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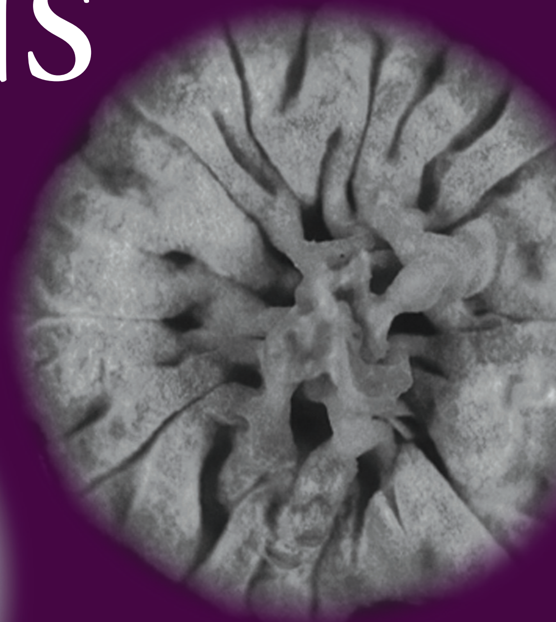

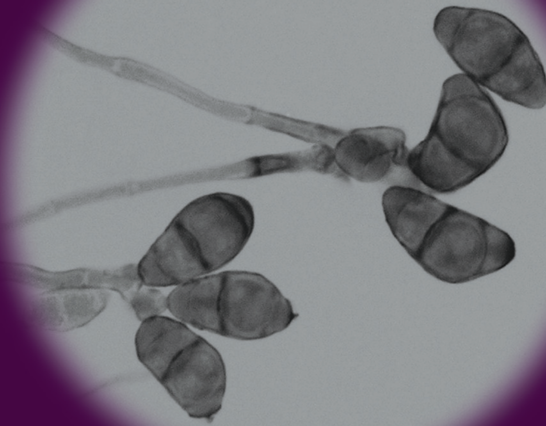

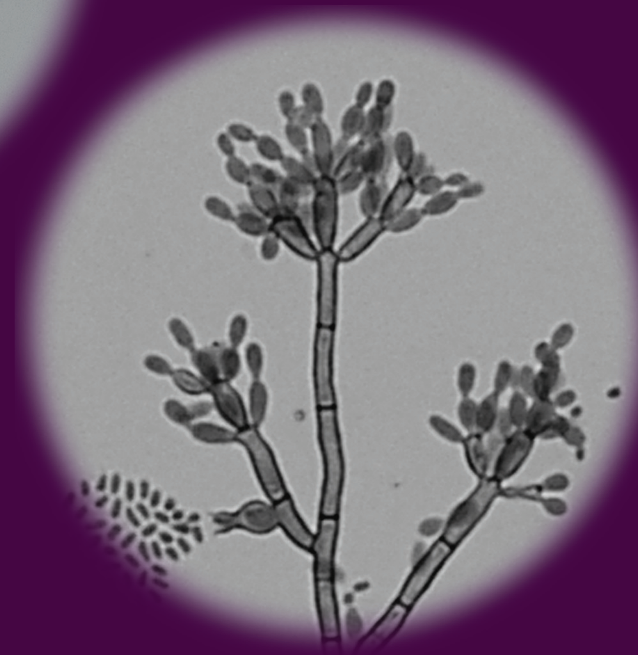

EDITED BY
DONGYOU LIU

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This volume is dedicated to a group of international mycologists, whose in-depth knowledge and technical expertise have made an all inclusive coverage of major human fungal pathogens possible.

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Preface

Fungi are a diverse group of eukaryotic organisms that range from yeasts, molds, mushrooms, lichens, rusts, smuts, to microsporidia. Forming a kingdom of their own and being ubiquitously distributed in all environments, most fungi are saprophytes involved in the decomposition and recycling of organic matters as well as in the formation of symbiotic relationship with plants and animals. However, some fungi have the capacity to cause diseases in plants, animals, and humans. Often occurring as a result of trauma or underlying immunosuppression, human mycoses may manifest as superficial, cutaneous, subcutaneous, or systemic diseases. The inability to distinguish human mycoses caused by various fungal pathogens on clinical ground necessitates the development and use of laboratory diagnostic procedures in order to facilitate their treatment and prevention.

Given their complex life cycle and their tendency to produce morphologically similar structures, fungi are notoriously difficult to identify on the basis of their macroscopic and microscopic features, even for an experienced mycologist. To increase the accuracy, sensitivity, and efficiency of fungal identification, molecular techniques such as PCR and nucleotide sequencing have been increasingly adopted and applied in research and clinical laboratories worldwide. Consequently, a large number of molecular protocols have been described in the literature for the identification and detection of fungal organisms. As the saying goes, one person's medicine could easily turn into another's poison. There is certainly no exception here. The overabundance of original protocols and subsequent modifications has created a dilemma for anyone who was not directly involved in their developments to know which are most appropriate to use for streamlined identification and detection of fungal organisms of interest.

With contributions from international scientists in respective fungal pathogen research and diagnosis, this book aims to provide a reliable and comprehensive source relating the molecular detection and identification of major human fungal pathogens. Each chapter consists of a brief review on the classification, epidemiology, clinical features, and diagnosis of one or a group of related fungal species; an outline of clinical sample collection and preparation procedures; a selection of representative stepwise molecular protocols; and a discussion on additional research for further improving the diagnosis. This book represents an indispensable tool for both upcoming and experienced medical, veterinary, and industrial laboratory scientists engaged in fungus characterization and provides an essential reference for undergraduate and graduate students majoring in mycology.

An all-encompassing book such as this clearly demands a concerted team's efforts. I am fortunate and extremely honored to have had a large group of international mycologists as chapter contributors, whose in-depth knowledge and technological insights into human fungal pathogen detection have significantly enriched this book. Additionally, the professionalism and dedication of executive editor Barbara Norwitz and senior project coordinator Jill Jurgensen at CRC Press have enhanced its presentation. Finally, without the understanding and support of my family, Liling Ma, Brenda, and Cathy, the compilation of this comprehensive book would have been unimaginable.

Editor

Dongyou Liu, PhD, undertook his veterinary science education at Hunan Agricultural University, Changsha, China. Upon graduation, he received an overseas postgraduate scholarship from the Chinese Ministry of Education to pursue further training at the University of Melbourne, Melbourne, Victoria, Australia, where he worked toward improved immunological diagnosis of human hydatid disease. During the past two decades, he has crisscrossed between research and clinical laboratories in Australia and the United States, with focuses on molecular characterization and virulence determination of microbial pathogens such as ovine footrot bacterium (*Dichelobacter nodosus*), dermatophyte fungi (*Trichophyton, Microsporum*, and *Epidermophyton*), and listeriae (*Listeria* species). He is the first author of more than 50 original research and review articles in various international journals and the editor of the recently released *Handbook of* Listeria monocytogenes (2008), *Handbook of Nucleic Acid Purification* (2009), *Molecular Detection of Foodborne Pathogens* (2009), *Molecular Detection of Human Viral Pathogens* (2010), and *Molecular Detection of Human Bacterial Pathogens* (2011), as well as the forthcoming *Molecular Detection of Human Parasitic Pathogens* (2012), all of which are published by CRC Press.

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1 Introductory Remarks

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1.1 PREAMBLE

Fungi (singular fungus, meaning "mushroom" in Latin) are a diverse group of eukaryotic organisms (ranging from yeasts, molds, mushrooms, lichens, rusts, smuts to microsporidia) that constitute one of the five kingdoms (i.e., Prokaryotae, Fungi, Protista, Plantae, and Animalia) in the current classification system for living organisms. Similar to other eukaryotic kingdoms (Protista, Plantae, and Animalia), fungi harbor membrane-bound nuclei with chromosomal DNA, which consists of noncoding regions (introns) and coding regions (exons), possess membrane-bound cytoplasmic organelles (e.g., mitochondria), sterol-containing membranes, and 80S ribosomes and produce a variety of soluble carbohydrates and storage compounds, including sugar alcohols, disaccharides, and polysaccharides. Furthermore, Fungi resemble Protista and Animalia by the lack of chloroplasts and thus the requirement of preformed organic compounds as energy sources. Although both Fungi and Plantae possess a cell wall and vacuoles, reproduce by sexual as well as asexual means, generate spores (as in ferns and mosses), and have haploid nuclei (as in mosses and algae), Fungi differ from Plantae

by the presence of chitin (which also exists in the exoskeleton of arthropods), instead of cellulose in the cell walls, and the absence of chloroplasts. On the other hand, Fungi differ from Prokaryotae by having nuclear membrane, plasma membrane, and cell wall.

In this introductory chapter, a brief overview is presented on the classification, biology, and genetics of fungal organisms, and clinical manifestations in human hosts resulting from their infections. This is followed by a summary of laboratory approaches that are useful for phenotypic characterization of fungi, including sample collection and processing, microscopic examination, in vitro cultivation, biochemical and anti-fungal testing. The subsequent section focuses on key attributes relating to molecular characterization of fungi, such as nucleic acid extraction, target gene selection, template amplification, and amplicon detection. Finally, the importance of rational result interpretation, standardization, and quality control and assurance in the molecular fungal testing is emphasized.

1.2 CLASSIFICATION, BIOLOGY, GENETICS, AND CLINICAL PRESENTATION

1.2.1 Classification

Fungi are an extremely diverse and abundant group of eukaryotic organisms, whose sizes range from single-celled aquatic chytrids to large mushrooms and whose number has been estimated to be between 700,000 and 1.5 million species, with nearly 100,000 species being described to date [1–8]. However, fewer than 500 of the recognized fungal species (including about 200 yeast species) have been shown to cause human infections.

Based on morphological criteria, fungi are often divided into two major categories: filamentous fungi (true fungi) and yeasts. Accounting for the bulk of fungal species, filamentous fungi produce tubular, elongated, and thread-like (filamentous) cellular structures (known as hyphae), which contain multiple nuclei and extend at their tips. With about 700 known species, yeasts are single-celled organisms that reproduce by budding or binary fission. In addition, a few fungal species are able to switch between a yeast phase and a hyphal phase in response to environmental conditions and are referred to as dimorphic fungi. Despite their relatively insignificant proportion in relation to filamentous fungi, about 200 of the 700 recognized yeast species are responsible for a majority of clinical cases of human mycoses.

Using a combination of morphological characteristics and mechanisms of reproduction, fungi have been traditionally separated into five phyla: Ascomycota (sac fungi), Basidiomycota (club fungi), Mycophycophyta (lichens fungi), Zygomycota (conjugation fungi), and Deuteromycota (imperfect fungi, or mitosporic fungi, which are fungi with no known sexual cycle) [9].

Recent phylogenetic analyses of 18S rRNA, 28S rRNA, 5.8S rRNA, *rpb1*, *rpb2*, and *tef1* genes indicate that the kingdom Fungi consists of one subkingdom (Dikarya including phyla Ascomycota and Basidiomycota), seven phyla (all with the suffix -mycota except Microsporidia; i.e., Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Blastocladiomycota, Neocallimastigomycota, and Microsporidia, in addition to Fungi incertae sedis, which encompasses fungi with indeterminate taxonomical status) (Table 1.1), 10 subphyla (with the suffix -mycotina), 35 classes (with the suffix -mycetes), 12 subclasses (with the suffix -mycetidae), and 129 orders (with the suffix -ales) [10–13]. A most notable feature of this taxonomical scheme is the reorganization of the former phylum Zygomycota into the phylum Glomeromycota and four separate subphyla (Mucoromycotina, Entomophthoromycotina, Zoopagomycotina, and Kickxellomycotina), which may form independent phyla upon further confirmation (Table 1.1). However, this scheme does not take into account of organisms such as oomycetes and slime molds that were formerly included in the kingdom Fungi [12]. Also, the genera *Caulochytrium*, *Olpidium*, *Rozella* (formerly of Chytridiomycota), and *Basidiobolus* (formerly of Entomophthorales, Zygomycota) are not included in any higher taxa in this scheme, pending further taxonomical resolutions [12]. In addition, a clade (i.e., Symbiomycota) sharing similarity between Glomeromycota and Dikarya is not included in the current scheme as Symbiomycota may possibly constitute a rank between kingdom and subkingdom [12].

Most human pathogenic fungi are found in the phyla Ascomycota, Basidiomycota, and Microsporidia as well as Fungi incertae sedis (principally Mucoromycotina and Entomophthoromycotina of the former phylum Zygomycota). From a medical mycologist's perspective, human pathogenic fungi are conveniently separated into eight subgroups: (i) dermatophytes (represented by *Epidermophyton*, *Microsporum*, and *Trichophyton*); (ii) yeasts (represented by *Blastoschizomyces*, *Candida*, *Cryptococcus*, *Lacazia*, *Malassezia*, *Rhodotorula*, *Saccharomyces*, and *Trichosporon*); (iii) dimorphic fungi (represented by *Blastomyces*, *Coccidioides*, *Histoplasma*, and *Paracoccidioides*); (iv) hyaline hyphomycetes (hyaline molds) (represented by *Acremonium*, *Aspergillus*, *Beauveria*, *Chrysosporium*, *Cylindrocarpon*, *Fusarium*, *Geotrichum*, *Gliocladium*, *Graphium*, *Madurella*, *Malbranchea*, *Onychocola*, *Paecilomyces*, *Penicillium*, *Scedosporium*, *Scopulariopsis*, *Sepedonium*, *Trichoderma*, *Trichothecium*, and *Verticillium*); (v) dematiaceous hyphomycetes (dematiaceous molds) (represented by *Acrophialophora*, *Alternaria*, *Aureobasidium*, *Bipolaris*, *Cladophialophora*, *Cladosporium*, *Curvularia*, *Drechslera*, *Exophiala*, *Exserohilum*, *Fonsecaea*, *Hortaea*, *Lecythophora*, *Ochroconis*, *Phaeoacremonium*, *Phialophora*, *Ramichloridium*, *Rhinocladiella*, *Scedosporium*, *Sporothrix*, *Ulocladium*, and *Veronaea*); (vi) coelomycetes (represented by *Colletotrichum*, *Lasiodiplodia*, *Nattrassia*, and *Phoma*); (vii) zygomycetes (represented by *Apophysomyces*, *Basidiobolus*, *Conidiobolus*, *Cunninghamella*, *Mortierella*, *Mucor*, *Absidia*, *Rhizomucor*, *Rhizopus*, *Saksenaea*, and *Syncephalestrum*); and (viii) basidiomycetes [7].

TABLE 1.1
Classification of the Kingdom Fungi

Phylum (Subkingdom)	Subphylum (Former Classification)	Brief Description
Ascomycota (*Dikarya*)	Pezizomycotina	Ascomycota (commonly known as sac fungi) represents the largest phylum of Fungi, with over 64,000 species grouped under three subphyla (Taphrinomycotina, Saccharomycotina, and Pezizomycotina). Ascomycota produce *ascus* (from *Greek askos*, meaning "sac" or "wineskin"), in which nonmotile *spores* (a *sexual structure* also known as *ascospores*) are formed. However, some Ascomycota (formerly belonging to *Deuteromycota*) are *asexual*, do not have a sexual cycle, and thus do not form asci (or ascospores). Forming part of Ascomycota, Pezizomycotina consist of *Orbiliomycetes*, *Pezizomycetes*, *Dothideomycetes*, *Arthoniomycetes*, Eurotiomycetes, *Laboulbeniomycetes*, Lichinomycetes, *Lecanoromycetes*, Leotiomycetes, and *Sordariomycetes*, as well as three unassigned orders (Lahmiales, Medeolarials, and Triblidiales). Pezizomycotina cover all ascomycetes that produce *ascocarps* (fruiting bodies), except for *Neolecta* in Taphrinomycotina.
	Saccharomycotina	Forming part of Ascomycota, Saccharomycotina consist of the "true" yeast class *Saccharomycetes*
	Taphrinomycotina	Forming part of Ascomycota, Taphrinomycotina consist of four classes: *Neolectomycetes* (hyphal fungi), *Pneumocystidomycetes* (mammalian lung pathogen Pneumocystis), *Schizosaccharomycetes* (fission yeasts), and *Taphrinomycetes* (hyphal fungi).
Basidiomycota (*Dikarya*)	Pucciniomycotina (Urediniomycetes)	Basidiomycota (commonly known as club fungi) is the second largest phylum of Fungi, with 31,515 species grouped under three subphyla (Pucciniomycotina, Ustilaginomycotina, and Agaricomycotina, in addition to two separate classes Wallemiomycetes and Entorrhizomycetes). Forming part of *Basidiomycota*, Pucciniomycotina consist of eight classes of rust fungi (i.e., *Classiculomycetes*, *Cryptomycocolacomycetes*, *Mixiomycetes*, *Atractiellomycetes*, *Agaricostilbomycetes*, *Cystobasidiomycetes*, *Pucciniomycetes*, and *Microbotryomycetes*).
	Ustilaginomycotina (Ustilaginomycetes)	Forming part of *Basidiomycota*, Ustilaginomycotina consist of two smut fungus classes *Exobasidiomycetes* and *Ustilaginomycetes*, as well as a separate smut fungus order Malasseziales.
	Agaricomycotina (Basidiomycetes)	Forming part of *Basidiomycota*, Agaricomycotina consist of three classes: *Agaricomycetes* (hymenia-forming fungi), *Dacrymycetes* (hymenia-lacking fungi), and *Tremellomycetes* (jelly fungi).
Chytridiomycota		Consisting of two classes Chytridiomycetes (with three orders: Chytridiales, Spizellomycetales, and Rhizophydiales) and Monoblepharidomycetes (with one order Monoblephariales), *Chytridiomycota* include more than 1000 known species. Chytridion (meaning "little pot") describes the structure containing unreleased *spores*. Chytrids are mostly primitive, *aquatic*, *saprobic* fungi involved in the degradation of *chitin* and *keratin*, have *coenocytic thalli*, and usually form *rhizoids* (instead of true mycelium).
Neocallimastigomycota	(Chytridiomycota)	Consisting of Neocallimastigales, a traditional member of Chytridiomycota, *Neocallimastigomycota* include a small group of anaerobic fungi that inhabit the digestive system of larger herbivorous mammals and possibly other terrestrial and aquatic environments. Although lacking *mitochondria*, *Neocallimastigomycota* possess *hydrogenosomes* of mitochondrial origin. Similar to chrytrids, neocallimastigomycetes form zoospores with posteriorly uniflagellate or polyflagellate. However, neocallimastigomycetes are distinct from other chytrids on the basis of both morphology and molecular phylogeny.
Blastocladiomycota	(Chytridiomycota)	Consisting of Blastocladiales, also a traditional member of Chytridiomycota, *Blastocladiomycota* are *saprotrophs*, and also parasites of all eukaryotic groups. *Blastocladiomycota* undergo *sporic meiosis* in contrast to chytrids, which mostly exhibit *zygotic meiosis*.
Glomeromycota	(*Zygomycota*)	Forming part (commonly known as "sugar" and "pin" molds) of former *Zygomycota*, and consisting of one class *Glomeromycetes* (with four orders: *Glomerales*, *Diversisporales*, *Paraglomerales*, and *Archaeosporales*) with about 200 described species (all of which reproduce asexually), Glomeromycota produce *arbuscular mycorrhizas* with roots or thalli, and are obligate *biotrophs*, dependent on symbiosis with land plants for carbon and energy.
Fungi *incertae sedis*	*Mucoromycotina* (*Zygomycota*)	Fungi that were placed in Zygomycota are now being reassigned to Glomeromycota, and Fungi *incertae sedis* (including four subphyla *Mucoromycotina*, *Entomophthoromycotina*, *Zoopagomycotina*, and *Kickxellomycotina*). Consisting part of Fungi *incertae sedis*, *Mucoromycotina* cover three orders: *Mucorales*, *Endogonales*, and *Mortierellales*.
	Entomophthoromycotina (*Zygomycota*)	Consisting part of Fungi *incertae sedis*, *Entomophthoromycotina* (with one order Entomophthorales) are pathogens of *insects*, *nematodes*, and *tardigrades*, as well as free-living *saprotrophs*.

(*continued*)

TABLE 1.1 (continued)
Classification of the Kingdom Fungi

Phylum (Subkingdom)	Subphylum (Former Classification)	Brief Description
	Zoopagomycotina (*Zygomycota*)	Consisting part of Fungi *incertae sedis*, *Zoopagomycotina* (with one order Zoopagales) are pathogens of microscopic animals such as amoebae.
	Kickxellomycotina (*Zygomycota*)	Consisting part of Fungi *incertae sedis*, *Kickxellomycotina* include four orders: *Asellariales*, *Kickxellales*, *Dimargaritales*, and *Harpellales*.
Microsporidia		Microsporidia cover about 150 genera (containing >1200 species) that were previously considered as protozoa, of which 12 species (representing 8 genera) have been shown to cause opportunistic infections in humans. Microsporidia lack mitochondria and motile structures (e.g., flagella) and produce highly resistant spores, the morphology (oval or pyriform, occasionally rod-shaped or spherical) of which is often used for their differentiation.

Sources: James, T.Y. et al., *Nature*, 443, 818, 2006; Hibbett, D.S. et al., *Mycol. Res.*, 111, 509, 2007.

1.2.2 Biology

Filamentous fungi are characterized by the production of hyphae, which are cylindrical, thread-like structures of 2–10 µm in diameter and up to several centimeters in length. Hyphae can be either septate (with two or more compartments separated by right-angled internal cell walls called septa) or aseptate (or coenocytic, with each compartment containing one or more nuclei). Septa have pores that facilitate passage and interchange of cytoplasm, organelles, and at times nuclei. Hyphae are important for penetration/invasion into the host cells and for the uptake of nutrients from living hosts and other substrates. New hyphae typically emerge from hyphal tips (apices), arise from existing hyphae by a process called branching, or occasionally grow hyphal tips bifurcate (fork) giving rise to two parallel-growing hyphae. The combined effects of apical growth and branching/forking result in the formation of an interconnected network of hyphae (with high surface area to volume ratios) known as mycelium (plural mycelia), which is also commonly called mold. Mycelia grown on solid agar media are referred to as colonies, which may exhibit a variety of sizes, shapes, and colors (pigmentations) [9].

