4. Microscopic reproductive features: **(A)** Dictyospores of *Alternaria alternata* UMIP 1958.90, **(C)** macroconidia of *Fusarium graminearum* UMIP 1412.82, **(E)** biseriate conidial head and conidia of *Aspergillus parasiticus* UMIP 1142.76. Colony growth of **(B)** *Aspergillus amoenus* UMIP 219.52, **(D)** *Aspergillus westerdijkiae*, and **(F)** *Acremonium egyptiacum* UMIP 2334.95 grown for 14 days on SDA media. SDA, Sabouraud Dextrose Agar; UMIP, Unité de Mycologie at Institut Pasteur.

freeze-dried or in sawdust and rice bran with 10% glycerol at −20°C.[21] In sum, methods of preservation should be adapted and validated for each type of organism when optimal quality attributes are needed. Liquid nitrogen is commonly used for long-term storage; a study by Homolka et al. indicated that this is an efficient way of preserving several basidiomycetes over the years when samples are supplemented with perlite.[22]

The freeze-drying method has been optimized over the years to improve strain viability and stability. One area of improvement concerns the nature of the cryoprotectant. The study of Stefanello et al. is a good example, among others, of the effort made in investigating the impact of several cryoprotectants on the preservation of microorganisms.[17] The authors demonstrated that the stability of *Wickerhamomyces anomalus* IAL 4533 strongly differs when sucrose and skimmed milk powder are used as cryoprotectants, with no revival and more than 70% of revival, respectively.

Beyond the assessment of the stability of the strains, our phenotypic and molecular analyses show that the strains are suitable for downstream morphological and genetic applications.

Our morphological analyses allowed us to characterize the strains and assess the accuracy of the initial identification. The examination of yeasts, for example, with India ink mounts revealed that all strains with a polysaccharidic capsule belonged to the genus *Cryptococcus sensu lato*. At the same time, the presence of arthroconidia was associated with *Geotrichum* conidiogenesis. To identify molds, we used microscopic wet mount preparations and slide cultures on specific media, as well as the observation of their specific sporulation-specific structures. We were able to authenticate strains labeled as *Aspergillus*, *Fusarium*, *Trichoderma*, and *Penicillium/Talaromyces* at the genus level. As morphological characteristics alone are insufficient for accurately identifying mold cultures to the species level, we followed a sequence-based approach to achieve the final strain identity.

As a result of our molecular analysis, we were able to assign strain identity values at the species level for most of the strains. This nomenclature monitoring allowed us to update the taxonomic name of the revived strains.

Over the years, some species have seen their name change because of the attribution of a unique name for the species' teleomorph and anamorph states (One Fungus, One Name) and their reallocation to different genera to obtain monophyletic clades. In our study, the yeasts *C. brumptii*, *C. glabrata, C. krusei, C. albidus var. kuetzingii*, *G. capitatum,* and *P. anomala* and molds *A. repens, P. viridis, P. cava*, and *T. mentagrophytes var. interdigitale* have been subjected to a nomenclature update.[23] *T. raubitschekii* has also been synonymized as *T. rubrum.*[24]

The disparity between the initial and current identifications is almost certainly because of the use of non-discriminant methods to characterize the strains at the time of their initial integration, or to the most recent descriptions of a new species that better matches the strain attributes. For instance, we believe that the UMIP1041.72 strain, now known to be *A. westerdijkiae*, a species first described in 2004, was initially described as *A. ochraceous,* as both are related species and belong to the section *Circumdati.*[25] Such a change could also apply to UMIP1043.72 (labeled *A. terreus*), UMIP505.60 (labeled as *F. pedrosoi*), and UMIP2381.96 (labeled *P. purpurogenum*)as their identification matches with the *A. citrinoterreus*, *F. monophora,* and *T. stollii*, three species that have been described recently.[26–28]

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On the other hand, it is possible that strains were initially misidentified based on features shared by other species. For example, we believe that UMIP2093.92, now known as *S. magnum*, could have been mistaken for a *Cryptococcus* species because of the similar characteristics under laboratory growth conditions. Such an identification error could also apply to the yeasts UMIP1972.91 and UMIP1973.91 (labeled as *C. laurentii)* and molds UMIP2334.95 (*A. strictum*), UMIP219.52 and UMIP689.63 (*Aspergillus versicolor*), and UMIP2381.96 (*T. flavus*).

Finally, UMIP1940.90, UMIP1939.90, and UMIP1938.90 (labeled as *C. neoformans var. gattii*) and UMIP 1464.83 and UMIP 2376.96 (labeled as *T. rubrum*) were identified within species complexes in our study. Therefore, further molecular analyses such as sequencing additional loci and/or in-depth phylogenetic studies must be performed to determine their current species name. In the same manner, additional analyses are needed for the UMIP2064.92 and UMIP2194.93 strains, originally labeled as *M. pachydermatis*, as our sequencing data did not fully match with species in the available databases (identity matches: 96.7% and 96.6% with the ITS region of CBS 10527). Resolution of strain misidentifications in legacy collections is critical not only for reproducible research results, maintenance of biodiversity but also for biosafety and biosecurity reasons and for clear communication in the scientific community.

Conclusions

Our proof-of-concept study demonstrated that yeasts and molds remain viable after more than 20 years in the freeze-dried status and can be fully qualified by a polyphasic approach. It enabled us to carry on toward the goal of preserving the fungal diversity present at the UMIP collection, which we know is critical for research, education, and environmental and industrial applications and will pave the way for the collection's future reopening.

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Authors' Contributions

M.E.G.: Conceptualization (supporting); project administration (equal); investigation (lead); formal analysis (equal); writing—original draft (lead); and visualization (equal). A.C.: Project administration (equal) and visualization (equal). S.B.: Funding acquisition (equal); project administration (equal); conceptualization (equal); and writing—review and editing (equal). F.B.: Project administration (equal) and writing—review and editing (equal). D.G.-H.: Funding acquisition (equal); project administration (equal); resources (lead); conceptualization (equal); formal analysis (equal); validation (lead) and writing—review and editing (equal).

Author Disclosure Statement

All authors declare that they have no conflicts of interest.

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