In general, fungi reproduce by means of microscopic propagules called spores (conidia) as a result of an asexual process. Near a third of all fungi reproduce by different modes of propagation, showing two well-differentiated stages (i.e., the teleomorph or sexual stage and the anamorph or asexual stage) within the life cycle of a species. Achieved via vegetative spores or mycelial fragmentation, asexual reproduction helps clonal populations to adapt to a specific niche and allows more efficient dispersal than sexual reproduction. Sexual reproduction through meiosis involves various sexual structures (e.g., fruiting bodies) and reproductive strategies. Compatible fungi may fuse their hyphae into an interconnected network in a process known as anastomosis, which is required for the initiation of the sexual cycle.

Yeasts commonly undergo asexual reproduction (mitosis) by budding or fission, although some have the capacity to reproduce both asexually and sexually. During the budding process, a small bud (or daughter cell) forms on the parent cell, and the nucleus of the parent cell splits into a daughter nucleus which migrates into the daughter cell. The growing bud eventually separates from the parent cell to become a new cell.

Fungi are widespread in all environments and habitats, including soil, plants, insects, animals, humans, air, deserts, and deep-sea sediments [14]. Most fungi are saprophytes that play an essential environmental role in the decomposition and recycling of organic matters, and form symbiotic relationship with plants and animals; some have the capacity to cause diseases in plants, animals, and humans. Furthermore, some fungi have other properties that can be exploited for bread/beverage making, insect pest control, medicine, and scientific research. For instance, yeasts have been employed in (i) the two-hybrid screening systems for the general detection of protein–protein interactions; (ii) the yeast artificial chromosomes (YACs) for cloning large fragments (200–800 kb) of DNA; and (iii) expression systems for heterologous proteins.

1.2.3 Genetics

Relative to other higher level eukaryotes (e.g., mammals), fungal genomes are simple and compact, with sizes ranging from 12,068 kb in *Saccharomyces cerevisiae*, 22,540 kb in *Trichophyton verrucosum* HKI 0517 (GenBank ACYE00000000), 28,467 kb in *Penicillium marneffei* ATCC 18224 (GenBank ABAR00000000), 32,228 kb in *Penicillium chrysogenum* Wisconsin 54-1255 to 51,230 kb in *Nectria haematococca* (anamorph *Fusarium solani*) (GenBank ACJF00000000).

The 12 Mb genome of baker's yeast *Saccharomyces cerevisiae* is clustered into 16 chromosomes (of 200–2200 kb in size), with a total of 6183 open-reading frames (ORFs), of which 5885 are predicated to be protein-coding genes. Its ribosomal RNA (rRNA) genes are coded by about 140 genes of a single tandem array on chromosome XII; small nuclear RNAs are coded by 40 genes; and transfer RNAs

(tRNAs) are coded by 275 genes. *S. cerevisiae* mitochondrial DNA encodes components of the mitochondrial translational machinery and about 15% of the mitochondrial proteins [15].

Whereas the 22 Mb genome of *Penicillium marneffei* ATCC 18224 harbors 10,136 ORF; the 32 Mb genome of *Penicillium chrysogenum* Wisconsin 54-1255 contains 13,911 ORF, with 12,791 being protein-coding genes [16]. As a member of the "*Fusarium solani* species complex" that encompasses >50 species, *Nectria haematococca* MPVI (anamorph *Fusarium solani*) has been shown to possess a 51 Mb genome, which is organized in 17 chromosomes (of 530 kb–6.52 Mb in size) with 15,707 predicted genes [17].

On the other hand, microsporidia possess extremely reduced eukaryotic genomes, which may be as small as 2.6 Mb with 2000 genes. These organisms have remnant mitochondria and show unique morphologies related to parasitism, including polar tube to penetrate host cells and initiate infection.

1.2.4 Clinical Presentation

Although most fungal species are saprophytic organisms with a very low inherent virulence, some have the ability to take advantage of the weakened host defense (e.g., trauma and immunosuppression) and invade the host cells, causing a variety of clinical diseases, ranging from (i) superficial, (ii) cutaneous, (iii) subcutaneous to (iv) systemic mycoses [18].

Superficial mycoses: As cosmetic fungal infections of the skin or hair shaft, superficial mycoses do not invade the living tissue nor elicit cellular response from the host. Patients with superficial mycoses seeking medical advices are largely for social or cosmetic reasons. Examples of such superficial mycoses include pityriasis versicolor and seborrhoeic dermatitis due to *Malassezia furfur*, tinea nigra due to *Hortaea werneckii*, white piedra due to *Trichosporon* species, and black piedra due to *Piedraia hortae*.

Cutaneous mycoses: Being another form of superficial fungal infections of the skin, hair, or nails, cutaneous mycoses do not invade the living tissue but may cause a variety of pathological changes in the host due to the presence of the infectious agent and its metabolic products. Examples of such cutaneous mycoses comprise dermatophytosis due to *Epidermophyton*, *Microsporum*, and *Trichophyton*; candidiasis (of skin, mucous membranes, and nails) due to *Candida* species; and dermatomycosis due to non-dermatophyte molds such as *Onychocola*, *Scopulariopsis*, and *Scytalidium*.

Subcutaneous mycoses: As chronic, localized infections of the skin and subcutaneous tissue following the traumatic implantation of a soil saprophyte, subcutaneous mycoses may present a variety of clinical symptoms. These range from sporotrichosis due to *Sporothrix*; chromoblastomycosis due to *Cladosporium*, *Fonsecaea*, and *Phialophora*; phaeohyphomycosis due to *Bipolaris*, *Cladosporium*, *Curvularia*, *Exophiala*, and *Exserohilum*; eumycetoma due to *Acremonium*, *Madurella*, and *Pseudallescheria*; subcutaneous zygomycosis due to *Basidiobolus* and *Conidiobolus*; rhinosporidiosis due to *Rhinosporidium*; and lacaziosis (or lobomycosis) due to *Lacazia loboi*.

Systemic mycoses: Some fungi, especially dimorphic fungi, have the capacity to breach the physical and immunological defenses of the human host, causing pulmonary and other infections after the inhalation of conidia. Examples of such systemic mycoses include histoplasmosis due to *Histoplasma capsulatum*; coccidioidomycosis due to *Coccidioides immitis*; blastomycosis due to *Blastomyces dermatitidis*; and paracoccidioidomycosis due to *Paracoccidioides brasiliensis* [7].

1.3 PHENOTYPIC CHARACTERIZATION

Due to the fact that clinical presentations of human mycoses caused by various fungal species are nonspecific and indistinguishable, and that different fungal pathogens demonstrate varied resistance to commonly used antifungal drugs, there is a need to identify the causative agents to genus and species level in order to implement effective control and prevention strategies.

Traditionally, laboratory identification and characterization of fungi rely mainly on morphological (e.g., the size and shape of spores or fruiting structures), biochemical (e.g., the ability to metabolize certain biochemicals, or the reaction to chemical tests), biological (e.g., the ability to mate), and other phenotypic criteria. Apart from some mycotic/hyphal elements, most fungi present in the clinical samples are impossible to distinguish upon direct microscopic examination. Therefore, in vitro cultivation is vital for isolation of the fungal pathogens of interest, permitting subsequent determination on the basis of distinct colonial (macroscopic) and microscopic features [19–21].

1.3.1 Sample Collection and Processing

1.3.1.1 General Guidelines for Specimen Handling

Fungal pathogens are capable of spreading through spores and may pose danger to laboratory personnel if sufficient caution is not heeded. Therefore, when dealing with fungal specimens in laboratory, it is essential to (i) wear a protective gown or laboratory coat while in the laboratory, (ii) wear gloves when handling clinical and culture materials, (iii) transport cultures in a rack or canister, (iv) disinfect specimen containers contaminated on the outside by wiping with gauze before opening, (v) open specimens in laminar flow safety cabinet, (vi) use mechanical pipetting devices for any material or reagent, and (vii) clean the work area with a 2% amphyl solution when work is completed.

A diverse range of clinical and environmental samples can be utilized for fungal testing. In order to ensure accurate and consistent results, samples intended for mycological investigations need to be collected and processed in such a way that offers the best chance for isolation and identification of causative fungal agents.

1.3.1.2 Sputum, Bronchial Washings, and Throat Swabs

Collection: (i) Collect sputum (5–10 mL, as a result of a deep cough not saliva) in sterile container in the early morning. Patients are advised not to eat before sputum collection. Thick sputum can be emulsified by the addition of 12–20 sterile glass beads and 3–5 mL of sterile distilled water followed by shaking. (ii) Collect bronchial washings (tracheal lavage or bronchial lavage) aseptically by physicians. (iii) Obtain throat specimens by rolling a moist sterile swab over the affected area. For suspected *Candida* specimen, scrape the affected area with a sterile tongue depressor. (iv) Store samples at 4°C in case of short delays in processing. *Processing*: (i) Make wet mounts in KOH (1 drop) and Gram stained smears (1 drop) for direct microscopy. Use periodic acid-Schiff (PAS) stain if KOH preparation is unsatisfactory. (ii) Inoculate sample onto Sabouraud's dextrose agar with chloramphenicol and gentamicin and incubate duplicate cultures at 26°C and 35°C. (iii) Inoculate sample onto brain heart infusion agar (BHIA) supplemented with 5% sheep blood and incubate at 35°C. Maintain cultures for 4 weeks.

1.3.1.3 Blood, Bone Marrow, and Body Fluids

Collection: (i) Collect blood (8–10 mL aseptically using vacutainer tube [#4960, containing 1.7 mL of 0.35% sodium polyanethol sulfonate (SPS) as an anticoagulant]). Clean the collection site with a disinfectant at the time of collection. (ii) Collect bone marrow and body fluids (pleural, synovial, and peritoneal) aseptically by physicians. Add SPS or heparin as an anticoagulant. *Processing*: (i) Prepare smears for Giemsa, Gram, and PAS staining. (ii) Inoculate 0.5–1.0 mL of buffy coat (after centrifugation of 5–8 mL of blood) onto the surface of culture media (Sabouraud's dextrose agar with chloramphenicol and gentamicin, and BHIA supplemented with 5% sheep blood) with a loop, or 1 part blood to 10–20 parts brain/heart infusion broth. (iii) Inoculate bone marrow and body fluids (pleural, synovial, and peritoneal) on culture media. Maintain cultures at 26°C and 35°C for 4 weeks.

1.3.1.4 Pus, Exudate, and Drainage

Collection: (i) Aspirate material from undrained abscesses using a sterile needle and syringe. (ii) Express pus using a sterile, sharp-pointed scalpel. (iii) Place the material in a sterile container.

1.3.1.5 Vaginal Swabs

Collection: (i) Collect material from the vagina using several sterile swabs. (ii) Insert swabs into a sterile tube. *Processing:* Smear swab onto heat-sterilized glass slide for Gram stain.

1.3.1.6 Urine

Collection: (i) Collect an early morning, mid-stream catch of >2.0 mL (do not process more than 50.0 mL) in a sterile container when aspiration or cystoscopy cannot be done. (ii) Store urine at 4°C for up to 12–14 h if necessary. *Processing*: (i) Centrifuge the urine for 10–15 min at 2000 rpm. Decant the supernatant and pool the sediment if necessary. (ii) Prepare a direct smear of the sediment in KOH for direct microscopy. PAS, Gram, or India ink preparations may also be helpful.

1.3.1.7 Cerebrospinal Fluid

Collection: (i) Collect 2–5 mL CSF aseptically by clinician. (ii) Leave CSF at room temperature or incubate at 30°C if there is a delay in processing. (iii) Centrifuge CSF and process the sediment as follows. *Processing*: (i) Use 1 drop of the sediment to make an India ink mount. (ii) Resuspend the remaining sediment in 1–2 mL of CSF and inoculate onto Sabouraud's dextrose agar with chloramphenicol and gentamicin and incubate at 26°C and 35°C. (iii) Inoculate sediment onto BHIA supplemented with 5% sheep blood and incubate at 35°C. Maintain cultures for at least 4 weeks.

1.3.1.8 Tissue Biopsies from Visceral Organs

Collection: (i) Collect tissue from the center and edge of the lesion aseptically by clinician. Include normal tissue for comparison. (ii) Keep a portion of the tissue in formalin for rapid frozen sectioning with staining by hematoxylin and eosin (H&E), Grocott's methenamine silver (GMS), and PAS. (iii) Keep tissue samples moist with sterile water, saline, or BHI broth. Do not refrigerate at 4°C for more than 8–10 h. *Processing*: (i) Tease apart tissue specimens aseptically in a sterile Petri dish. (ii) Perform a smear for direct microscopic examination with staining by H&E, GMS, and PAS (as well as Gram stain, Ziehl Neelsen stain, and modified Ziehl Neelsen stain if necessary) and inoculate directly onto the isolation media, if areas of pus and necrosis are present. (iii) Mince tissue specimen with a sterile scalpel blade, or grind in a sterile glass tissue grinder, if no areas of pus or necrosis are present, and inoculate the minced or homogenized material onto the isolation media. (iv) Inoculate onto Sabouraud's dextrose agar with chloramphenicol and gentamicin and incubate duplicate cultures at 26°C and 35°C; (v) Inoculate onto BHIA supplemented with 5% sheep blood and incubate at 35°C. Maintain cultures for 4 weeks.

1.3.1.9 Nail, Hair, Skin Scraping, and Swabs

Collection: For nail, (i) clean nail with 70% alcohol. (ii) Scrape outer dorsal plate surface and discard. (iii) Scrape the deeper portion and remove a portion of debris from under the nail with a scalpel. (iv) Collect whole nail or nail clippings. (v) Place all material in a clean envelope labeled with the patient's data. For hair, (i) Select infected areas and with forceps, epilate at least 10 hairs. (ii) Use a scalpel or a blade knife for hairs broken off at the scalp level. (iii) Place hairs between two clean glass slides or in a clean envelope labeled with the patient's data. For skin scraping and swabs, (i) wipe lesions and interspaces between the toes with alcohol sponge or sterile water. (ii) Scrape the entire lesion and both sides of interspaces with a sterile scalpel and place scrapings between two clean glass slides or place in a clean envelope labeled

with the patient's data. (iii) Swab the lesion with a sterile swab (wetted in distilled water if necessary) and place the swab into a clean tube. *Processing*: (i) For nail, hair, and skin scrapings, make a wet mount preparation in KOH for direct microscopy. Calcofluor-stained mount may also be useful. (ii) For skin swabs, smear swab onto heat-sterilized glass slide for Gram stain. (iii) Inoculate skin scraping and swab specimens onto Sabouraud's dextrose agar slopes containing chloramphenicol and gentamicin, but NO cycloheximide and incubate at 35°C. (iv) For suspected secondary bacterial infection, inoculate the swab onto a blood agar plate, followed by the Sabouraud's agar containing the antibiotics and then place into brain heart infusion broth. Incubate at 35°C. Maintain the cultures for 4 weeks.

1.3.2 Microscopic Examination

In addition to macroscopic assessment of colonial size, shape, and color, direct microscopic observation of mycotic elements in clinical specimens and subsequent examination of fungal structures of cultured isolates are critical for correct identification of causal fungal agents and accurate diagnosis of mycoses. Direct microscopy not only facilitates the selection of the proper portion of clinical specimen, the appropriate media, and inoculation techniques for enhanced recovery of the fungus, but also alerts the physician as to the likely etiology of the disease. In general, all fungal specimens of sufficient quantity are examined microscopically and inoculated on culture media. However, when the quantity of a fungal sample is insufficient, culture should take priority over direct microscopy due to its higher sensitivity.

A number of stains (e.g., KOH and its derivatives, lactophenol cotton blue, India ink, and Southgate's mucicarmine stain) can be applied for improved identification and recognition of mycotic elements in clinical samples and structural details of fungal isolates (Table 1.2) [22,23]. Ocular lens containing a micrometer disc may be employed on light microscope for the measurement of hyphae, conidia, and other fungal structures. For detection of fungal elements in tissue biopsies, PAS stain, GMS stain, Fontana-Masson stain, Gridley's stain, and H&E may be utilized. In particular, GMS stain represents an essential stain for detection of fungal elements in tissue sections. Fontana-Masson stain is indispensable for detection of melanin in the cell walls of dematiaceous fungi. Besides standard light or phase-contrast microscopy, transmission electronic microscopy (TEM) enables visualization of fine structural details (e.g., the outer wall layers of conidia and ascospores) of fungal organisms, leading to more accurate speciation.

Cellotape flag preparation may be utilized for rapid mounting and keeping intact of the reproductive structures of sporulating fungi. This is performed by (i) using clear 2 cm wide cellotape and a wooden applicator stick (orange stick) to make a small cellotape flag (2 × 2 cm), (ii) using sterile technique to gently press the sticky side of the flag onto the surface of the culture, (iii) applying a drop of 95% alcohol to the flag to act as a wetting agent and also to dissolve the adhesive glue holding the flag to the applicator stick, (iv) placing the flag onto a small drop of lactophenol cotton blue on a clean glass slide, removing the applicator stick and discarding, and (v) adding another drop of stain, covering with a coverslip, gently pressing and moping up any excess stain before microscopy [7].

For macroscopic examination of colonial morphology, attention should be paid to (i) surface texture (e.g., glabrous, suede-like, powdery, granular, fluffy, downy, cottony, and velvety); (ii) surface topography (e.g., flat, raised, heaped, folded, domed, radial, and grooved); (iii) surface pigmentation (e.g., white, cream, yellow, brown, green, grey, and black); (iv) reverse pigmentation (e.g., none, yellow, brown, red, and dark); (v) growth rate (e.g., fast, moderate, and slow); and (vi) growth temperature (e.g., 25°C, 37°C, 40°C, and 45°C). For microscopic assessment of cultured isolates, the morphologic characteristics of conidia are recognized in terms of septation, shape (e.g., spherical, pyriform, clavate, and ellipsoidal), size, color (hyaline and darkly pigmented), surface texture (e.g., smooth, rough, verrucose, and echinulate), type (microconidia and macroconidia), and arrangement (e.g., single, in mass) [7].

1.3.3 In Vitro Cultivation

In vitro cultivation remains a critical step for the phenotypic characterization of fungi. Macroscopic examination of colonial size, shape, and color followed by microscopic investigation of final structures of fungal isolates allows proper determination of fungal organisms implicated in human mycoses in most cases. Further characterization of fungal isolates is possible by using various biological and biochemical techniques as well as antifungal drug resistance testing. The composition, preparation, and application of common mycological media are summarized in Table 1.3.

Slide culture preparation allows observation of the precise arrangement of the conidiophores and conidial ontogeny (the way the conidia are produced). This is conducted by (i) using a sterile blade to cut out an agar block (7 × 7 mm) from a plate of cornmeal agar or Czapek dox agar that is small enough to fit under a coverslip, (ii) flipping the block up onto the surface of the agar plate, (iii) inoculating the four sides of the agar block with spores or mycelial fragments of the fungus, (iv) placing a flamed coverslip centrally upon the agar block, (v) incubating the plate at 26°C until growth and sporulation occur, (vi) removing the cover slip from the agar block, (vii) applying a drop of 95% alcohol as a wetting agent, (viii) gently lowering the coverslip onto a small drop of lactophenol cotton blue on a clean glass slide, (ix) leaving the slide overnight to dry and resealing later with fingernail polish, and (x) applying a coat of clear polish followed by one coat of red-colored polish before microscopy [7].

Apart from in vitro culture, in vivo animal models (e.g., rodents and rabbits) have been occasionally applied to compare diagnostic procedures for the estimation of fungal burdens in blood, bronchoalveolar lavage (BAL), and tissue samples.

TABLE 1.2
Composition, Preparation, and Application of Common Mycological Stains

Stain	Composition	Preparation	Application
15% KOH	KOH 15 g Glycerol 20 mL Distilled water 80 mL	1. Place a portion of specimen onto a clean glass microscope slide. 2. Add a drop of 15% KOH to specimen and mix. 3. Pace a cover glass over the preparation. 4. Leave slide at room temperature until the material is cleared. The slide may be warmed for speedy clearing. 5. Observe slide under microscopy. 6. Slides that appear negative for fungi may be reexamined the following day.	For direct microscopic examination of skin scrapings, hairs, nails, and other clinical specimens for fungal elements.
10% KOH with Calcofluor white	Solution A KOH 10 g Glycerin 10 mL Distilled water 90 mL Solution B Calcofluor white (fluorescent brightener 28, F6259, Sigma) 0.1 g Distilled water 100 mL	1. Mix one drop of solution A and a drop of solution B on the center of a clean microscope slide. 2. Place specimen in the solution and cover with a coverslip, squash the preparation with the butt of the inoculation needle and then blot off the excess fluid. 3. Gently heat the slide and examine microscopically for the presence of fungal elements that fluoresce a chalk-white or brilliant apple green color, depending on the filter combination.	For direct microscopic examination of skin scrapings, hairs, nails, and other clinical specimens for fungal elements. Calcofluor white (or Uvitex 2B, Blankophor) binds to cellulose and chitin and fluoresces blue-white or apple-green when exposed to ultraviolet light.
KOH with Parker Ink	KOH 10 g Glycerol 10 mL Parker Quink permanent blue ink 10 mL Distilled water 80 mL (dissolve the KOH in water; add glycerol and Parker ink. The glycerol prevents crystallization of the reagent and prevents the specimen from drying out).	1. Remove a portion of specimen with an inoculation needle and mount in a drop of KOH on a clean microscope slide. 2. Cover with a coverslip, squash the preparation with the butt of the inoculation needle, and then blot off the excess fluid. 3. Gently heat by passing through a flame two or three times. 4. When the specimen is cleared (from 20 min for skin scrapings to several hours for nail scrapings), examine microscopically for faintly blue-stained fungal elements.	For direct microscopic examination of skin scrapings, hairs, nails, and other clinical specimens for fungal elements. Keep negative specimens and reexamine the next day to avoid reporting false-negative. Preparations may be kept until culture result is known.
KOH-DMSO preparation	Dimethyl sulfoxide (DMSO) 40 mL Distilled water 60 mL KOH 10 g	1. Remove a small portion of the specimen with an inoculation needle and mount in a drop of KOH-DMSO on a clean microscope slide. 2. Cover with a coverslip, squash the preparation with the butt of the inoculation needle, and then blot off the excess fluid. Do not heat the preparation. 3. Examine the mount within 20 min microscopically for unstained refractile fungal elements.	For direct microscopic examination of skin scrapings, hairs, and nails for fungal elements. DMSO gives more rapid maceration and clearing, but preparations do not keep long.
Lactophenol cotton blue	Cotton blue (Aniline blue) 0.05 g Phenol crystals ($C_6H_5O_4$) 20 g Glycerol 40 mL Lactic acid ($CH_3CHOH\ COOH$) 20 mL Distilled water 20 mL	1. Dissolve the cotton blue in the distilled water overnight; 2. Add phenol crystals to the lactic acid in a glass beaker, mix to dissolve, and then add glycerol; 3. Filter the cotton blue and distilled water solution into the phenol/glycerol/lactic acid solution, mix, and store at room temperature.	For direct microscopic examination of skin scrapings, hairs, nails, and other clinical specimens for fungal elements.

India Ink	India Ink (colloidal carbon)	Place a drop of India Ink on the specimen, mix well with a sterilized loop, and cover with a coverslip.	For direct microscopic examination of *Cryptococcus neoformans* and other encapsulated fungi in a cell suspension (e.g., CSF sediment).
Southgate's Mucicarmine stain	Carmine 1 g Aluminium hydroxide 1 g 50% alcohol 100 mL (mix above by shaking) Aluminium chloride (anhydrous) 0.5 g (boil in water bath for 2–3 min, cool, make up to original volume with 50% alcohol and filter. The stain is stable for a few months).	1. Take sections to water. 2. Stain nuclei with hematoxylin. 3. Differentiate in acid-alcohol and blue in tap water. 4. Stain with Southgate's mucicarmine solution for 30 min and rinse in distilled water. 5. Dehydrate, clear, and mount.	For detection of capsular material in *Cryptococcus neoformans*. Mucicarmine stains acidic mucins pink.
Periodic acid-Schiff (PAS) and PAS digest stain	1% periodic acid (50%) Periodic acid 2 mL Distilled water 98 mL Schiff's reagent Basic fuchsin (C.I. 42500) 1 g Potassium metabisulfite 2 g Distilled water 200 mL HCl concentrate 2 mL Deactivated charcoal 1–2 g (add basic fuchsin slowly to boiling distilled water, mix, and cool to 50°C. Add potassium metabisulfite, mix, and cool to room temperature before adding HCl. Keep in the dark overnight. Add charcoal and filter through coarse filter paper, then fine filter paper. Store in fridge).	1. Take sections to water. 2. For PAS digest only, digest glycogen with saliva for 15 min, wash in water. 3. Treat with 1% periodic acid for 5 min, rinse in water. 4. Treat with Schiff's reagent for 10 min, wash in running tap water for 5 min (to help develop the color). 5. Counterstain nuclei lightly with Mayer's hematoxylin for 1 min, wash, and "blue up" in Li2Co3. 6. Dehydrate, clear, and mount.	For demonstration of glycogen and neutral mucins, and for detection of fungal elements in tissue sections. As a counter stain, PAS reveals the background host cellular detail, tissue architecture, and inflammatory response. PAS-positive material appears magenta; nuclei appear blue; PAS digest material appears colorless.
Gomori's methenamine silver stain (GMS) [or Grocott-Gomori silver stain]	5% aqueous chromic acid 1% aqueous sodium bisulfite 5% aqueous borax 0.1% aqueous gold chloride 2% aqueous sodium thiosulfate Stock methenamine silver solution (add 5 mL of 5% silver nitrate to 100 mL of 3% hexamine, mix, and keep at 4°C for 2 months). Working silver solution (filter before use). (stock methenamine silver solution 25 mL, distilled water 25 mL, 5% borax 1–2 mL)	1. Take sections to water. 2. Oxidize in 5% chromic acid for 1 h and wash in running tap water for 10 min. 3. Treat with sodium bisulfite for 1 min to remove any residual chromic acid, and wash in tap water then distilled water. 4. Place section in the working silver solution at 60°C in a water bath and rinse in distilled water. 5. Tone in 0.1% gold chloride for 5 min and rinse in distilled water. 6. Treat with 2% sodium thiosulfate for 1–2 min to remove unreduced silver and wash thoroughly. 7. Counterstain with light green. 8. Dehydrate, clear, and mount.	An essential stain for detection of fungal elements in tissue sections. As a counter stain, GMS removes the fine details of background host cells and tissues, but provides a more sensitive stain for detecting small fragments of cell wall. Fungi stain black; cell walls stain brown to black; background stains pale green.
Fontana-Masson stain	10% silver nitrate 0.1% gold chloride 5% hypo Nuclear-Fast Red	1. Deparaffinize and hydrate to distilled water. 2. Add 10% silver nitrate, place in 60°C oven for 1 h, and rinse in distilled water. 3. Add 0.1% gold chloride, leave for 10 min, and rinse in distilled water. 4. Add 5% hypo, leave for 5 min, wash in tap water, and rinse in distilled water. 5. Add nuclear-Fast Red, leave for 5 min, and wash in tap water. 6. Dehydrate, clear, and coverslip.	For staining cell walls of dematiaceous fungi and *Cryptococcus neoformans* in tissue sections. Melanin, argentaffin cells stain black; nuclei stain red; background stains pale pink.

(*continued*)

TABLE 1.2 (continued)
Composition, Preparation, and Application of Common Mycological Stains

Stain	Composition	Preparation	Application
Gridley's stain	Chromic acid Metabisulfite bleach Schiff's reagent Aldehyde fuchsin Metanil yellow	1. Bring sections to water via xylene and ethanol. 2. Place in chromic acid for 1 h, wash with tap water. 3. Treat with the metabisulfite bleach for 1 min, wash with tap water, and rinse with distilled water. 4. Place in Schiff's reagent for 20 min, wash with tap water, and rinse with 70% ethanol. 5. Place in aldehyde fuchsin 30 min, rinse off excess with 95% ethanol, and wash with tap water. 6. Counterstain with metanil yellow for 1 min, and rinse with distilled water. 7. Dehydrate, clear and mount in a resinous medium.	For enhanced visualization of fungi and their morphology in tissue sections. Cell walls stain purple to magenta; yeasts stain rose to purple; capsules stain deep purple; background stain yellow.
Hematoxylin and eosin	Alum hematoxylin 0.3% acid alcohol Scott's tap water substitute Eosin/phloxine	1. Bring sections to distilled water. 2. Stain nuclei with alum hematoxylin, rinse in running tap water. 3. Differentiate with 0.3% acid alcohol, rinse in running tap water, then in Scott's tap water substitute, and again in tap water. 4. Stain with eosin/phloxine for 2 min. 5. Dehydrate, clear, and mount.	For visualization of host response to fungus in tissue sections. Nuclei stain blue, cartilage; calcium deposits stain dark blue; cytoplasm and other components stain shades of red; erythrocytes stain bright red.

Sources: McGinnis, M.R., *Laboratory Handbook of Medical Mycology*, Academic Press, New York, 1980; Schwarz, J., *Human Pathol.*, 13, 519, 1982; Koneman, E.W. and Roberts, G.D., *Practical Laboratory Mycology*, The Williams and Wilkins Co., Baltimore, MD, 1985.

TABLE 1.3
Composition, Preparation, and Application of Common Mycological Media

Medium	Composition	Preparation	Application
Potato dextrose agar (PDA)	Potato infusion 200 g Dextrose 20 g Bacto agar 15 g Distilled water 1000 mL (potato infusion, dextrose, and Bacto agar may be replaced with 39 g PDA, Oxoid CM139).	1. Mix ingredients in 1000 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes as required. 4. Autoclave at 121°C for 15 min; slope or pour for plates as required.	For routine cultivation and identification of fungi.
Cornmeal agar (CA)	CA (BD) 8.5 g Distilled water 500 mL	1. Mix agar in 500 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes as required. 4. Autoclave at 121°C for 10 min; slope or pour for plates as required.	For routine cultivation and identification of fungi.
Oatmeal agar (OA)	Oatmeal (Difco) 60 g Agar 12.5 g Distilled water 1000 mL (oatmeal and agar may be replaced with 72.5 g OA, Difco 255210).	1. Mix oatmeal and agar in 1000 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes as required. 4. Autoclave at 121°C for 15 min; slope or pour for plates as required.	For routine cultivation and identification of fungi.
Malt extract agar (MEA)	Malt extract 20 g Peptone 1 g Dextrose 20 g Bacto agar 15 g Distilled water 1000 mL (malt extract, peptone and dextrose may be replaced with 20 g malt extract, Oxoid L39)	1. Mix malt extract, peptone and dextrose in 1000 mL water (adjust to pH 6.5 with NaOH if necessary). 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes as required. 4. Autoclave at 121°C for 10 min, slope or pour for plates as required.	For routine cultivation and identification of fungi. MEA is a useful alternative to bread and BHI broth for recovery of zygomycetes.
Inhibitory mould agar (IMA)	Pancreatic digest of casein 3.0 g Sodium phosphate 2.0 g Peptic digest of animal tissue 2.0 g Magnesium sulfate 0.8 g Yeast extract 5.0 g Ferrous sulfate 0.04 g Dextrose 5.0 g Sodium chloride 0.04 g Starch 2.0 g Manganese sulfate 0.16 g Dextrin 1.0 g Agar 15.0 g Chloramphenicol 0.125 g Distilled water to 1000 mL	IMA is an enriched medium with inorganic salts, chloramphenicol, and gentamicin and can be obtained commercially.	For isolation of fungi and inhibition of bacteria.

(*continued*)

TABLE 1.3 (continued)
Composition, Preparation, and Application of Common Mycological Media

Medium	Composition	Preparation	Application
1% Peptone agar (PA)	Bacto peptone (BD) 5 g Bacto agar (BD) 10 g Distilled water 500 mL	1. Mix agar and peptone in 500 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes as required. 4. Autoclave at 121°C for 10 min; slope or pour for plates as required.	For cultivation and differentiation of fungi.
Sabouraud's dextrose agar (SDA) with chloramphenicol and gentamicin	SDA (Oxoid CM41) 65 g Chloramphenicol 1× 250 mg capsule Distilled water 1000 mL Gentamicin (40 mg/mL) 0.65 mL	1. Mix SDA and chloramphenicol in 1000 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Add gentamicin, mix, dispense for slopes as required. 4. Autoclave at 121°C for 10 min; slope, or pour for plates as required.	For primary isolation and cultivation of yeasts and moulds.
SDA with cycloheximide, chloramphenicol, gentamicin, and yeast extract.	SDA (Oxoid CM41) 65 g Cycloheximide (actidione) 0.5 g Chloramphenicol 1× 250 mg capsule Yeast extract 5 g Distilled water 1000 mL Gentamicin (40 mg/mL) 0.65 mL	1. Mix the first four ingredients in 1000 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Autoclave at 121°C for 10 min and cool to 50°C. 4. Add gentamicin, dispense for slopes, or plates as required.	For primary isolation and cultivation of dermatophytes. Mycosel (BBL) and mycobiotic (Difco) agars (containing SDA, 1% glucose, chloramphenicol, and cycloheximide) are commercially available.
SDA with 5% NaCl	SDA (Oxoid CM41) 32.5 g NaCl 25 g Distilled water 500 mL	1. Mix agar and NaCl in 500 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes as required. 4. Autoclave at 118°C for 10 min; slope or pour for plates as required.	For the cultivation and differentiation of dermatophytes especially *T. rubrum* from *T. mentagrophytes*.
Dermatophyte test medium (DTM)	Papaic digest of soybean meal 10.0 g Dextrose 10.0 g Phenol red 0.2 g Cycloheximide 0.5 g Agar 20.0 g Distilled water 1000 mL	1. Mix ingredients to 1000 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Autoclave at 121°C for 15 min.	For recovery of dermatophytes from heavily contaminated clinical specimens and for presumptive indication of the presence of a dermatophyte. Dermatophytes and a few other fungi/bacteria turn medium from pink to red.
Trichophyton agar nos. 1–7	Trichophyton agar nos. 1–7 (BD) 11.8 g Distilled water 200 mL	1. Mix agar in water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes, autoclave at 118°C for 10 min, and slope.	For differentiation of *Trichophyton* species. (i) *Trichophyton mentagrophytes* grows well on agar nos. 1,2,3, and 4 (ii) *Trichophyton tonsurans* grows well on agar nos. 3 and 4; poorly on agar nos. 1 and 2. (iii) *Trichophyton verrucosum* grows well on agar nos. 2 and 3; poorly on agar nos. 1 and 4.
Lactrimel agar (LA)	Dutch Jug skimmed milk powder 7 g Glycine 10 g Honey 10 g CA (BD) 17 g Chloramphenicol 1× 250 mg capsule Distilled water 1000 mL	1. Mix milk powder with 150 mL water, add other ingredients, and 850 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes as required. 4. Autoclave at 115°C for 10 min, slope, or pour plates as required.	For production of pigment by *Trichophyton rubrum*.

Hair perforation test (HPT)	Autoclaved pre-pubital hair (blonde if available) cut into short pieces (1 cm) Sterile distilled water 5 mL in vial	1. Place autoclaved hair in a vial containing 5 mL sterile water. 2. Inoculate with small fragments of the test fungus and incubate at room temperature. 3. Remove individual hairs at intervals up to 4 weeks and examine microscopically in lactophenol cotton blue.	For differentiation of dermatophytes. *Trichophyton mentagrophytes* and its variants produce marked localized areas of pitting and marked erosion whereas *T. rubrum* does not.
Urease agar (UA) with 0.5% glucose	Bacto agar (BD) 1.5 g Distilled water 91 mL Sterile urea solution (Oxoid SR20) 5 mL 10% sterile glucose solution 4 mL	1. Autoclave agar in 100 mL bottle at 115°C for 20 min and cool to 50°C. 2. Add aseptically 5 mL of sterile urea solution and 4 mL of 10% sterile glucose solution. 3. Dispense for slopes.	For differentiation of dermatophytes.
Rice grain slopes (RGS)	Rice 1/2 teaspoon Distilled water 8 mL	1. Add 1/2 teaspoon rice grains into wide neck 20 mL vials containing 8 mL distilled water to each vial. 2. Close lid, autoclave at 121°C for 15 min, and slope.	For induction of sporulation and for differentiation of *M. audouinii*, *M. canis*, and *M. distortum*.
Littman oxgall agar (LOA)	LOA (BD) 27.5 g Distilled water 500 mL	1. Mix agar in 500 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes as required. 4. Autoclave at 121°C for 10 min; slope or pour for plates as required.	For routine inoculation of specimens from skin, nails, and hair, etc.
CGB (L-canavanine, glycine, 2 bromthymol blue) agar	Solution A Glycine 10 g KH_2PO_4 1 g $MgSO_4$ 1 g Thiamine HCl 1 mg L-canavanine sulfate 30 mg Distilled water 100 mL Solution B Bromthymol blue 0.4 g 0.01 N NaOH 64 mL Distilled water 36 mL CGB agar Bacto agar 20 g Solution B 20 mL Distilled water 880 mL Solution A 100 mL	Solution A 1. Dissolve ingredients in beaker and adjust to pH 5.6. 2. Filter sterilize solution using 0.22 µm filter; store in refrigerator. Solution B 1. Dissolve the bromthymol blue in 64 mL of 0.01 N NaOH. 2. Add 36 mL water. CGB agar 1. Mix 20 g agar and 20 mL solution B, autoclave to 121°C for 15 min, and cool to 48°C. 2. Add 100 mL of the filtered solution A, mix, and dispense in plates.	For differentiation between *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gattii*.
Brain-heart infusion agar (BHIA) with 5% sheep blood	BHIA (Oxoid) 52 g Chloramphenicol 1× 250 mg capsule Distilled water 1000 mL Sheep blood 50 mL Gentamicin (40 mg/mL) 0.65 mL	1. Mix BHI agar and chloramphenicol in 1000 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Autoclave at 121°C for 15 min, cool to 50°C. 4. Add sheep blood and gentamicin, dispense for slopes, or plates as required.	For recovery of *Cryptococcus neoformans* from sterile specimens such as CSF, and for yeast-mould conversions of *Sporothrix* and *Paracoccidioides*. BHIA is an alternative to chocolate agar for isolation of fungi and bacteria, and used routinely by some ophthalmologists for corneal scrapings. Ready-to-use BHIA is commercially available.
Brain heart infusion (BHI) broth	BHI agar (Oxoid) 52 g Distilled water 1000 mL Penicillin G (20 U/mL) 1 mL	Aseptic addition of sterile penicillin to BHI broth inhibits bacteria.	BHI broth with penicillin is useful for isolation of zygomycetes.

(*continued*)

TABLE 1.3 (continued)
Composition, Preparation, and Application of Common Mycological Media

Medium	Composition	Preparation	Application
Dixon's agar (DA)	Malt extract (Oxoid L39) 18 g Peptone (BDH 44075) 18 g Bacto agar (BD) 7.25 g Ox-bile desiccated (Oxoid L50) 10 g Tween 40 5 mL Glycerol monooleate 2.5 mL Distilled water 500 mL	1. Mix ingredients in 500 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes. 4. Autoclave at 121°C for 10 min and then slope.	For primary isolation and cultivation of *Malassezia furfur*.
Czapek dox agar (CDA)	CDA (Oxoid CM97) 45.4 g Distilled water 1000 mL	1. Mix agar in 1000 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes as required. 4. Autoclave at 121°C for 10 min, slope or pour for plates as required.	For routine cultivation of fungi, especially *Aspergillus*, *Penicillium*, and non-sporulating moulds.
Bird seed agar	Niger seed (*Guizotia abyssinica*) 50 g Glucose 1 g KH_2PO_4 1 g Creatinine 1 g Bacto agar (BD) 15 g Distilled water 1000 mL Penicillin G (20 U/mL) 0.5 mL per 500 mL Gentamicin (40 mg/mL) 0.5 mL per 500 mL	1. Grind *Guizotia abyssinica* seeds finely and add to 1000 mL distilled water. 2. Boil for 30 min, pass through filter paper and adjust volume to 1000 mL. 3. Add the remaining ingredients except Bacto agar to filtrate and dissolve. 4. Cool to room temperature, adjust to pH 5.5, and dispense into 500 mL bottles. 5. Add 7.5 g Bacto agar to each 500 mL reagent bottle and autoclave at 110°C for 20 min. 6. Cool to 48°C, add 0.5 mL Penicillin G and 0.5 mL gentamicin to each 500 mL, mix, and pour into 90 mm plastic Petri dishes.	For selective isolation of *Cryptococcus neoformans*.
Cornmeal glucose sucrose yeast extract agar	CA (BD) 17 g Dextrose (glucose) 2 g Sucrose 3 g Yeast extract (Difco) 1 g Distilled water 1000 mL	1. Mix dry ingredients into 1000 mL water. 2. Heat with frequent stirring, bring to boil for 1 min. 3. Dispense for slopes. 4. Autoclave at 120°C for 10 min and slope.	For sporulation of some zygomycetes, such as *Saksenaea* and *Apophysomyces*.
Sterile bread	Bread Glass Petri dish	1. Sterilize a piece of bread in a humidified glass Petri dish. 2. Inoculate specimens from non-contaminated sites directly; treat contaminated specimens with antibacterial agents before inoculation.	Sterile bread without preservatives is superior to other media for recovery of zygomycetes from clinical specimens.

Sources: McGinnis, M.R., *Laboratory Handbook of Medical Mycology*, Academic Press, New York, 1980; Schwarz, J., *Human Pathol.*, 13, 519, 1982; Koneman, E.W. and Roberts, G.D., *Practical Laboratory Mycology*, The Williams and Wilkins Co., Baltimore, MD, 1985.

1.3.4 Biochemical and Antifungal Testing

Many fungi demonstrate varied tolerance to temperature, which can be exploited as a complementary tool in the differentiation of dematiaceus fungi. Examination of primary metabolites such as ubiquinones (coenzyme Q) has proven useful for the taxonomy of black yeasts and filamentous fungi. Secondary metabolites (e.g., steroids, terpenes, alkaloids, cyclopeptides, and coumarins) produced by fungal organisms may be examined by thin-layer chromatography. The resulting pattern of secondary-metabolite production provides a reliable approach for identification and classification of lichens. Using pyrolysis gas chromatography, pyrolysis mass spectrometry, gas chromatography, and partition aqueous polymer two-phase systems, the cellular fatty acid composition of fungi can be determined, which represents another useful means for differentiating filamentous fungi. In addition, the structure and composition of the cell wall may be targeted for the definition of fungi. For example, chitin and glucan are present in ascomycetes and basidiomycetes whereas chitosan and polyglucuronic acid are found in zygomycetes. The presence or absence of polysaccharides (e.g., fucose, galactose, rhamnose, and xylose) in the walls of yeast cells allows their differentia on. Further, isoenzyme patterns generated by electrophoretic techniques (zymograms) enable the determination of generic relationships of fungi [9]. Various serological tests have been also described for specific detection of fungal antigens in clinical specimens. Matrix-assisted laser desorption/ionization time-of-flight intact cell mass spectrometry (MALDI-TOF ICMS) has been used to identify fungal organisms, including (i) the terverticillate penicillia, (ii) aflatoxigenic, black, and other aspergilli, (iii) *Fusarium*, (iv) *Trichoderm*a, (iv) wood rotting fungi (e.g., *Serpula lacrymans*), and (v) dermatophytes [24]. Moreover, assessment of the sensitivity of fungal isolates to various antifungal drugs offers an additional way to their discrimination as well as treatment [25–27].

1.4 GENOTYPIC CHARACTERIZATION

Phenotypic characterization of fungal organisms on the basis of morphological, biological, and biochemical features suffers from the drawbacks of laborious, time-consuming, and variable, especially for poorly differentiated filamentous fungi. For improved taxonomic resolution and epidemiological study of fungal organisms, molecular techniques detecting the nucleic acids have been increasingly utilized [28–38].

1.4.1 Nucleic Acid Purification

Due to the presence of a tough cell wall in fungi, it is often necessary to undertake several steps to purify the nucleic acids before molecular testing becomes feasible. These include (i) disruption of cell walls, (ii) denaturation of nucleoprotein complexes, (iii) inactivation of endogenous DNase/RNAse, and (iv) removal of contaminating proteins, polysaccharides, polyphenolic pigments, and other compounds.

Grinding lyophilized or fresh mycelia in liquid nitrogen with a mortar and pestle represents a common way to disrupt the fungal cell walls. Because of its time-consuming and laborious nature and its potential for cross-contamination between samples, this technique is not suitable for dealing with a large number of samples. Another means to mechanically break the fungal cell walls is through the use of glass beads with a vortex mixer. In addition, sonicator may be employed for disruption of fungal cell walls. Alternative methods to disrupt the fungal cell walls include enzymatic digestion (using a combination of lyticase, zymolase, chitinase, gluculase, and proteinase K), acid, and alkali treatments. Subsequent treatment with organic solvents (e.g., phenol/chloroform) and detergents (e.g., sodium dodecyl sulfate, SDS; hexadecyltrimethylammonium bromide, CTAB; and *N*-lauroylsarcosine) denatures cytosolic proteins and lipid membranes and inactivates endogenous DNase/RNAse, facilitating their removal. Subsequent precipitation using ethanol or isopropanol results in the isolation of high-purity nucleic acids.

The development of various easy-to-use commercial kits has eliminated the use of hazardous organic solvents in the isolation of DNA/RNA from fungi. For the preparation of fungal DNA, Qiagen DNeasy Plant Kit (Qiagen), UltraClean™ Microbial DNA kit (Mo Bio Laboratories), DNAzol® (Invitrogen), and Whatman FTA cards (Whatman) are highly efficient [39]. Furthermore, automated DNA extraction systems have become increasingly sophisticated and affordable, contributing to the reduction of potential cross-contamination during manual handling.

1.4.2 Target Genes

For accurate and efficient identification of fungal organisms, a number of gene regions have been proven valuable. The most versatile and widely used target is rRNA gene [40–42]. This is followed by *rpb1*, *rpb2*, *tef1a*, and *atp6* genes. Other genes of interest include those encoding β-tubulin, actin, chitin synthase, acetyl coenzyme A synthase, glyceraldehyde-3-phosphate dehydrogenase, isoepoxydon dehydrogenase (idh), lignin peroxidase, and orotidine 5′-monophosphate decarboxylase genes [43,44].

Ribosome is an essential cellular organelle that is involved in protein synthesis in all living organisms. Being the key component of the ribosome, rRNA molecules consists of two complex folded subunits of differing sizes (small and large), whose main functions are to provide a mechanism for decoding messenger RNA (mRNA) into amino acids (at the center of small ribosomal subunit) and to interact with tRNA during translation by providing petidytransferase activity (large subunit [LSU]). The two rRNA subunits in eukaryotes including fungi have sedimentation coefficiency values of 40S (Svedberg units) and 60S. The small rRNA subunit (40S) in eukaryotes contains a single RNA species (i.e., 18S rRNA), whereas the large rRNA subunit (60S) in eukaryotes comprises three RNA species (5S, 5.8S, and 25S–28S rRNA). On the other hand, the two rRNA subunits in prokaryotes

FIGURE 1.1 Organization of fungal ribosomal RNA (rRNA) genes. Considerable variations exist in the small and large subunits of rRNA genes among different fungal groups. Notably, the small subunit (SSU) of rRNA gene in filamentous fungi and yeasts is 18S in size, while that in microsporidia is 16S. Similarly, the large subunit (LSU) of rRNA gene in filamentous fungi is 28S in size, that in yeasts is 25S and that in microsporidia is 23S. ITS, internal transcribed spacer; IGS, intergenic spacer.

measure 30S and 50S, respectively. While the small rRNA subunit (30S) in prokaryotes contains a single RNA species (i.e., 16S rRNA), the large rRNA subunit (50S) in prokaryotes contains two RNA species (5S and 23S rRNA). Interestingly, despite their recent redesignation as a phylum in the kingdom Fungi, microsporidia possess rRNA subunits (30S and 50S) and RNA species (16S and 23S) that are characteristic of prokaryotes, with a notable absence of 5.8S rRNA in microsporidia.

The fungal rRNA genes exist as a family of multiple-copy genes that are arranged in a head-to-toe manner, with each copy (of 8–12 kb in size) consisting of 18S RNA (small subunit [SSU]), ITS 1 (internal transcribed spacer 1), 5.8S RNA, ITS 2, 25S–28S RNA (LSU), IGS 1 (intergenic spacer), 5S, and IGS 2 (Figure 1.1). The tandemly repeated copies of rRNA genes have been homogenized by concerted evolution and contain highly similar nucleotide sequence. Therefore, they are almost always treated as a single-locus gene. Along with ITS, the 18S, 5.8S, and 25S–28S rRNAs are transcribed as a 35S–40S precursor, with all spacers being later spliced out of the transcript. A nontranscribed or IGS region exists between the copies of the 18S, 5.8S, and 25S–28S rRNA repeats, serving to separate the repeats from one another on the chromosome. A 5S RNA gene takes a position within the IGS region and is transcribed in the opposite direction. In filamentous ascomycetes, the 5S RNA gene is absent [45–49].

Much of the 25–28S RNA (LSU) gene is conserved across widely divergent taxa, and only the first 600–900 bases contain three divergent domains (D1, D2, and D3) that are useful for phylogenetic study of fungal organisms. Typically, the first 900 bases of LSU are amplified with primers 5.8SR (located in the 5.8S RNA) and LR7 (located in the 28S RNA), and the resulting amplicon is sequenced with primers LR5, LR16, LR0R, and LR3R (see Table 1.4) [43]. In yeasts, the D1 and D2 variable regions of 25S rRNA regions are often targeted [50].

The 18S rRNA (SSU) gene also includes alternating regions of sequence conservation and heterogeneity. The conserved regions are often targeted for phylogenetic analysis of higher taxonomic orders (e.g., phylum, family, and genus), while the regions of sequence diversity are valuable for characterization of isolates to the genus or species level (with isolates showing sequence identity >97% in the 18S rRNA gene being considered as the identical species).

Compared to the rRNA genes, the rRNA ITS1 and ITS2 regions are not as essential, and thus tend to be more variable, offering extremely valuable targets for fungal speciation and identification. Frequently, the ITS1 and ITS2 regions together with 5.8S RNA are amplified with primers ITS1 (located at the end of 18S RNA) and ITS4 (located at the beginning of 28S RNA). The resulting product is sequenced with primers ITS1, ITS2, ITS3, and ITS4 (see Table 1.4) [43,51]. The identity of the testing fungus is defined by its ITS sequence similarity (%) to the type strain or control isolate: species, ≥99%; genus, 93%–99%; and inconclusive, ≤93%.

Similar to ITS1 and ITS2 regions, the rRNA IGS regions (of 2–5 kb in length, depending on fungal taxa) also experience more genetic drift and consequently are not as highly conserved. This makes the rRNA IGS regions another potential target for fungal identification. The rRNA IGS regions can be amplified with primers LR12R (located at the end of 28S RNA) and invSR1R (located at the beginning of 18S RNA); the resulting product is sequenced with primers LR12R, 5SRNA, 5SRNAR, and invSR1R for all basidiomycetes and some ascomycetous yeasts, and with primers LR12R and invSR1R plus other internal primers for filamentous ascomycetes (as 5S RNA is nonexistent in filamentous ascomycetes) (see Table 1.4) [43,52].

1.4.3 Template Amplification

Prior to the advent of polymerase chain reaction (PCR) in the mid-1980s, molecular procedures for fungal identification were insensitive and cumbersome. With the development of PCR and other nucleic acid amplification technologies (such as ligase chain reaction [LCR], nucleic acid sequence-based amplification [NASBA], transcription-mediated amplification [TMA], strand displacement amplification [SDA], rolling circle amplification [RCA], cycling probe technology [CPT], branched DNA [bDNA], and loop-mediated isothermal amplification [LAMP]), it has become possible to rapidly and specifically detect a single copy of nucleic acid in a matter of hours [38].

Due to their efficiency, simplicity, robustness, and versatility, PCR and its derivatives have been widely adopted in both research and clinical laboratories for identification and determination of fungi and other microbial organisms. From the original version using a pair of primers and gel-based detection, improvements have been made in the forms of nested PCR, multiplex PCR, reverse transcription-PCR (RT-PCR), real-time PCR, quantitative PCR (Q-PCR), and arbitrarily primer PCR (or random amplified polymorphic DNA, RAPD), etc. [38,53]. These developments have not only enhanced the assay sensitivity (nested PCR) and versatility (multiplex PCR detecting multiple genes and/or organisms and RT-PCR detecting RNA instead of DNA), but also enabled the accurate quantitation of fungal organisms (Q-PCR) and elimination of manual handling post-amplification (real-time PCR).

TABLE 1.4
Identity and Sequence of Common rRNA, ITS, and IGS Primers for PCR Amplification and Sequencing Analysis of Fungal Organisms

Gene Region	Primer	Sequence (5′–3′)	Orientation	Nucleotide Positions in *M. grisea* (Nucleotide Positions in *S. cereviseae*)
18S rRNA	NS1	GTA GTC ATA TGC TTG TCT C	Forward	413–422
18S rRNA	NS1R	GAG ACA AGC ATA TGA CTA C	Reverse	413–422
18S rRNA	NS2	GGC TGC TGG CAC CAG ACT TGC	Reverse	943–963
18S rRNA	NS3	GCAAGTCTGGTGCCAGCAGCC	Forward	943–963
18S rRNA	NS4	CTT CCG TCA ATT CCT TTA AG	Reverse	1525–1544
18S rRNA	NS5	AAC TTA AAG GAA TTG ACG GAA G	Forward	1523–1544
18S rRNA	NS6	GCA TCA CAG ACC TGT TAT TGC CTC	Reverse	1806–1829
18S rRNA	NS7	GAG GCA ATA ACA GGT CTG TGA TGC	Forward	1806–1829
18S rRNA	NS8	TCC GCA GGT TCA CCT ACG GA	Reverse	2162–2181
18S rRNA	NS17	CAT GTC TAA GTT TAA GCA A	Forward	447–465
18S rRNA	NS18	CTC ATT CCA ATT ACA AGA CC	Reverse	887–906
18S rRNA	NS19	CCG GAG AAG GAG CCT GAG AAA C	Forward	771–792
18S rRNA	NS20	CGT CCC TAT TAA TCA TTA CG	Reverse	1243–1262
18S rRNA	NS21	GAA TAA TAG AAT AGG ACG	Forward	1193–1210
18S rRNA	NS22	AAT TAA GCA GAC AAA TCA CT	Reverse	1687–1706
18S rRNA	NS23	GAC TCA ACA CGG GGA AAC TC	Forward	1579–1598
18S rRNA	NS24	AAA CCT TGT TAC GAC TTT TA	Reverse	2143–2162
18S rRNA	SR1R	TAC CTG GTT GAT TCT GC	Forward	394–410 (1–21)
18S rRNA	SR1	ATT ACC GCG GCT GCT	Reverse	(578–564)
18S rRNA	SR2	CGG CCA TGC ACC ACC	Reverse	(1277–1263)
18S rRNA	SR3	GAA AGT TGA TAG GGC T	Reverse	696–711 (318–302)
18S rRNA	SR4	AAA CCA ACA AAA TAG AA	Reverse	(838–820)
18S rRNA	SR5	GTG CCC TTC CGT CAA TT	Reverse	(1146–1130)
18S rRNA	SR6	TGT TAC GAC TTT TAC TT	Reverse	(1760–1744)
18S rRNA	SR6R	AAG WAA AAG TCG TAA CAA GG	Forward	(1744–1763); similar to ITS 1
18S rRNA	SR7	GTT CAA CTA CGA GCT TTT TAA	Reverse	(617–637)
18S rRNA	SR7R	AGT TAA AAA GCT CGT AGT TG	Forward	(637–617)
18S rRNA	SR8R	GAA CCA GGA CTT TTA CCT T	Forward	(732–749)
18S rRNA	SR9R	QAG AGG TGA AAT TCT	Forward	(896–910)
18S rRNA	SR10R	TTTG ACT CAA CAC GGG	Forward	(1181–1196)
18S rRNA	SR11R	GGA GCC TGA GAA ACG GCT AC	Forward	779–798
18S rRNA	SSU1Fd	CTG CCA GTA GTC ATA TGC TTG TCT C	Forward	407–431
18S rRNA	SSU1Rd	CTT TGA GAC AAG CAT ATG AC	Reverse	416–435
18S rRNA	SSU2Fd	GAA CAA YTR GAG GGC AAG	Forward	930–947
18S rRNA	SSU2Rd	TAT ACG CTW YTG GAG CTG	Reverse	974–991
18S rRNA	SSU3Fd	ATC AGA TAC CGT YGT AGT C	Forward	1389–1407
18S rRNA	SSU3Rd	TAY GGT TRA GAC TAC RAC GG	Reverse	1397–1416
18S rRNA	SSU4Fd	CCG TTC TTA GTT GGT GG	Forward	1670–1686
18S rRNA	SSU4Rd	CAG ACA AAT CAC TCC ACC	Reverse	1682–1699
18S rRNA	SSU5Fd	TAC TAC CGA TYG AAT GGC	Forward	2037–2054
18S rRNA	SSU5Rd	CGG AGA CCT TGT TAC GAC	Reverse	2148–2165
18S rRNA	SSU6Fm	GCT TGT CTC AAA GAT TAA GCC ATG CAT GTC	Forward	423–452
18S rRNA	SSU6Rm	GCA GGT TAA GGT CTC GTT CGT TAT CGC	Reverse	1707–1733
18S rRNA	SSU7Fm	GAG TGT TCA AAG CAG GCC TNT GCT CG	Forward	1153–1178
18S rRNA	SSU7Rm	CAA TGC TCK ATC CCC AGC ACG AC	Reverse	1921–1943
18S rRNA	SSU8Fm	GCA CGC GCG CTA CAC TGA C	Forward	1848–1866
18S rRNA	V9G	TTA CGT CCC TGC CCT TTG TA	Forward	2002–2021
ITS	ITS1	TCC GTA GGT GAA CCT GCG G	Forward	2162–2180
ITS	ITS1F	CTT GGT CAT TTA GAG GAA GTA A	Forward	2124–2145; similar to ITS 1
ITS	ITS1Fd	CGA TTG AAT GGC TCA GTG AGG C	Forward	2043–2064
ITS	ITS1Rd	GAT ATG CTT AAG TTC AGC GGG	Reverse	2671–2691

(*continued*)

TABLE 1.4 (continued)
Identity and Sequence of Common rRNA, ITS, and IGS Primers for PCR Amplification and Sequencing Analysis of Fungal Organisms

Gene Region	Primer	Sequence (5′–3′)	Orientation	Nucleotide Positions in *M. grisea* (Nucleotide Positions in *S. cereviseae*)
ITS	ITS2	GCT GCG TTC TTC ATC GAT GC	Reverse	
ITS	ITS3	GCA TCG ATG AAG AAC GCA GC	Forward	
ITS	ITS4	TCC TCC GCT TAT TGA TAT GC	Reverse	2685–2704
ITS	ITS4B	CAG GAG ACT TGT ACA CGG TCC AG	Reverse	
ITS	ITS4S	CCT CCG CTT ATT GAT ATG CTT AAG	Reverse	2680–2703
ITS	ITS5	GGA AGT AAA AGT CGT AAC AAG G	Forward	2138–2159; similar to ITS 1
ITS	ITS5R	CCT TGT TAC GAC TTT TAC TTC C	Reverse	
5.8S rRNA	5.8S	CGC TGC GTT CTT CAT CG	Forward	(51–35)
5.8S rRNA	5.8SR	TCG ATG AAG AAC GCA GCG	Reverse	(34–51)
5.8S rRNA	5.8S1Fd	CTC TTG GTT CBV GCA TCG	Forward	2333–2350
5.8S rRNA	5.8S1Rd	WAA TGA CGC TCG RAC AGG CAT G	Reverse	2451–2472
28S rRNA	F377	AGA TGA AAA GAA CTT TGA AAA GAG AA	Forward	3005–3030
28S rRNA	LR0R	GTA CCC GCT GAA CTT AAG C	Forward	2668–2686
28S rRNA	LR1	GGT TGG TTT CTT TTC CT	Reverse	(73–57); similar to ITS 4
28S rRNA	LR2	TTT TCA AAG TTC TTT TC	Reverse	3009–3025
28S rRNA	LR2R	AAG AAC TTT GAA AAG AG	Forward	3012–3028
28S rRNA	LR3	GGT CCG TGT TTC AAG AC	Reverse	3275–3291
28S rRNA	LR3R	GTC TTG AAA CAC GGA CC	Forward	3275–3291
28S rRNA	LR5	TCC TGA GGG AAA CTT CG	Reverse	3579–3595
28S rRNA	LR5R	GAA GTT TCC CTC AGG AT	Forward	3580–3596
28S rRNA	LR6	CGC CAG TTC TGC TTA CC	Reverse	3756–3772
28S rRNA	LR7	TAC TAC CAC CAA GAT CT	Reverse	4062–4078
28S rRNA	LR8	CAC CTT GGA GAC CTG CT	Reverse	4473–4489
28S rRNA	LR8R	AGC AGG TCT CCA AGG TG	Forward	4473–4489
28S rRNA	LR9	AGA GCA CTG GGC AGA AA	Reverse	4799–4815
28S rRNA	LR10	AGT CAA GCT CAA CAG GG	Reverse	5015–5031
28S rRNA	LR10R	GAC CCT GTT GAG CTT GA	Forward	5013–5029
28S rRNA	LR11	GCC AGT TAT CCC TGT GGT AA	Reverse	5412–5431
28S rRNA	LR12	GAC TTA GAG GCG TTC AG	Reverse	5715–5731
28S rRNA	LR12R	CTG AAC GCC TCT AAG TCA GAA	Forward	5715–5735
28S rRNA	LR13	CAT CGG AAC AAC AAT GC	Reverse	5935–5951
28S rRNA	LR14	AGC CAA ACT CCC CAC CTG	Reverse	5206–5223
28S rRNA	LR15	TAA ATT ACA ACT CGG AC	Reverse	2780–2796
28S rRNA	LR16	TTC CAC CCA AAC ACT CG	Reverse	3311–3327
28S rRNA	LR17R	TAA CCT ATT CTC AAA CTT	Forward	3664–3681
28S rRNA	LR20R	GTG AGA CAG GTT AGT TTT ACC CT	Forward	5570–5592
28S rRNA	LR21	ACT TCA AGC GTT TCC CTT T	Reverse	3054–3072
28S rRNA	LR22	CCT CAC GGT ACT TGT TCG CT	Reverse	2982–3001
28S rRNA	LSU1Fd	GRA TCA GGT AGG RAT ACC CG	Forward	2655–2674
28S rRNA	LSU1Rd	CTG TTG CCG CTT CAC TCG C	Reverse	2736–2754
28S rRNA	LSU2Fd	GAA ACA CGG ACC RAG GAG TC	Forward	3280–3299
28S rRNA	LSU2Rd	ATC CGA RAA CWT CAG GAT CGG TCG	Reverse	3379–3402
28S rRNA	LSU3Fd	GTT CAT CYA GAC AGC MGG ACG	Forward	3843–3863
28S rRNA	LSU3Rd	CAC ACT CCT TAG CGG ATT CCG AC	Reverse	3876–3898
28S rRNA	LSU4Fd	CCG CAG CAG GTC TCC AAG G	Forward	4469–4487
28S rRNA	LSU4Rd	CGG ATC TRT TTT GCC GAC TTC CC	Reverse	4523–4545
28S rRNA	LSU5Fd	AGT GGG AGC TTC GGC GC	Forward	3357–3373
28S rRNA	LSU5Rd	GGA CTA AAG GAT CGA TAG GCC ACA C	Reverse	5355–5379
28S rRNA	LSU6Fd	CCG AAG CAG AAT TCG GTA AGC G	Forward	5499–5520
28S rRNA	LSU6Rd	TCT AAA CCC AGC TCA CGT TCC C	Reverse	5543–5564
28S rRNA	LSU7Fd	GTT ACG ATC TRC TGA GGG TAA GCC	Forward	5943–5966
28S rRNA	LSU7Rd	GCA GAT CGT AAC AAC AAG GCT ACT CTA C	Reverse	5927–5954

TABLE 1.4 (continued)
Identity and Sequence of Common rRNA, ITS, and IGS Primers for PCR Amplification and Sequencing Analysis of Fungal Organisms

Gene Region	Primer	Sequence (5′–3′)	Orientation	Nucleotide Positions in *M. grisea* (Nucleotide Positions in *S. cereviseae*)
28S rRNA	LSU8Fd	CCA GAG GAA ACT CTG GTG GAG GC	Forward	3469–3491
28S rRNA	LSU8Rd	GTC AGA TTC CCC TTG TCC GTA CC	Reverse	4720–4742
28S rRNA	LSU9Fm	GGT AGC CAA ATG CCT CGT CAT C	Forward	4882–4903
28S rRNA	LSU9Rm	GAT TYT GCS AAG CCC GTT CCC	Reverse	4979–4999
28S rRNA	LSU10Fm	GGG AAC GTG AGC TGG GTT TAG A	Forward	5543–5564
28S rRNA	LSU10Rm	CGC TTA CCG AAT TCT GCT TCG G	Reverse	5499–5520
28S rRNA	LSU11Fm	TTT GGT AAG CAG AAC TGG CGA TGC	Forward	3753–3776
28S rRNA	LSU12Fd	GTG TGG CCT ATC GAT CCT TTA GTC C	Forward	5355–5379
IGS	LR12R	GAA CGC CTC TAA GTC AGA ATC C	Forward	
IGS	5SRNA	ATC AGA CGG GAT GCG GT	Reverse	
IGS	5SRNAR	ACQ GCA TCC CGT CTG AT	Forward	
IGS	invSR1R	ACT GGC AGA ATC AAC CAG GTA	Reverse	

Sources: White, T.J. et al., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in Innis, M.A. et al. (eds.), *PCR Protocols: A Guide to Methods and Applications*, Academic Press, New York, 1990, pp. 315–322; Bruns, T.D. et al., *Ann. Rev. Ecol. Syst*., 22, 525, 1991; Bruns, T.D. et al., *Mol. Phylog. Evol*., 1, 231, 1992; Gardes, M. and Bruns, T.D., *Mol. Ecol*., 2, 113, 1993; Vilgalys, R. et al., *Mycol. Helvet*., 6, 73, 1994; Gargas, A. and DePriest, P.T., *Mycologia*, 88, 745, 1996; Crous, P.W. et al., *Stud. Mycol*., 64, S17, 2009.

Notes: Primer names with a "d" ending denote degenerate primers, whereas those with an "m" ending denote specific primers. The nucleotide positions of the primers refer to the rRNA gene sequence of *Magnaporthe grisea* (GenBank accession *AB026819*) or that of *Saccharomyces cereviseae* in the 5′–3′ direction [43,61].

1.4.4 Product Detection

In its standard form, the products generated by PCR are separated by agarose gel electrophoresis with or without modification (e.g., enzymatic digestion), stained with a DNA-binding dye (e.g., ethidium bromide or gel star), and visualized under UV light. For PCR products <100 bp or for distinction of products with minor size differences, polyacrylamide gel electrophoresis and its derivatives (e.g., single-strand conformational polymorphism [SSCP] analysis, denaturing gradient gel electrophoresis [DGGE], and temperature DGGE [TGGE]) may be used.

To improve the sensitivity of PCR product detection, enzymatic signal amplification (e.g., ELISA and flow cytometry) can be applied. In a common version of PCR-ELISA, streptavidin-coated microtiter plate is incubated with a biotinylated capture probe (or oligonucleotide) with specificity for a fungal gene. Aliquots of a PCR products generated with digoxigenin-labeled primers (derived from the same gene) are denatured in NaOH and subsequently hybridized to the capture probe. Specific hybridization products are then visualized by a colorimetric detection system based on an anti-digoxigenin horseradish peroxidase conjugate in the presence of a chromogenic substrate solution. After stopping the enzyme reaction with H_2SO_4, the absorbance is measured in an ELISA reader with a 450 nm filter.

PCR amplicons can be also detected by DNA microarray (also known as DNA chip, gene or genome chip, or gene array). Typically, a collection of microscopic DNA spots (either in the form of oligonucleotides or amplified DNA fragments with specificity for unique portions of the 18S rRNA gene) are affixed to a solid surface (e.g., glass, plastic, or silicon chip) as probes, forming an array for simultaneous identification of fungal organisms. Universal 18S rRNA gene primers (one of which contains a fluorescent label) are used in PCR to amplify all the 18S rRNA genes present in a sample. The resulting PCR products are added to the array and will only bind to the probes for which they have a complementary sequence. Pathogens are identified by the pattern of fluorescing spots in the array. Line probe assay (LiPA) is another nucleic acid hybridization test that is modified from DNA microarray. Instead of a glass, silicon, plastic chip, LiPA is conducted on a nitrocellulose strip, on which specific oligonucleotide probes are attached at known positions as parallel lines and are hybridized with biotin-labeled PCR products.

Recent advances in instrument automation and fluorescent dye chemistry permit instant monitoring of PCR amplicons (so-called real-time PCR) without additional manual handling. In one form of real-time PCR, a double-stranded DNA intercalating dye (e.g., SYBR Green) is used. SYBR Green increases its emission spectrum by 50- to 100-fold when binding to double-stranded DNA. As the double-stranded DNA is synthesized during PCR, an increase in fluorescence correlates to an increasing concentration of PCR products, which can be determined real time with reference to a standard sample. Discrimination of amplicons generated by multiplex PCR from different genes is also possible if these gene products have sufficiently different T_m values.

A melting curve analysis is performed post-PCR, using the SYBR Green as a fluorescent marker. As the melting point is reached, the DNA denatures and the fluorescence decreases sharply. The plotting of fluorescence versus temperature in a graph assists calculation of the melting temperature for each product. Other forms of real-time PCR employ specifically designed probes that target a region of amplicon and incorporate a fluorescent dye. Examples of these probes include hydrolysis dual-labeled probes (TaqMan®), hybridization probes (LightCycler), molecular beacons, peptide nucleic acid (PNA) probes, TaqMan minor groove-binding (MGB™) probes, locked nucleic acid (LNA®) primers and probes, and scorpions™ [38].

DNA sequencing analysis provides a most accurate way to ascertain the identity of PCR amplicons generated from fungi and other organisms. The classical chain termination sequencing method (or Sanger method) utilizes primers or dideoxynucleotides that are labeled with radioactive isotope or fluorescent tag, and the sequencing products are detected by exposure to x-rays or UV light. More recently, pyrosequencing, Roche 454, and Illumina Solexa platforms have been adopted for high-throughput sequencing analysis of PCR products. The nucleotide sequences of the PCR amplicon are then compared with those stored at reference databases such as GenBank, and the phylogenetic relationships of related fungi are displayed in the form of trees, constructed with distance matrix methods (resulting in phenograms) and maximum-parsimony methods (resulting in cladograms) [54].

1.5 RESULT INTERPRETATION, STANDARDIZATION, QUALITY CONTROL, AND ASSURANCE

1.5.1 Key Performance Characteristics

The performance of a diagnostic assay is often evaluated by using several key parameters, including detection limit, sensitivity, specificity, accuracy, intra-assay precision, inter-assay precision, and linearity (as in the case of a quantitative assay). Detection limit (or limit of detection) is the lowest concentration or quantity of bacteria that can be detected by a given assay. Sensitivity is the percentage of samples containing bacteria of interest that are identified by the assay as positive for the bacteria. Specificity is the percentage of samples without bacteria of interest that are identified by the assay as negative for the bacteria. Accuracy (or trueness) is the degree of conformity of an assay's measurements to the actual (true) value. It is often estimated by analyses of reference materials or comparisons of results with those obtained by a reference method. The closer an assay's measurements to the accepted value, the more accurate the assay is. Precision is the degree of mutual agreement among a series of assay's individual measurements, values, or results. Usually characterized in terms of the standard deviation of the measurements, precision can be stratified into (i) repeatability—the variation arising using the same instrument and operator in a single rune (i.e., intra-assay precision) or repeating during a short time period; and (ii) reproducibility—the variation arising using the same measurement process among different instruments and operators from one run to another (i.e., inter-assay precision) or over longer time periods. Linearity refers to the tendency of measurements by a quantitative assay to form a straight line when plotted on a graph. Data from linearity experiments may be subjected to linear regression analysis with an ideal regression coefficient of 1. In case of a nonlinear curve, other objective, statistically valid methods may be utilized.

1.5.2 Result Interpretation

A positive result by a molecular assay for a given pathogen normally confirms the etiologic relationship if the clinical syndrome is compatible with the pathogen identified. Considering the sensitive nature of the amplified methods such as PCR, it is important to rule out the possibility of a false-positive result. Occasionally, false-positive results may originate from the low diagnostic specificity of the assay, in which primers bind to irrelevant sequences and occasionally a homologous sequence that is shared among related or unknown bacteria. More often, false-positive results in the molecular testing come from contamination, which may arise during manual handling of the samples in the testing laboratory either at the pre- or post-extraction (while setting up the PCR) stages. This risk is heightened when a high copy-number polynucleotide (or plasmid) is used as a quantification standard and distributed around the laboratory, contaminating reaction source. Additionally, contamination may be attributable to samples that are referred from other laboratories, which do not utilize manipulation techniques that are mandatory for the molecular testing. These may include the use of unplugged pipette tips, infrequent changing of gloves, and using pipette for long periods without decontamination. Another cause of contamination is by amplification products from previous tests. Contamination may also occur by leakage from tubes or microtiter plates with lids not tightly closed or by breakage of glass capillaries leading to spillage of the amplification mixture. Besides the adoption of stringent laboratory practice, the risk of contamination with PCR products may be reduced by replacing nucleotide dTTP with dUTP in PCR and implementing a digestion step with uracil-DNA-glycosylase (UNG) to remove previous PCR products containing dUTP prior to each amplification reaction. Furthermore, inclusion of multiple negative controls, such as no-template controls (NTC) and no-amplification controls (NAC), may help identify the likely source of contamination and prevent false-positive results. Moreover, microbial DNA may come with PCR reagents.

Similarly, a negative result by a molecular assay for a given pathogen normally indicates the absence of the pathogen. However, it is equally important to rule out the possibility of false-negative results. One possible cause is due to the low sensitivity of the assay employed. Alternatively, insufficient amount of bacteria may be present in the sample (due

to sample degradation or prior antibiotic treatment). Another may be due to the impurity of the processed sample. Enzymes (e.g., DNA polymerase, reverse transcriptase) used in PCR and RT-PCR are impeded by components in blood and feces (e.g., heme, hemoglobulin, lactoferrin, immunoglobulin G, leukocyte DNA, polysaccharides, and urea), in foods (e.g., phenolics, glycogen, calcium ions, fat, and other organic substances), in environmental specimens (e.g., phenolics, humic acids, and heavy metals), and in added anticoagulants (e.g., EDTA and heparin) as well as nucleic acid purification reagents (e.g., detergents, lysozyme, NaOH, alcohol, EDTA, EGTA, phenol, and high salt concentrations). Any impurities and contaminations present in the samples after nucleic acid isolation may contribute to false-negative results. A useful way to determine the effective of nucleic acid purification procedure for removing inhibitory substances is to spike samples with well-defined DNA or RNA prior to and after sample preparation (as process and amplification internal controls). In light of the high sensitivity of PCR, the occurrence of false-negative results is probably a truly underestimated problem [55].

Because few species-specific molecular assays are available for fungal organisms, PCR amplification and DNA sequencing analysis of the rRNA genes, ITS and other gene regions have remained a most useful tool for fungal identification. As this approach is contingent on sequence comparison, inaccuracy of data deposited in reference databases may lead to incorrect identification. For example, a lack of pigmentation in *Alternaria infectoria* may contribute to its misidentification using macroscopic characteristics. Sequence data from the incorrectly identified isolates that are stored in reference databases may result in erroneous determination of testing isolates. Indeed, de Hoog and Horré [56] demonstrated that about 14% of the *Alternaria infectoria* sequences deposited in GenBank were found to be misidentified. In a separate study, Nilsson et al. [57] reported that about 20% of the entries related to fungi in the International Nucleotide Sequence Database might have been incorrectly identified to species level.

1.5.3 Standardization and Validation

As molecular tests such as PCR and sequencing offer improved sensitivity, specificity, accuracy, precision, and result availability for fungal identification and diagnosis, they have been increasingly adopted and applied in routine diagnostic laboratories. Considering the possibility of false-positive and false-negative results that may occur in these highly sensitive tests, it is essential to properly standardize and validate them prior to their adoption, and to put in place appropriate quality control measures to ensure their consistent performance.

Standardization of molecular tests addresses the need for standardized reagents and common units, contamination control mechanisms, inhibition control mechanisms, clinically relevant dynamic ranges and internal controls, etc. Validation helps to verify the sensitivity, specificity, accuracy, repeatability (intra-assay precision), reproducibility (inter-assay precision), detection limit, and linearity (if quantitative) of molecular tests.

Before validating a method, it is important to have all instruments calibrated and maintained throughout the testing process. The validation process may involve a series of steps including (i) testing of dilution series of positive samples (or plasmid construct) to determine the limits of detection of the assay and their linearity over concentrations to be measured in quantitative test (using minimal number of reference calibrators such as previously tested patient samples or pooled sera); (ii) evaluating the sensitivity and specificity of the assay, along with the extent of cross-reactivity with other genomic material; (iii) establishing the day-to-day variation of the assay's performance; (iv) assuring the quality of assembled assays using quality control procedures that monitor the performance of reagent batches; and (v) aligning the in-house primer and probe sequences with a genome sequence databank to avoid extended specificity testing [25–27,58].

1.5.4 Quality Control and Assurance

1.5.4.1 Quality Control

Quality control strategies for nucleic acid-based tests include (i) designation of a "clean" area for reaction setup (e.g., room under negative air pressure; positive-displacement pipettes; aerosol-block pipette tips; UV-equipped PCR cabinet); (ii) use of personal protective equipment (PPE) (e.g., disposable gloves and laboratory coats to prevent the introduction of contaminating DNA or nucleases); (iii) use of uracil-*N*-glycosylase (UNG) in real-time PCR (to eliminate cross-over amplicon contamination); (iv) use of a "hot-start" method (to minimize false priming events by withholding a crucial reaction component until appropriate temperature is reached); (v) use of external positive and negative controls (to monitor reaction performance and contamination) and homologous or heterologous internal controls (to monitor presence of inhibitors).

A variety of test controls may be considered for diagnostic PCR. These include (i) internal amplification control (IAC) (negative sample spiked with sufficient pathogen and processed throughout the entire protocol); (ii) processing positive control (PPC) (negative sample spiked with sufficient closely related, but non-target, strain processed throughout the entire protocol.); (iii) reagent control (blank) (containing all reagents, but no nucleic acid apart from the primers.); (iv) premise control (tube containing the master mixture left open in the PCR setup room) to detect possible contaminating DNA in the environment (carried out at regular intervals as part of the quality assurance program); (v) standard (three to four samples containing 10-fold dilution series of known number of target DNA copies in a range) [31,59].

1.5.4.2 Quality Assurance

One way to assess preparedness of the diagnostic laboratories is through the conduct of an external quality assurance (EQA) program providing characterized specimens

containing pathogens of interest. The design of a quality assurance program has the following components: (i) internal quality control (IQC) materials are distributed every month and comprising three pools of clinical samples of known pathogen status (typically one negative, one positive containing 1 log 10 over the lower limit of detection of the assay, and one low positive containing up to 1 log 10 of the lower limit of detection of the assay). These are incorporated in test runs on a weekly basis. The purpose of IQC is to provide samples of known status for repeated testing in parallel with clinical samples to ensure reproducibility of the test system in an individual laboratory. (ii) EQA distributions of panels of five unknown samples distributed quarterly. Results are returned to the QA laboratory for assessment. EQA compares the performance of different testing sites using specimens of known but undisclosed content. (iii) Aliquots of all samples sent from the reference laboratory are posted back to Site A for repeat testing to check for integrity of the pools and for transport problems. (iv) A final element of the pilot program involves Sites B, C, and D sending an aliquot of every 50th sample to Site A to check for reproducibility. (v) A detailed record of distributions is kept to provide an audit trail.

1.6 CONCLUSIONS

Fungi are a diverse group of eukaryotic organisms that are ubiquitously distributed in all environments. Although most fungi are saprophytes involved in the decomposition and recycling of organic matters as well as in the formation of symbiotic relationship with plants and animals, some have the capacity to infect plants, animals, and humans. Human mycoses often occur as a result of trauma or underlying immunosuppression, with clinical symptoms ranging from superficial, cutaneous, subcutaneous, to systemic mycoses. Since human mycoses caused by various fungal species are almost impossible to distinguish clinically, it is important to identify the causative agents to genus and species level for implementation of appropriate treatment and prevention measures.

Due to their complex life cycle and their production of morphologically similar structures, fungi are notoriously difficult to identify on the basis of macroscopic and microscopic features [60]. Use of biochemical, biological, and other phenotypic procedures to assess cultured isolates adds further delay in the laboratory speciation of fungal organisms. With recent development and application of molecular techniques, especially PCR amplification and sequencing analysis of the rRNA gene, ITS, and other gene regions, rapid, sensitive, and accurate determination of fungal organisms has become a reality [61]. Moreover, molecular characterization of fungal organisms is much less technically demanding than phenotypic procedures.

Considering that sequence-based approach for fungal identification is dependent on the reliability and accuracy of reference databases for comparison with other related organisms, it is critical to be aware that inaccuracy in reference databases that hold fungal nucleotide sequences may lead to incorrect identification. In addition, frequent taxonomical changes for many fungal organisms in the past as well as in recent times represent another potential source of misidentification if care is not taken. Therefore, future identification and characterization of novel taxon-specific gene markers will contribute to the increased accuracy in the molecular determination of fungal species/varieties implicated in human mycoses. These gene markers may come in the forms of previously uncharacterized, uniquely present genes, or of taxon-specific probes recognizing distinct portions of the shared genes. The latter category is exemplified by the development of species-specific probes from the internal transcribed spacer (ITS) regions that are common to all dermatophytes [62].

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Part I

Ascomycota

Pezizomycotina: Dothideomycetes

2 Alternaria

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2.1 INTRODUCTION

2.1.1 Classification, Morphology, and Biology

Alternaria is a genus of asexual or imperfect fungi assigned with the class hyphomycetes. Fungi in this genus are anamorphs of ascomycetes, including members of the genus *Pleospora*. *Alternaria* is a dematiaceous mold, which includes, at present, about 50 species and varieties. However, only eight species have been implicated as human pathogens: *A. alternata*, *A. brassicicola*, *A. chartarum*, *A. stemphylioides*, *A. dianthicola*, *A. infectoria*, *A. pluriseptata*, and *A. tenuissima*. *A. iridis* is mentioned only as an allergic strain.[1,2]

In 1817 Nees described this telluric fungus is characterized by chains of spores with apical beak under the name of *Alternaria tenuis*. Subsequently Fries noted in 1832 described that *Alternaria* species are characterized by very distinctive large multicellular dictyospores that have a beak and are produced in chains. Species of *Alternaria* occur as parasites on a number of crop plants, causing early blight or leaf spot diseases, or as saprobes on a wide variety of organic substrates. This genus is prominent in aerobiological literature because it is recognized as an important aeroallergen as well as plant pathogen.[3,4]

Many pathogenic species of *Alternaria* have a worldwide distribution and are infective to a variety of plants including potato, tomato, onion, and members of Brassicacee.[5] In general, *Alternaria* attacks plants under stress, especially those affected by drought, insect infestation, or senescence. *Alternaria* spores are passively dispersed from infected leaves by moderate to strong gusty wind, with velocities of 2–3 m/s required for spore release. As a component of the dry air spora, dispersal typically occurs during dry weather that immediately follows periods of rain or heavy dew.

Alternaria is also commonly isolated from soil, food, and indoor air environment, in particular on carpets and house dust.[6,7]

Alternaria spp. grow rapidly and the colony size reaches a diameter of 3–9 cm following incubation at 25°C for 7 days on potato glucose agar. The colony is flat, downy to woolly, and is covered by grayish, short, aerial hyphae in time. The surface is grayish white at the beginning, which later darkens and becomes greenish black or olive brown with a light border. The reverse side is typically brown to black due to pigment production.[8–10]

On microscopic examination, *Alternaria* spp. have septate, brown hyphae. The conidiophores arise singly or in small groups, simple or branched, straight or flexible, sometimes geniculate, pale to mid-olivaceous or golden brown, smooth, up to 50 µm long, 3–6 µm thick with one or several conidial scars. They bear simple or branched large conidia (7–10 × 23–34 µm) that have both transverse and longitudinal septations. These conidia may be observed singly or in acropetal chains and may produce germ tubes. They are ovoid to obclavate, darkly pigmented, muriform, and smooth or roughened. The end of the conidium nearest to the conidiophore is

round while it tapers toward the apex. This gives the typical beak or club-like appearance of the conidia.[8–10]

2.1.2 Clinical Features

The genus *Alternaria* comprises a large number of mostly saprobic or plant-pathogenic species and infects mainly immunocompromised hosts. Although infections in immunocompetent hosts have also been reported, these rarely involve invasive disease.[11] *Alternaria* can be found on normal human and animal skin[12] and conjunctiva.[13] This fungus has been associated frequently with hypersensitivity pneumonitis, bronchial asthma, and allergic sinusitis and rhinitis.[14–21] It can also cause several different types of human infections, for example, paranasal sinusitis, ocular infections, onychomycosis,[22] cutaneous, and subcutaneous infections,[23–25] and in some cases also soft palate perforation[26] and disseminated disease.[27,28]

The portal of entry of the infection is usually through corneal trauma or breakdown of the skin barrier.

2.1.2.1 Ocular infections

The incidence of *Alternaria* spp. in oculomycosis ranges from 2.3% to 10.4%[29–32] but it can vary according to the geographical location and is probably related to the risk of trauma caused by organic matter. The majority of cases are keratitis, although endophthalmitis is frequently associated.[32] Patients, in general, are farmers or gardeners who have been exposed to soil and garbage. Accidental or surgical ocular traumas are the predisposing factors.[33,34] The causative agent was generally identified as *A. alternata* or *A. infectoria*, although identification to the species level has not been performed on many occasions.

2.1.2.2 Rhinosinusitis

Sinusitis, or more accurately rhinosinusitis (RS), is a common disorder affecting approximately 20% of the population.[35] A report by the Centers for Disease Control and Prevention has estimated that the prevalence of sinusitis is up to 14.1% of the U.S. adult population, and the fact that there is no treatment for this disorder approved by the U.S. Food and Drug Administration emphasizes the profound impact of this disease.[36] Acute RS is well categorized and is often attributed to bacterial or viral causal agents. However, controversies surround chronic rhinosinusitis (CRS) and the role of fungi in this condition. CRS accounts for >90% of all cases of RS, and the correct diagnosis of each category of CRS is important for optimum therapy and predicting the course. Patients with CRS suffer from long-term nasal congestion, thick mucus production, loss of sense of smell, and intermittent acute exacerbations secondary to bacterial infections.

CRS is characterized histologically by an intense eosinophilic infiltration into the nasal mucosa.[37] The role of fungi in CRS is noninvasive, and it is not a fungal infection. It needs to be differentiated from other forms of fungal sinusitis, such as fungus balls (noninvasive) and invasive fungal sinusitis (acute, fulminant, or chronic form). Recently, striking progress has been made in the development of better techniques to detect fungi in nasal secretion or in ethmoid sinus. Polymerase chain reaction (PCR) with specific fungal primers has been used to find fungal DNA in polypoid nasal tissue,[38] in ethmoid sinus mucus, and nasal fluids, and it has provided greater sensitivity in detecting fungal elements. *Alternaria* DNA was detected in surgical specimens from the mucosa of the middle meatus and the paranasal sinuses or in ethmoid sinus mucosal specimens in almost all patients with CRS but in none of the normal controls.[38,39] It has been postulated that fungi act as non-IgE-mediated immunologic targets which initiate and maintain the inflammatory reaction in paranasal sinuses.[40] It has been shown that patients with CRS have exaggerated cytokine and humoral immune response to fungi, particularly to *Alternaria*.[41] The fungal DNA detected in the mucosa by PCR has been assumed to be related to an antigen-presenting process involving cells such as dendritic cells.[38]

2.1.2.3 Onychomycosis

Different epidemiological studies reported *Alternaria* as a causative agent of onychomycosis only in the 0.08%–2.5% of the cultured-proved clinical cases of nail infections.[42–47]

As with onychomycosis caused by other fungi, a history of contact with soil or trauma in the nail exists in most of the cases. Nails appear dystrophic and distal subungual hyperkeratosis and onycholysis are frequent manifestations. More than one nail could be affected, and in some cases both fingernails and toenails show infections.[42–47] Identification to the species level is not always reported, although *A. alternata* is the prevalent identified species, with some reports of *A. humicola*, *A. pluriseptata* and *A. chlamydospora* as causative agents.[47]

2.1.2.4 Cutaneous and Subcutaneous Infections

Most of clinical presentations concerned localized cutaneous infections resulting from direct, traumatic inoculation, even if in a compromised host a systemic spread is possible. Most of *Alternaria* infections are in patients with immunosuppression, and transplant recipients are one of the groups at risk for developing cutaneous alternariosis.[23–25] Phaeohyphomycosis caused by *Alternaria* may be difficult to recognize because lesions appear variable in size and aspect, ranging from crusted lesions, papules to erythematous macules or subcutaneous nodules, and, rarely, as cellulitis with secondary ulceration. Lesions are located on exposed areas, mainly on upper or lower limbs. Histopathologically, *Alternaria* infection is sometimes misdiagnosed as yeast infection or blastomycosis and in vitro distinction of species may be problematic, since clinical isolates may remain degenerate and sterile. An inflammatory diffuse infiltrate with mixed cellularity (lymphocytes, neutrophils, plasma cells, and histiocytes) mixed with a sarcoid-like and suppurative granulomatous inflammation is usually observed. Biopsy specimens from early lesions (<3 months evolution) are often characterized by the presence of epidermal changes (pseudoepitheliomatous hyperplasia, acanthosis) and a diffuse dermal mixed

inflammatory infiltrate with lymphocytes, plasma cells, histiocytes, and neutrophils. Histiocytic cells often presented with intracytoplasmic hyphae that adopted a typical aspect of round-to-oval, thick refractile walls (spore-like structures). In biopsy specimens from more advanced lesions (>3 months evolution), the observation of an inflammatory granulomatous infiltrate is a constant feature. Round, yeast-like structures and branched hyphae are found within the granulomas and microabscesses.

Two different routes of cutaneous infection have been reported: (1) from an exogenous source either as a consequence of traumatic inoculation of fungal elements (after injury by a plant spine) or to colonization of pathologically altered skin, or (2) from an exogenous focus (inhalation of fungal conidia and systemic spread) resulting in secondary cutaneous involvement. Some authors have also defined a "dermatopathic" cutaneous alternariosis, consisting of secondary colonization by Alternaria of preexisting lesions such as steroid-treated eczema of the face.[48]

An altered host resistance appears to be the prerequisite in the majority of affected patients. The majority of the reported patients with cutaneous alternariosis are solid-organ transplant recipients, although many of these patients were receiving systemic corticosteroids. Some authors have postulated that cutaneous fragility induced by corticosteroids may increase the risk of percutaneous inoculation from the environment.[49]

Rarely, *Alternaria* species may cause a systemic infection involving other organs apart from the skin. This endogenous cutaneous variant is manifested by multilocular involvement without having a site of predilection. Visceral and mucosal infection is more common among patients with HIV infection.[50] The observation of cutaneous alternariosis progressing to systemic involvement seems to be an exceedingly rare phenomenon.[25,51]

Differentiation down to the species level is recommended, because species may have different virulence or resistance to antimycotic therapy due to the occurrence of chlamydospores.[52,53] Moreover blastomycosis, sporotrichosis, cryptococcosis, and other subcutaneous mycosis that may present similar clinical features require different therapeutical approaches.

2.1.3 Pathogenesis

Melanins play an important role in the evasion of host defence by fungal pathogens, including dematiaceous molds. Melanins are believed to be composed of polymerized phenolic and/or indolic compounds. Melanins are dark in color, insoluble in aqueous or organic fluids, resistant to concentrated acid, and susceptible to bleaching by oxidizing agents.[54] At this moment these pigments remain poorly characterized because current biochemical and biophysical techniques are unable to provide a chemical structure for this complex polymer. Melanin in dematiaceous molds such as *Alternaria alternata* is synthesized through the dihydroxynapththalene (DHN) polyketide pathway.[55,56] The production of this pigment may protect the fungi from diverse environmental insult. The microbiota at the site of the Chernobyl nuclear reactor accident provides one of the most striking associations between fungal melanogenesis and the ability of these organisms to survive in an extreme environment. The extreme conditions present in the Chernobyl environment, radiation levels over 10,000 times the lethal human dose, select a radiation tolerant *A. alternata* clone.[57]

Melanin synthesis is associated with virulence for mammals in several pathogenic fungi including *C. neoformans* and *Exophiala dermatitidis* by comparing the relative pathogenicity of wild-type strains to mutants incapable of melanization, and it seems that melanin formation occurs in vivo. Melanins contribute to virulence with different mechanisms: they give protection against oxidants because they are highly effective scavengers of free radicals and have electron transfer properties that can facilitate redox cycling. Moreover DHN melanin in *A. alternata* protects against permanganate and hypoclorite and protects against neutrophil oxidative burst. Melanization interferes with phagocytosis and protects against killing by macrophages.

Melanin induces resistance to antimicrobial compounds in *Cryptococcus neoformans*, binds amphotericin B and caspofungin, and prevents them from reaching their target sites.[58]

Melanins are immunologically active compounds; they can elicit a vigorous inflammatory reaction inducing granuloma formation. It is conceivable that cell-wall-associated melanin in tissue provides an indigestible material that serves as "foreign body"-like material that interferes with clearance of infection while at the same time stimulating intense inflammation.

Factors other than 1,8-DHN melanin contribute to the virulence of dematiaceous fungi, although most of the pathogenic virulence factors are studied in *Wangiella dermatitidis* used as a model for black molds.[59] Among the many possibilities, chitin is receiving the most attention. The cell wall content of chitin is significantly enriched in most of the alternative morphotypes of *W. dermatitidis* compared to that of its budding yeast.[59] Furthermore, additional chitin is delocalized from predominantly yeast septal regions to all cell wall locations in hyphae and sclerotic forms. Chitin could act as a virulence factor by adding additional strength to the cell walls of black molds, whenever conditions are encountered in hosts that temporarily retard rapid yeast growth or induce transition of yeast cells to other morphotypes. Many studies using chitin synthase gene disruption showed morphological changes like abnormal hyperpigmentation or pseudohyphal-like growth with defective septa. Tissue sections from patients and animals with chronic cutaneous and subcutaneous infections frequently show yeast and hyphae with abnormally thickened walls. Even brain and other tissue from systemically infected humans and animals with rapidly progressing disease often show many types of thickwalled forms, which are variously described as yeast, chain and clusters of yeast, sclerotic cells, and cells with internal septa. Whether these different in vivo tissue forms are actually enriched with chitin is not known,

but chemical and cytochemical analyses of identical morphotypes produced in vitro indicate that they are.[60]

2.1.4 Laboratory Diagnosis

2.1.4.1 Conventional Diagnosis

The collection, transport, and processing of clinical specimens encompass some of the most important considerations in determining the etiology of fungal disease. Only with the appropriate handling of specimens can the recovery of fungal organisms be clearly associated with a disease process. As with all disease processes, the best specimen for determining the etiologic agent is the one taken from the active infection site. The collection details for fungal cultures are very similar to those for bacterial cultures, although the volume of material required for recovery of fungi may exceed those necessary for bacteria. The presence of more material for primary inoculum and concentration of large volumes of fluid greatly increases the likelihood of recovery of fungal species. Appropriate transport and storage of specimens are necessary for fungal elements to remain viable. Fungal viability may be affected by excessive heat and cold; room temperature transport and storage, within 2 h of collection, are recommended.

The most frequently submitted specimens for the recovery of dematiaceous fungi include aspirates, scrapings, and surgical tissue. Transport media should not be used, but specimens must not be allowed to become desiccated prior to processing. Specimen portions that are necrotic, purulent, or caseous should be selected for microscopic examination and inoculation onto isolation media. Tissue specimens should be minced with scalpels into 0.5 mm pieces and used to directly inoculate culture media. The use of tissue homogenizer for fungal cultures is discouraged because some molds do not have regularly septate hyphae and thus can be killed easily during homogenizing.

Media for primary isolation must support fungal growth while inhibiting bacteria, especially with specimens from nonsterile sites. The most commonly used media are brain heart infusion (BHI) plus antibiotics, Sabouraud dextrose agar alone or plus antibiotics, and selective media containing cycloheximide, a eukaryotic protein synthesis inhibitor, as suppressor of many saprobic fungi. In facilities where a large portion of the patient population may be neutropenic or immunocompromised, the rapid identification of opportunistic fungi, which may quickly disseminate in these individuals, is critical. The addition of cycloheximide and/or antibiotics to potato dextrose agar may be more appropriate in this setting, since it facilitates the identification of these organisms by providing plates for tape mounts of diagnostic structures from primary media. Sheep blood on BHI may inhibit conidiation, so is useful for primary isolation but not for identification.

Microscopic examination of a clinical specimen is essential to detect fungal elements. In laboratories that use fluorescence microscopy, bright-field examination of a positive field must be done to evaluate the amount of melanin present. Heavily melanized fungal cells are not reliably detected by using calcofluor white. Fluorescent-antibody-specific conjugates are not available for *Alternaria* sp.

Colonies of *Alternaria* grow rapidly and appear flat, downy to woolly, and are covered by grayish, short, aerial hyphae in time. The surface is grayish white at the beginning, which later darkens and becomes greenish black or olive brown with a light border. The reverse side is typically brown to black due to pigment production.

Until recently, the identification of *Alternaria* rested upon microscopic morphology, with the most significant characteristics being the morphology of the conidia and the formation of conidial chains. Morphology evaluations normally are based upon cultures that have been grown on a medium such as potato dextrose agar or cornmeal dextrose agar at 25°C–30°C for approximately 2 weeks. Slide culture preparation using cornmeal dextrose agar is ideal for conidiogenesis because these nutritionally minimal media usually stimulate the formation of spores.[61]

The morphological characteristics useful for distinction among the three species more frequently isolated are the following: *A. alternata* shows medium-brown conidia with a short, cylindrical beak, forming long and profusely branched chains, usually 10 or more conidia; in *A. tenuissima* the conidia are golden-brown, frequently tapering gradually into a beak that is up to half the length of the conidium, and usually occur in unbranched chains of three to five conidia; *A. infectoria* species group comprises more than 30 named anamorph taxa. Morphologically, the *A. infectoria* species group differs from other Alternaria species in the three-dimensional sporulation pattern and has more scarce conidia, as this species usually sporulates poorly in common media, and its small conidia (up to 70 µm in length) occur in branched chains with long, geniculate multiseptate secondary conidiophores (up to 120 µm) between conidia.[62]

2.1.4.2 Molecular Techniques

Identification of pathogenic dematiaceous fungi such as *Alternaria* spp. is typically done by morphological and physiological procedures.[63,64] However, these procedures are time-consuming, require technical expertise, and are ineffective for identification of species with poor conidia production and a wide diversity in anamorphic life cycles.[65]

The use of molecular techniques facilitates the identification of rare pathogenic fungi, such as *Alternaria* spp., and the most immediate need for nucleic acid detection methods is for the immunocompromised patient group. In this context, rapid diagnosis of mycological infection by in vitro amplification and detection of fungal DNA is a common method used in clinical laboratories.

2.1.4.2.1 Selection of Target DNA

Two avenues are available for this application; first is the selection of the target DNA using specific sequence information from databases, allowing primers to be designed across conserved and variable regions, and second is cloning and sequencing of arbitrary parts of the fungal genome.

Ribosomal RNA is an essential component of protein synthesis, thus ubiquitous. The sequence of rRNA has highly conserved as well as variable regions. Examination of this sequence reveals the relatedness between the species or the genetic distance between the organisms in question. An additional advantage of this is that these genes are not horizontally transferred, like other prokaryotic genes, for example, drug resistance genes.

Nuclear ribosomal RNA is coded by ribosomal DNA (rDNA), which is organized in ribosomal operons (usually 100–200 identical copies in fungi) located in chromosomes. The nuclear-encoded rRNA genes of fungi exist as a multiple-copy gene family consisting of highly similar DNA sequences (typically from 8 to 12 kb each) arranged in a head-to-toe manner. Each operon codes for the large subunit (LSU-rRNA; 28S rRNA), small subunit (SSU-rRNA; 18S rRNA), 5.8S rRNA and 5S rRNA. The position of 5S rRNA varies, but the organization of the rest of the genes is the same in all fungi (Figure 2.1).

The fungal identification is based on detection of conserved sequences in 5.8S and 28S rDNA that enable the amplification of the ITS region between these two regions and detection of D1/D2 domain contained in 28S rDNA. Then the tools for molecular investigation are represented by (i) DNA sequencing of the full length internal transcribed spacer (ITS) region ITS1–2 and (ii) DNA sequencing of the D1/D2 region of LSU gene in 28S rDNA.

The main area for the development of fungal diagnostics is ribosomal genes,[66] present in all organisms and at high copy numbers aiding detection and the sensitivity of PCR. The fungal nuclear rDNA consists of three genes: the large subunit gene (28S), the SSU gene (18S), and the 5.8S gene, separated by ITS regions, in a unit repeated many times. The ITS region is an area of particular importance to fungal diagnostics. It has areas of high conservation and areas of high variability and is an ideal starter for the development of specific PCR primers for identification of fungal species. Universal primers[66] are available for fungi that isolate the regions of the ITS. Once cloned these sequences can be compared to the wealth of other sequences in the sequence database and diagnostic primers developed for a particular fungus. The MicroSeq D2 large-subunit rDNA sequencing kit appears to be accurate and useful for the identification of filamentous fungi, even those that are relatively uncommon that are seen in the clinical laboratory. However, the library includes more of the common *Alternaria* spp. and other environmental flora that cause disease in immunocompromised patients.

2.2 METHODS

2.2.1 Sample Preparation

A key step in the detection of fungi using PCR is the ability to efficiently extract DNA from hyphae and/or conidia. The efficiency of this step is even more critical when attempting to detect small quantities of fungal material in biological samples such as human blood, mucus, and tissues.

The detection of fungal pathogens in clinical samples by PCR requires the use of extraction methods that efficiently lyse fungal cells and recover DNA suitable for amplification. The DNA extraction stage of the sample is critical processing because fungi have cell walls that impede lysis and the recovery of nucleic acids. Because of the structure and composition of the fungal cell wall, consisting of thick layers of chitin, (1–3)-β-D-glucan, (1,6) β-glucans, mannan, mannoproteins, lipids, and peptides, and the presence of a melanin complex in the cell wall, the release of fungal DNA usually requires additional lysis steps, such as mechanical disruption and enzymatic digestion or use of toxic chemicals.[54] In general, the sample preparation method should release intracellular DNA from the fungal cell wall and/or thick capsule; it must concentrate DNA targets that may be present in very small amounts; and it must eliminate protein debris, contaminants, potential inhibitors, and other extraneous materials without degrading the target DNA.

Alternaria molecular detection test can be used indirectly on molds isolated from a patient's specimen or directly on clinical samples. Several commercial kits are available for mold DNA extraction in a clinical microbiology laboratory.[67] Many extraction methods use a bead matrix and lysis buffer to destroy mycelial cells, followed by adsorption of DNA to a spin filter, a wash step, and the elution of DNA in buffer, prior to amplification steps.

(i) Clinical sample pretreatment—Step 1

Tissue: For DNA extraction, all tissue samples are incubated for ≥2 h in proteinase K and digestion buffer at 55°C, and the DNA is extracted using the miniMAG™ or easyMAG instrument (bioMérieux) according to the manufacturer's instructions. The DNA could be stored at −20°C prior to use.

(Note: Digestion buffer: 50 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS, pH 8.0. Proteinase K: 0.5 mg/mL in digestion buffer).

1. Place 20–25 mg of tissue into a polypropylene microfuge tube.

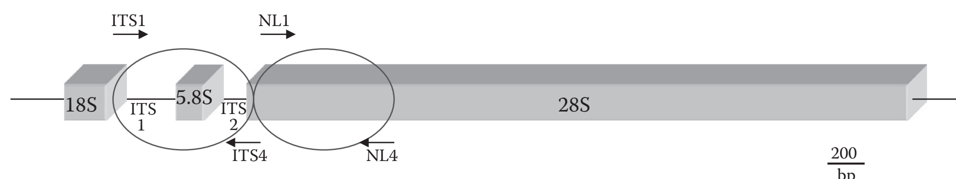

FIGURE 2.1 Map of fungal rRNA from the 3′ end of the 18S rRNA gene to the 3′ end of the 28S rRNA gene. PCR primers for sequencing and PCR assay development (arrows).

2. Add 0.5 mL of DNA digestion buffer with proteinase K.
3. Incubate for a minimum of 3 h to overnight at 55°C with gentle shaking.

   Corneal scrapings are collected from all of the patients while they are under local anesthesia by use of a slit-lamp microscope and a flame-sterilized Kimura spatula.[68] One part of each scraping is directly transferred to lysis buffer for DNA extraction.

   Cutaneous swab is immersed in lysis buffer in a microcentrifuge tube and incubated at room temperature for 5 min with occasional agitation.

(ii) Clinical sample pretreatment—Step 2

1. Up to 1 mL of lysis buffers each pre-treated sample containing fungal cells or conidia.
2. Add 300 mg of MicroBeads in each sample tube.
3. Continue DNA purification as described below in step 3 for Method UC (UltraClean DNA isolation kit [MoBio, Inc., Solana Beach, CA]) of DNA extraction from culture.

   The supernatant solution (above the beads) is transferred to a mini-MAG for a semi-automated magnetic extraction or an easy MAG for automated magnetic extraction.

(iii) DNA extraction from culture

Method UC (UltraClean DNA isolation kit [MoBio, Inc., Solana Beach, CA]) uses a bead matrix and lysis buffer to pulverize cells by horizontal shaking on a vortex mixer, followed by adsorption of DNA to a spin filter, a wash step, and the elution of DNA in buffer.[69] Microcentrifuge tubes with sample and bead matrices were attached to a horizontal platform on a vortex mixer and agitated vigorously for 40 min. Each sample is eluted in a TE1X buffer. In detail the method:

1. Add 300 µL of MicroBead solution to a MicroBead tube. MicroBead Solution contains salts such as guanidine thiocyanate and a buffer which stabilizes and homogeneously disperses the microbial cells prior to lysis.
2. Use an inoculation loop to collect approximately half of the mycelia covering the cellophane square. Transfer mycelia into the MicroBead tube.
3. Add 50 µL of solution MD1 to the MicroBead tube. Solution MD1 contains sodium dodecyl sulfate (SDS) and other disruption agents required for cell lysis.
4. Place MicroBead tubes in a liquid nitrogen safe container (such as a Styrofoam cup) and cover with liquid nitrogen. Incubate for 1 min. Remove MicroBead tubes from the liquid nitrogen using forceps. Immediately vortex tubes for 10 min.
5. Repeat previous step two times.
6. Vortex MicroBead tubes an additional 10 min.
7. Centrifuge the MicroBead tubes for 2 min at 10,000 × *g*.
8. Add 100 µL of solution MD2 to a clean 2.0 mL microcentrifuge tube. Solution MD2 contains ammonium acetate, a reagent to precipitate non-DNA organic and inorganic material including cell debris and proteins.
9. Transfer the supernatant from step 7 to the tube containing solution MD2. Vortex briefly. Incubate at 4°C until tubes are completely chilled (at least 15 min).
10. Centrifuge tubes for 1 min at 10,000 × *g*.
11. Add 900 µL of solution MD3 to a clean 2.0 mL microcentrifuge tube. Solution MD3 contains guanidine hydrochloride/isopropanol which is a highly concentrated salt solution necessary to bind DNA to the spin filter membrane in the following step.
12. Transfer the supernatant in step 10 to the tube containing solution MD3. Vortex briefly and spin down. During transfer of supernatant, do not touch the protein pellet with the pipette tip.
13. Transfer 650 µL to a clean spin filter. Centrifuge for 30 s at 10,000 × *g*. Discard the flow through. Replace the filter into the same collection tube.
14. Repeat step 13 with remaining supernatant MD3.
15. Add 300 µL of solution MD4 to the spin filter and centrifuge for 30 s at 10,000 × *g*. Solution MD4 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the spin filter. Discard the flow through. Replace the filter into the same collection tube.
16. Centrifuge the empty spin filter for 60 s at 10,000 × *g*. Discard the collection tube.

    (*Note*: Alcohol is a PCR inhibitor. Make sure no residual alcohol remains near the filter basket).
17. Place the spin filter into a clean 2.0 mL microcentrifuge tube.
18. Add 35 µL of solution MD5 onto the center of the filter. Solution MD5 is 10 mM Tris pH 8. Incubate for 2 min. Centrifuge for 30 s at 10,000 × *g*.
19. Be careful not to touch the filter with the pipette tip.
20. Discard the spin filter. Extracted DNA is contained in the flow through and is ready for use. Store DNA at −20°C.

(iv) Semi-automated extraction

DNA could be isolated using a semi-automated magnetic extraction method, NucliSens® miniMAG, and the NucliSens Magnetic Extraction Reagents (bioMérieux S.A.).

In the first step, 2 mL of NucliSens lysis buffer containing guanidine thiocyanate (5 mol/L) is added

to each 1.5 mL tube containing a single pretreated sample.

The tubes are pulse vortexed for 15 s, and then incubated for 10 min at room temperature. The NucliSens lysis buffer contains a chaotropic salt for efficient lysis and inactivation of nucleases. In the second step, 50 µL of Magnetic Silica Particles (bioMérieux) are added to allow DNA binding during a 10 min incubation period at room temperature.

Next, the silica particles are washed twice with 400 µL of wash buffer 1 (5 mol/L guanidine thiocyanate, Tris/HCl, Triton X 100, EDTA), twice with 400 µL of wash buffer 2 (MES hydrate), and once with 500 µL of wash buffer 3 (component: disodium tetraborate) using the NucliSens miniMAG instrument to remove any contaminants from the biological specimen.

Removal of wash buffer is done by vacuum aspiration.

Finally, the DNA is recovered from the particles using 40 µL of wash buffer 3 during a 10 min incubation period at 70°C and under constant shaking (1400 rpm). Thirty microliter of each supernatant is used as template for PCR amplification.

(v) Automatic extraction

Extraction with the easyMAG (bioMérieux S.A.) is done according to the manufacturer's recommendations. Up to 1 mL of each pretreated sample is placed in the disposable sample vessel and it is loaded onto the extractor.

After the initial lysis incubation, 100 µL of magnetic silica particles, prepared as recommended by the manufacturer, are added to each sample, and the extractor restart.

Samples are eluted in 55–110 µL. All samples are transferred to a 1.5 mL microcentrifuge tube and stored at 4°C for 24 h, or −20°C for no more than 2 months.

The internal control is added after the initial incubation step, immediately before the magnetic silica is added.

2.2.2 Detection Procedures

PCR mixes (50 µL) are set up as follows: 10 mM Tris–HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM (each) dATP, dCTP, dGTP, and dTTP; 1.25 U of *Taq* DNA polymerase (Amplitaq Gold (Applied Biosystems), 0.2 µM (each) of the appropriate primers targeting both the D1/D2 region NL1 (forward) 5-GCATATCAATAAGCGGAGGAAAAG-3 and NL4 (reverse) 5-GGTCCGTGTTTCAAGACGG-3′, as well as the larger 18S-ITS1-5.8SITS2-28S (ITS1/ITS4), ITS1 (forward) 5-TCCGTAGGTGAACCTGCGG-3′ and ITS4 (reverse) 5-TCCTCCGCTTAT TGATATGC-3, as previously described,[70] and 5 µL of DNA template. The reaction mixtures are subjected to the following thermal cycling parameters in a MJ PTC 20 thermocycler: 95°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min.

To validate the presence of amplifiable DNA and absence of inhibitory substances a PCR is performed using the primer set GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PC04 (5′-CAACTTCATCCACGTTCACC-3′) targeting the human β globin gene.[71] The conditions are as described above except that 5 µL plasmid DNA containing partial β globin gene are used, in noncellular sample. When the internal control result is negative, DNA extraction must be repeated if enough material is available.

Following amplification, aliquots (10 µL) are removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 2% (w/v) agarose (Gibco, United Kingdom) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.3), stained with ethidium bromide (5 µg/100 mL).

The two most important clinical species, *A. alternata* and *A. infectoria*, can easily be differentiated according to the length of the ITS1-4 amplicon, with the ITS spacer domain being ca. 570 bp in the former species and ca. 600 bp in the latter. *A. tenuissima* cannot reliably be distinguished from *A. alternata* using this method.

All PCR products are purified before DNA sequence analysis particularly to remove dNTPS, polymerases, salts, and primers using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Purified amplicons were then sequenced on both strands using the same primers as described above. BigDye terminator cycle sequencing Ready Reaction kits (Applied Biosystems) were employed as recommended by the manufacturer. All cycle sequencing reactions were performed on a MJ PTC200 thermal cycler using an initial denaturation at 96°C for 5 s, followed by 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Products are purified using a Dye-Ex spin kit (Qiagen), dried in a vacuum centrifuge, and resuspended in either template suppression reagent (Applied Biosystems; D1/D2R products) or formamide (Applied Biosystems; ITS products). Products are then analyzed on an automated capillary DNA sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems). Comparative sequence analysis and GenBank searches are assisted by the Genetics Computer Group software package (FASTA, BESTFIT, STRETCHER, and PILEUP algorithms; University of Wisconsin, Madison), the Clustal W alignment program,[72] and the nucleotide–nucleotide Basic Local Alignment Search Tool (BLAST) algorithm (blastn).[73]

2.3 CONCLUSION AND FUTURE PERSPECTIVES

Alternaria is a cosmopolitan mold, found in soil or more often on living and dead plants. This mold is a well-known phytopathogen fungal genus, but numerous published cases of human alternarioses are reported. The most frequent clinical presentations are cutaneous and subcutaneous infections, followed by oculomycosis, RS, and onychomycosis. Immunosuppression is frequently associated with clinical

manifestations, and solid organ transplantation is the most important risk factor. The portal of entry of this mold is usually through mechanical trauma or breakdown of the skin barrier.

The use of molecular techniques, the detection of fungal DNA by PCR has been described as an important tool in the early diagnosis of fungal infection. Moreover it facilitates the identification of rare pathogenic fungi, such as *Alternaria* spp., even by nonexperts. In fact, using BLAST, it is now easy to compare sequences of a given unidentified mold, even if it is a non-sporulating strain. Sequences deposited previously in GenBank are useful in order to find the region of similarity among different strains. However it is important to ensure that unknown sequences are compared with the sequence of reference strains that have been identified by experts.

Amplification of the ITS region with pan-fungal primers, followed by downstream sequencing, has been shown to be a useful method for identification of *Alternaria* spp. De Hoog and Horré[52] have provided an accurate procedure to distinguish between *Alternaria* spp. based on a PCR method using general primers, followed by restriction enzyme digestion of the amplicons. *A. alternata* and *A. infectoria* are easily differentiated with the length of the ITS1-4 amplicon, with 570 bp in the *A. alternata* and 600 bp in the *A. infectoria*.

There is a regular need for identification of *Alternaria* isolates, because they have acquired different abilities in nature, which affect the possibilities that the isolates could be an opportunistic human pathogen or plant pathogen or saprotrophic on cereals producing biologically active metabolites.

In medical mycology, molecular identification using ITS is fast, well-known, and often the only method to identify strains from human lesions that rapidly lose their ability to sporulate in vitro.

Concerning alternarioses in humans or animals, generally only identification to species-group level is needed, since the same medical treatment is likely to be applicable regardless of taxon identification. Otherwise the combination of real-time PCR and direct sequencing of positive PCR products obtained from clinical samples improves the diagnostic turnaround time.

Considering that molecular techniques offer the possibility of faster detection and identification of fungi in various samples, the application of PCR has to be evaluated as a supplementary method to mycological cultivation. Cultivation methods ensure the possibility to distinguish between colonization and acute disease and moreover allow to measure the antibiotic resistance.

In the modern medicine era, a critical use of molecular methods is essential in a microbiology laboratory. Application of DNA typing could provide rapid diagnosis and consequently results in the administration of specific and effective therapy. Moreover molecular methods could help in the near future to understand more on the pathogenic role of environmental fungi, even in the era of large-scale immunosuppressant usage, the most important risk factor in predisposing to mycotic disease.

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3 *Aureobasidium*

Miia Pitkäranta and Malcolm D. Richardson

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3.1 INTRODUCTION

Aureobasidium is a saprophyte distributed widely throughout the environment, commonly isolated from plant debris, soil, wood, textiles, and wet areas of bathrooms. In particular, it is found in osmotic environments, such as in food and on plant leaves. It is a frequent colonizer of damp stone and glass and commonly found in the clinical laboratory as a contaminant. Only exceptionally is *Aureobasidium* involved in human disease.

3.1.1 Classification, Morphology, and Environmental Sources

3.1.1.1 Classification

Hyphomycetes form the major part of the moulds encountered in the medical mycology laboratory. They are composed of regularly septate hyphae and produce asexual propagules directly on the hyphae, without fruit bodies.[1] Included in the hyphomycetes are the black yeasts[1] that are defined as asexual fungi potentially able to produce melanized budding cells (a yeast phase) in any stage of their life cycle. Often strictly filamentous relatives of pathogenic black yeasts are included in this group. Here the term "black yeast-like fungi" is applied. *Aureobasidium pullulans*, the only well-known species in the genus, is a dematiaceous fungus, characterized by the presence of melanin pigment in the cell wall and the production of hyaline blastoconidia that develop darkly pigmented chains of arthroconidia in culture.[2]

According to present taxonomy, *Aureobasidium* belongs to Ascomycota, order Dothideales, family *Dothioraceae*.[3] More than 20 species are listed under this genus in Mycobank (http://www.mycobank.org). As such, the genus is polyphyletic and many species are better known by their synonyms (Table 3.1). Most species are saprophytes on various substrates or are plant pathogens causing leaf spots.[2] The species of *Aureobasidium* and closely related genera *Hormonema* and *Kabatiella* are difficult to distinguish in culture by

TABLE 3.1
Members of the Genus *Aureobasidium* and Availability of Molecular Data

Species	Currently Used Synonym	Pathogenicity	Sequence Data
Aureobasidium aleuritidis (Vassiljevsky) Herm.-Nijh. 1977		Plant	N/A
A. apocryptum (Ellis & Everh.) Herm.-Nijh. 1977		Plant	N/A
A. australiense McAlpine 1896		N/A	N/A
A. bolleyi (Sprague) Arx 1957	*Microdochium bolleyi* (Sprague) de Hoog & Herm.-Nijh. 1977	Plant	18S, ITS
A. caulivorum (Kirchn.) Cooke 1962	*Kabatiella caulivora* (Kirchn.) Karak. 1923	Plant	18S, 28S, ITS, ELO1
A. dalgeri (Morelet) Herm.-Nijh. 1977		Plant	N/A
A. foliicola (Oudem.) Muell. 1964	*Lecythophora hoffmannii* (Beyma) Gams & McGinnis 1983	Human[14–16]	18S, 28S, ITS, Cytb
A. harposporum (Bres. & Sacc.) Herm.-Nijh. 1977	*Kabatiella harpospora* (Bres. & Sacc.) Arx 1957	Plant	N/A
A. indicum Pande & Ghate 1985		N/A	N/A
A. lilii Crisan & Hodisan 1964		Plant	N/A
A. lini (Laff.) Herm.-Nijh. 1977	*Kabatiella lini* (Laff.) Karak. 1950	Plant	18S, 28S, ITS, EF1, TUB, ELO1
A. mansonii (Castell.) Cooke 1962	*Exophiala castellanii* Iwatsu, Nishim. & Miyaji 1999	Human[17]	18S, 28S, RPB1, Cytb
A. microstictum (Bubák) Cooke 1962	*Kabatiella microsticta* Bubák 1907	Plant	18S, 28S, ITS, EF1, TUB, ELO
A. microstromoides (Moesz) Cooke 1962		N/A	N/A
A. nigricans (Atk. & Edgerton) Cooke 1962	*Kabatiella nigricans* (Atk. & Edgerton) Karak. 1923	Plant	N/A
A. nigrum (Marpmann) Cif. & Dalla Torre 1963	*Torula dematia* Berkhout 1923	N/A	N/A
A. prunicola (Ellis & Everh.) Herm.-Nijh. 1977		Plant	N/A
A. prunorum Dennis & Buhagiar 1973	*Hormonema prunorum* (Dennis & Buhagiar) Herm.-Nijh. 1977	Saprophytic	18S, ITS
A. pullulans (de Bary) Arnaud 1918		Human, other mammalian, saprophytic	18S, 28S, ITS, EF1, TUB, ELO, other
A. ribis (Vassiljevsky) Herm.-Nijh. 1977		Plant	N/A
A. salmonis (Carmich.) Borelli 1969	*Exophiala salmonis* Carmich. 1966	Fish	18S, 28S, ITS, mt ssu rRNA, RPB1, RPB2, EF1, Cytb
A. sanguinariae (Ellis & Everh.) Herm.-Nijh. 1977		Plant	N/A
A. slovacum Svob.-Pol., Chmel & Bojan. 1966	*Chmelia slovaca* (Svob.-Pol., Chmel & Bojan.) Svob.-Pol. 1966	Human[18]	N/A
A. thujae-plicatae Morelet 1978		Plant	N/A
A. umbellulariae (Harv.) Herm.-Nijh. 1977		Plant	N/A

TABLE 3.1 (continued)
Members of the Genus *Aureobasidium* and Availability of Molecular Data

Species	Currently Used Synonym	Pathogenicity	Sequence Data
A. vaccinii Richit. & Teodoru 1989		Plant	N/A
A. zeae (Narita & Hirats.) Dingley 1973	*Kabatiella zeae* Narita & Y. Hirats. 1959	Plant	ITS

Abbreviations: 18S, nuclear small subunit rRNA gene; 28S, nuclear large subunit rRNA gene; ITS, nuclear internal transcribed spacer region; ELO1, fatty acid elongase gene; Cytb, mitochondrial gene for cytochrome b; EF1, translation elongation factor 1-α-like gene; TUB, β-tubulin gene; RPB1, RNA polymerase II largest subunit gene; RPB2, RNA polymerase II second largest subunit gene; N/A, data not available. Synonym data: http://www.mycobank.org

morphology or nutritional physiology.[4] Several studies have assessed the taxonomy of the group using molecular methods. The genera *Aureobasidium* and *Kabatiella* are overlapping based on common micromorphological, physiological, and molecular features.[2,5,6] The taxonomy of the group remains largely uncertain.[7]

A. pullulans is a clonal anamorph species without a confirmed teleomorph. It has got no close relatives among human pathogenic fungi, but certain other species, that is, plant pathogenic *Kabatiella* spp., as well as *Selenophoma mahoniae*, a pycnidial fungus with *Aureobasidium*-like cultural synanamorph, and *Discosphaerina* (*Columnosphaeria*) *fagi*, a potential teleomorph of *A. pullulans*, show high genetic affinity with it. *Hormonema dematioides* resembles *Aureobasidium pullulans* phenotypically and these species are often mixed up.[1,4,6–11] Phytopathogenic species *A. bolleyi*, *A. prunorum*, and *A. zeae*, as well as *A. salmonis*, a pathogen of fish, show considerable genetic distance to *A. pullulans* complex (see Table 3.1 for synonyms in use). Unknown *Aureobasidium*-like strains have been isolated from various environments.[7,12] DNA sequence analysis is a powerful tool for characterizing these isolates, and novel entities belonging to *Aureobasidium* will probably be described in the future.[7,13] For most species in the genus *Aureobasidium*, pheno- and genotypic data is not available or is available only from a restricted number of strains.

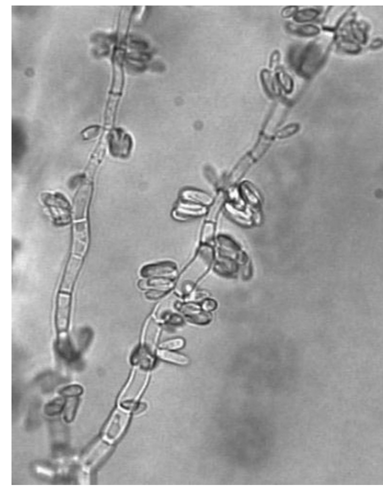

FIGURE 3.1 Septate, hyaline hyphae with groups of synchronous conidia of *Aureobasidium pullulans*. (From Andreoni, S. et al., *Medical Mycology Atlas*, 2004.)

3.1.1.2 Morphology

On malt extract agar (MEA) or glucose peptone agar, *Aureobasidium* grows rapidly when incubated at 30°C, reaching 30 mm in 1 week, appearing flat and smooth, and soon covered with a slimy exudate. The colonies are cream or pink and then later becoming brown or black. The reverse of the colony is cream, turning brown or black with age. The varieties show different pigmentation behavior, the entire colonies of var. *melanigenum* turning green to black usually within 1 week, while other varieties stay mainly light colored for at least 1–2 weeks. Bright color variants have been isolated from the tropics.[13] The microscopical appearance (Figure 3.1)[70] is vegetative hyphae 3–12 μm wide, hyaline, locally converting into blackish-brown, thick-walled chlamydospores, and expanding hyphae with irregular dichotomous branching. The conidia-bearing cells appear undifferentiated and mostly intercalary in hyaline hyphae. Conidia are initially produced synchronously in dense groups from small denticles. They are smooth, oval, and non-pigmented. Subsequently, the conidia are produced percurrently and adhere to one another in slimy heads. The conidia are hyaline, ellipsoidal, vary considerably in shape and size, $7.5–16.0 \times 3.5–7.0$ μm, and are one celled, often with an indistinct hilum. Polar budding of the conidia is often observed. An additional feature is the presence of endoconidia (asexual propagules formed inside a cell) in intercalary cells.

3.1.1.3 Environmental Sources

The environmental sources of *Aureobasidium* are varied. It is a ubiquitous microorganism that can be easily isolated from the phyllosphere and from plant residues, flowers, soil, wood, indoor and outdoor air, and even stone (reviewed in Cooke and Taylor et al.).[19–21]

3.1.2 Pathogenesis and Clinical Features

3.1.2.1 Pathogenesis

In *Aureobasidium*, *A. pullulans* is the most important species with respect of pathogenicity, but *A. foliicola* (*L. hoffmannii*), *A. mansonii* (*E. castellanii*), and *A. slovacum* (*C. slovaca*) have occasionally been implicated as causative agents of clinical conditions in compromised patients.[16–18] The latter three species are not related to other species of *Aureobasidia*,[8,22] and, regarding their close affinity to other genera these species are discussed in detail elsewhere. Individual cases of cutaneous phaeohyphomycosis, peritonitis, and fungemia caused by *H. dematioides* in immunocompromised patients have been described.[23–25]

Aureobasidium pullulans is an opportunistic pathogen able to cause disease in compromised patients (BSL-1). It may cause phaeohyphomycosis, keratomycosis, pulmonary mycosis with sepsis, peritonitis, and other opportunistic infections, as well as cutaneous mycoses such as eumycotic dermatitis. *Aureobasidium* may also colonize hair, skin, and nails in humans, but its ability to penetrate healthy tissues is limited. Patients with an *Aureobasidium* blood stream infection as a result of major trauma such as road traffic accidents exemplify the possibility of accidental inoculation of the pathogen into the host. Although the mechanism of infection remains unknown, it is likely that inoculation occurs as open fractures come into contact with contaminating material like soil during the accident. After entering the body, the fungus may be capable of survival or dissemination, depending on the general condition and immunological status of the patient and the virulence of the fungal strain. A similar mechanism of infection is possible in immunocompromised patients with peritoneal dialysis catheter, central venous line, or other piece of indwelling synthetic material. *A. pullulans* has affinity for synthetic materials, for example, Silastic devices and indwelling catheters which, when contaminated, may serve as the primary source and focus of infection. Removal of the contaminated device is generally needed to resolve the infection.[26] The pathogenicity in healthy subjects is low and the fungus is commonly considered as a contaminant in clinical specimens.

Aureobasidium pullulans is an agent of phaeohyphomycosis.[27] Clinically, it has been reported to cause a variety of localized infections, including peritonitis (among patients on peritoneal dialysis), cutaneous infection, pneumonia, meningitis, corneal and scleral infection, and abscesses in the spleen and jaw (reviewed in Hawkes et al. and Richardson and Warnock).[26,28] The underlying conditions of patients with reported *Aureobasidium* infection include carcinoma, leukemia, chronic renal failure, diabetes mellitus, disseminated lymphoma, multiple traumatic injuries, organ transplantation, congenital heart lesion repaired with intracardiac prosthetic material, and pregnancy. Many human infections by *Aureobasidium* have followed traumatic inoculation. Published reports have included keratitis, onychomycosis, cutaneous and subcutaneous phaeohyphomycosis, osteomyelitis of the mandible after tooth extraction, and systemic phaeohyphomycosis in both HIV-infected and non-HIV-infected individuals. Systemic infections have been reported including peritonitis and invasive disease in AIDS patients.

3.1.2.2 Hypersensitivity Pneumonitis (Extrinsic Allergic Alveolitis)

Aureobasidium pullulans is present in the environment and exposure may result in sensitization to the organism and is associated with the development of allergic diseases.[20,29,30] A study in a cohort of 405 children suggested that specific dust-borne fungi (*Aspergillus* and *Aureobasidium pullulans*) found at home in the first 3 months of life might be associated with an increased risk of developing allergic rhinitis by 5 years of age.[29] A further study showed that sensitivity to *A. pullulans* was significantly associated with more severe asthma.[31]

A. pullulans has been associated with an outbreak of hypersensitivity pneumonitis (extrinsic allergic alveolitis).[32] Hypersensitivity pneumonitis is a syndrome that is caused by a broad spectrum of inhaled organic dusts or chemical products that cause an immunologically mediated inflammatory response of the alveoli and bronchioles, frequently accompanied by systemic symptoms. This expression depends on several factors including immunological responsiveness of the host, intensity of the exposure, and antigenicity of the inhaled biological dust. Despite extensive studies, the exact immunological mechanisms are not known. Antigen exposure is associated with the presence of circulating IgG antibodies in exposed individuals. Various forms of hypersensitivity pneumonitis have been described including ventilation hypersensitivity pneumonitis caused by thermophilic actinbacteria, moulds such as *Aspergillus fumigatus*, and *Aureobasidium pullulans*. Cases of hypersensitivity pneumonitis secondary to residential exposure to *A. pullulans* and other fungi proliferating in wet, contaminated building constructions have also been described.[32–34]

3.1.2.3 Mycotic Keratitis

Rare cases of keratomycosis (corneal and scleral ulcer) caused by *Aureobasidium pullulans* have been described. An illustrative case report describes a patient who developed *Aureobasidium pullulans* keratitis following refractive laser epithelial keratomileusis (LASEK).[35] The patient was referred to a tertiary care center 1 month after LASEK for the treatment of a corneal ulcer that was unresponsive to conventional therapy. Mycology culture and direct microscopy identified *Aureobasidium* as the infectious organism. The infection responded well to treatment with topical natamycin and systemic itraconazole.

3.1.2.4 Disseminated Infections, Fungemia and Peritonitis

A. pullulans is a very rare cause of systemic infection in humans. Disseminated systemic infection has been reported in only four cases so far (reviewed in Joshi et al).[36] Of the four cases, one had acute myeloid leukemia, the second patient had ovarian carcinoma (both had Hickman catheters

in situ), the third patient had met with a road traffic accident with accidental inoculation of pathogen, and the fourth was a child with congenital heart disease who had undergone closure of an atrial septal defect with a Goretex patch. Very occasionally, cutaneous involvement by *Aureobasidium* in patients with systemic infection has been reported, for example, in kidney and liver transplant patients.[36]

Nosocomial infections caused by *A. pullulans* are rare and have been described only in some cases of peritonitis involving patients undergoing peritoneal dialysis and in one case of severe infection where the fungus was isolated from a splenic abscess (reviewed in Bolignano and Criseo[37]). Even more uncommon are infections where *A. pullulans* can be isolated from blood cultures. Catheter-related fungemia has been reported.[38]

Cases of *A. pullulans* fungemia after allogeneic stem cell transplantation have been reported.[36] In one case, the blood culture initially grew yeast-like colonies suggestive of *Candida* species.[36] However, on subculture, the characteristic colony morphology (moist and creamy colonies in 2 days, which matured into shiny brownish black colonies with a gray fringe and pigment production) was suggestive of *A. pullulans* fungemia. The source of fungemia in this patient was the central venous catheter.

A rare case of disseminated nosocomial fungal infection was seen in a patient who was severely traumatized following a road traffic accident.[37] The patient was diagnosed with diffuse cerebral edema, hemoperitoneum, multiple fractures of the left femur and tibia, a suspected pelvic fracture, and multiple ruptures of the liver's right lobe, which made immediate surgery mandatory. After surgery, the patient underwent mechanical ventilation and prolonged total parenteral nutrition. *Aureobasidium pullulans* was isolated from blood and urine samples. Isolation from urine was not achieved initially and became possible only after isolation from blood, thus suggesting a progressive colonization of the patient's organs. The patient was treated with fluconazole 400 mg/day. Subsequently, all cultures became negative. The isolates from this case were further identified as *A. pullulans* var. *melanigenum* on the basis of early black pigmentation of the colonies.[2] This appears to be the first reported case of disseminated nosocomial fungal infection by *A. pullulans* var. *melanigenum*.

3.1.2.5 Antifungal Treatment

There is no standard treatment for infection caused by *A. pullulans* because of the paucity of human cases reported in literature. Amphotericin-B alone or in combination with azoles has been tried with variable success. Combination therapy is probably the treatment of choice. The duration of treatment is not certain, though most patients received antifungal treatment for 4–8 weeks. *Aureobasidium* is susceptible to the majority of antifungal drugs with the exception of 5-fluorocytosine.[17,26]

3.1.3 Diagnosis

The diagnosis of *Aureobasidium* infection is seldom suspected until the fungus is isolated from blood, cutaneous lesions, or other clinical specimens.[28] Often, however, patients have died and the infection has remained unrecognized until postmortem material obtained has been investigated.

3.1.3.1 Conventional Techniques

There are no descriptions of direct microscopy being applied to primary clinical specimens from cases of presumed *Aureobasidium* infection. *Aureobasidium* can be isolated from clinical and environmental specimens using various media (see Section 3.2). Histopathological features are similar to those seen for other agents of phaeohyphomycosis. The pigment produced by these fungi is also produced in vivo. The pigment is often a complex phenol-derived substance. Dark-walled, short, septate hyphae may be observed in H&E-stained sections. If the pigment production is not prominent, the use of periodic acid-schiff stain (PAS), Gömöri methenamine silver stain (GMS), or the Fontana-Masson silver stain may be advantageous.[39] There are no serological tests for the diagnosis of *Aureobasidium* infection.

3.1.3.2 Molecular Techniques

Diagnostics, taxonomic placement, and intraspecies variability of *Aureobasidium pullulans* have been assessed by various molecular methods in addition to traditional morphological and physiological studies. These include fingerprinting techniques like restriction enzyme analysis of polymerase chain reaction (PCR)-amplified ribosomal DNA target area (PCR-ribotyping or restriction fragment length polymorphism (RFLP)-PCR),[4,40,41] universally primed PCR (UP-PCR),[40,42] arbitrary primed PCR (ap-PCR),[43] random amplified polymorphic DNA (RAPD-PCR),[43,44] fluorescent amplified fragment length polymorphism (fAFLP),[45] and repetitive-element PCR (rep-PCR) method,[41] as well as sequence analysis of rRNA genes and internal transcribed spacer (ITS) regions, and protein coding genes.[6,7,9,41] Intraspecies variability has also been assessed using whole-cell protein analysis with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) technique.[41] Strain-specific sequence-characterized amplification region (SCAR) primers,[46,47] species-specific oligonucleotide probes,[48] and PCR primers[49] have been developed for the detection of *A. pullulans*, as well as a quantitative PCR (qPCR) assay,[50] see http://www.epa.gov/nerlcwww/moldtech.htm. The applicability of some of these methods for clinical identification and strain typing of *A. pullulans* is discussed below.

Apart from qPCR and other specific PCR protocols, the methods described here require the isolation of the microbe in pure culture. Also a monoculture (in respect of fungi) in normally sterile medium can serve as identification target. At present there are no commercial tools for molecular diagnostics of *Aureobasidium*.

3.1.3.2.1 Molecular Identification of Aureobasidium pullulans

Both DNA sequencing-independent and sequencing-based methods can be used to identify *A. pullulans*. Due to the genetic distance between *A. pullulans* and other pathogens,

DNA-based separation is usually straightforward. This applies to the separation from other melanized hyphomycetes, as well as from yeasts like *Candida albicans*, *Candida parapsilosis*, and *Cryptococcus albidus*, which resemble *A. pullulans* in young culture. Distinguishing *A. pullulans* from nonpathogenic relatives is more challenging, but several unique genetic markers are nevertheless available. RFLP analysis of a PCR amplicon containing partial large subunit (lsu or 28S) rRNA gene and ITS2 region with *DdeI* restriction endonuclease creates unique banding pattern for *A. pullulans*, distinguishing it from phytopathogenic relatives.[4,41,51] For further identification of non-*A. pullulans* strains, enzymes *RsaI*, *HhaI*, *MspI*, and *AluI* can be used according to the scheme presented by Yurlova et al.[4] Since restricted amount of reference information for this region is available, the use of RFLP in diagnostics calls for further validation and may become obsolete for identification along with the reducing prices of more unambiguous DNA sequencing-based methods. It may, however, be used for screening large numbers of isolates for epidemiologic purposes. When more precise results of the identity of studied isolates are wanted, the ITS2 region of the obtained undigested PCR products can be sequenced.

Species specific oligonucleotide probe for small subunit (ssu or 18S) rRNA gene was published for *A. pullulans* in 1996.[48] The probe has been used to detect *A. pullulans* by fluorescent in situ hybridization on microscope slides and leaf surfaces,[52] but it has not been reportedly used in clinical applications. Along with increasing amount of available sequence data, the probe seems to be to some extent unspecific and the use of this probe in clinical settings calls for validation. Other species-wide *A. pullulans*-specific probes have not been published so far in the scientific literature, yet molecular data for the species suggests that this approach would be feasible. Recently, a PCR assay targeting ITS2 region was described for species wide detection of endophytic *A. pullulans* in grapewine.[49] Based on publicly available sequence data, the primer pair is prone to amplify most strains in *A. pullulans* var. *melanigenum* with lowered efficiency compared to var. *pullulans* due to suboptimal matching of the primers.

The U.S. Environmental Protection Agency (EPA) has developed validated qPCR assays for more than 130 major indoor air fungi, including *A. pullulans*. The assays are based on TaqMan chemistry.[53] The primer-probe set targets the ITS1 region, matching well with presently available *A. pullulans* nucleotide data, and has been used to detect and enumerate *A. pullulans* among other fungi in indoor environments.[21,50] The patented assays are available through a license.

Along with lowering costs of sequencing technology and an increasing amount of reference data in public databases, DNA sequencing-based identification is becoming one of the golden standards in mycology. At present, a relatively well covering set of molecular data exists from *A. pullulans* in public DNA databases, including DNA sequence information from about 200 distinct strains or isolates of the species, as well as from related fungi. Most data is retrieved from ribosomal genes and spacers, the ITS region being most widely represented (more than 150 sequences), followed by partial lsu (more than 85 seqs) and full or partial ssu rRNA genes (more than 45 seqs). DNA sequences from the genes elongase (*ELO*, 39 seqs), translation elongation factor (*EF1*α, 29 seqs), and β-tubulin (*TUB*, 29 seqs) are available, but reference data from other fungal species is limited compared to ribosomal targets. The disadvantage of the abundance of published ribosomal sequences is that public databases harbor also misannotated *A. pullulans* sequences as well as non-*A. pullulans* sequences annotated as *A. pullulans*, and hence interpretation of database query results must always be done with deliberation. (Data from EMBL Nucleotide database in April 2009.)

Several studies point out the potential of ITS region sequencing for fungal identification in clinical laboratory.[54–59] Due to the varying degree of inter- and intraspecies variability of ITS region among different fungal clades, few general rules have been suggested concerning the interpretation of sequence comparison results. The intraspecies variation of the whole ITS region is below 3% in *A. pullulans*, whereas the distance to closest relatives is 5% or more; ITS region sequencing readily distinguishes *A. pullulans* from all known pathogenic fungi, as well as from *Hormonema* spp., *Selenophoma mahoniae*, *Pringsheimia* sp., *Dothiora* sp., and *Kabatiella* spp. apart from *K. lini*.[7] ITS2 region is more variable than ITS1 in *Aureobasidium* and relatives,[6] and can be used for the identification alone or in combination with 5.8S rRNA gene and ITS1. Recently, a detailed ITS sequencing-based protocol was presented for the wide-spectrum identification of fungi in clinical laboratory. This included fast DNA extraction from pure culture, universal PCR, and ITS sequencing followed by comparison against a novel commercial database as well as against public DNA database (GenBank).[58] In evaluation study, the protocol provided an identical or more accurate identification compared to traditional identification in case of 84% of tested 244 random clinical isolates. The study included isolates that could not be identified to species level within 5 days by standard phenotypic criteria, including two strains of *A. pullulans*. The database and data handling platform are commercially available from SmartGene (SmartGene, Zug, Switzerland). The authors represent acceptable limits for sequence similarity between analyzed and database sequences for identification to species and genus level. The species-level limits can be applied to *A. pullulans* (see Section 3.2). ITS sequence comparison has been used to identify a pathogen cultivated from septic patient as *A. pullulans*.[38] The authors report the identification of two yeast-like isolates yielded from blood cultures of two septic patients. For one isolate, an initial identification using VITEK system (Yeast Biochemical Card, bioMerieux, France) revealed 90% similarity with *Cryptococcus laurentii*. ITS sequencing revealed 100% similarity with *A. pullulans* for both of the strains.[38]

A quick, culture-independent detection of fungal pathogens directly from clinical samples is achievable by a broad-range fungal real-time PCR reaction recently described by

Vollmer et al.[60] The assay utilizes TaqMan chemistry and is designed for the variable D1/D2 region of lsu rRNA gene. Species identification is attainable by sequencing the positive PCR product, yet only genus-level identification can be achieved in case of some species belonging to genera with little genetic variation within the region. *Aureobasidium pullulans* is detected by the assay with slightly lowered sensitivity due to suboptimal primer matching. The authors report results consistent with cultivation-based identification and a diagnostic turnaround time of 9 h for the identification of fungal pathogens in cervical swabs and tracheal secretion samples.[60] The method is not quantitative. Traditional PCR targeting the same region has commonly been applied to identifying numerous ascomycetous yeasts as well as medically important zygomycetes and dematiaceous fungi, including *A. pullulans*.[10,61–63]

3.1.3.2.2 Molecular Subtyping of *A. pullulans*

A. pullulans shows considerable variability in its vegetative morphology and physiological properties.[5] There is also a remarkable amount of genetic diversity inside *A. pullulans* that differentiates between distinct varieties, and can also be used for strain-specific monitoring.[7,41,45–47] Redefinition of *A. pullulans* and its varieties has recently been presented by Zalar et al.[7] Data from multigene analysis supports the division of *A. pullulans* into two well-known entities, *A. pullulans* var. *pullulans* and var. *melanigenum*, as well as into two novel varieties, var. *subglaciale* and var. *namibiae*.[7] Sequence comparison of variable D1/D2 region of lsu rRNA gene clusters strains with shared morphological and metabolic characteristics into the above-mentioned varieties with high confidence.[7] Even more precise separation is achieved by comparison of partial sequences of the ELO gene.[7] The genetic relationships of the four varieties as depicted by alignment of D1/D2 region of the lsu rRNA gene are shown in Figure 3.2. Potentially novel entities comprising tropical isolates of *Aureobasidium* were recently characterized from Thailand using sequence analysis of five gene loci. Combined data analysis of BT2, RPB2, EF1-1α, and ITS sequences produced 12 well-supported phylogenetic clades. The studied strains shared a good level of congruence in respect of colony characteristics and level of *pullulan* production and xylanase activity within each clade. The clades are separate from the var. *pullulans*, but their relation to other entities remains to be solved.[13] Certain strains of *A. pullulans* are of commercial interest due to their biocontrol abilities and production of specific metabolites like the water-soluble polysaccharide *pullulan* and β1.3–1.6 glucans with reported beneficial immunomodulatory properties.[13,46]

Several papers describe the design of strain-specific primer and probing systems for tracking biocontrol strains of *A. pullulans* in agricultural environment, especially in phylloplane.[46,47,52,64] Meanwhile, the genetic properties of clinical *Aureobasidium* isolates have not been assessed, and there is no information of genotypic differences between clinical strains and the rest of *A. pullulans*. According to available geno- and phenotypic data, practically all clinically significant strains of *A. pullulans* cluster to the var. *melanigenum*.[10,37,38,56,60,65] This is consistent with the thermophilic nature and strong

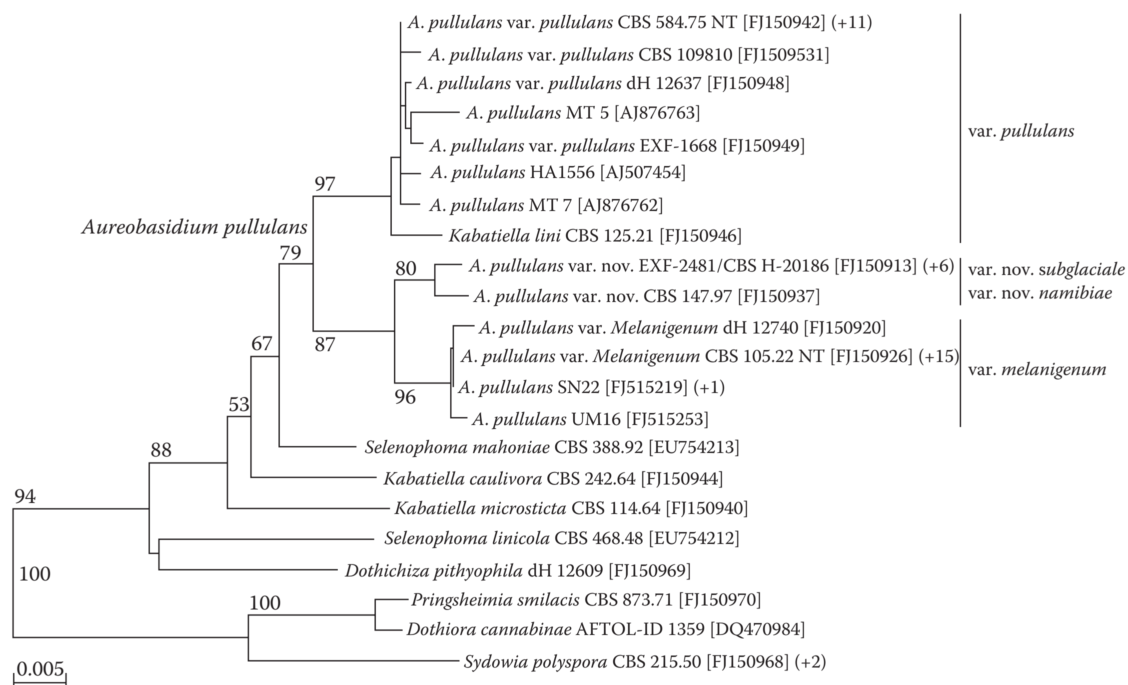

FIGURE 3.2 Neighbor joining tree of *Dothioraceae* showing relationships among varieties of *Aureobasidium pullulans* and relatives. The taxon and strain name, sequence id (in square brackets), and number of additional strains with identical sequence presently available (in brackets) are given for representative strains. Bootstrap values above 50% are shown.

pigmentation of *A. pullulans* var. *melanigenum* that may play a role in increased pathogenicity of the variety.[2,7]

3.2 METHODS

Like many fungi of low virulence, *A. pullulans* is considered a contaminant when isolated from a healthy host. It may be considered a pathogen when isolated from a normally sterile site, from multiple samples, in the presence of clinical signs of infection, or with pathological evidence of tissue-invasive disease.[26]

3.2.1 Sample Preparation

3.2.1.1 In Vitro Pure Culture

Aureobasidium can be isolated from clinical and environmental specimens using various media, for example, half-strength corn meal agar (CMA), rose bengal agar, dichloran rose bengal chloramphenicol (DRBC), MEA, sabourad dextrose agar (SDA), or brain heart infusion agar with gentamicin and chloramphenicol, and identified on yeast malt extract agar (YMA), MEA, glucose peptone agar, SDA with gentamicin and chloramphenicol, potato dextrose agar (PDA), or blakeslee's MEA.[7,13,58] On MEA or glucose peptone agar, *Aureobasidium* grows rapidly when incubated at 30°C, reaching 30 mm in 1 week, appearing flat and smooth, and soon covered with slimy exudates. The optimum temperature for growth is 25°C, maximum near 35°C. The var. *melanigenum* has higher optimum temperature for growth (30°C) than other varieties, and some strains can grow in 37°C. *Aureobasidium* does not grow on media containing cycloheximide. It tolerates salt concentrations up to 10%–15%. In the setting of fungemia, *Aureobasidium* can be isolated in blood culture systems, for example, the Bactec 9240, using pediatric aerobic culture media.[26]

3.2.1.2 DNA Extraction

Alternative DNA extraction protocols suitable for putative *Aureobasidium* and other pathogenic fungal isolates are presented here. They differ in the utilization of in-house methods and reagents, or fast but more costly commercial products and equipment. All used equipment must be DNA free. The methods below are for fungal pure cultures. For DNA extraction from clinical samples and direct PCR detection of fungi, see Vollmer et al.[60] For additional information about DNA extraction procedures, see Hebart et al.[66]

1. Cetyltrimethylammonium bromide (CTAB) extraction. Collect 1 cm^2 of mycelium from plate culture with disposable loop and transfer to 2 mL Eppendorf tube containing 2:1 mixture of silicagel and Celite 545 and 300 μL CTAB buffer (Tris–HCl 200 mM, pH 7.5, 200 mM Na-EDTA, 8.2% NaCl w/v, 2% CTAB w/v). Grind with micropestle for 1–2 min. Add 200 μL of CTAB buffer. Shake to homogenize. Incubate in 65°C water bath for 10 min. Add 500 μL of chloroform and vortex briefly. Centrifuge at 14,000 rpm for 5 min. Transfer the supernatant to clean Eppendorf tube, and add two volumes of cold (−20°C) 96% ethanol. Mix gently. Incubate in −20°C for 30 min to overnight. Centrifuge at 14,000 rpm for 5 min, discard supernatant, and wash the pellet twice with 500 μL cold 70% ethanol. Dry in room temperature until no liquid is visible on the pellets (~5–30 min). Resuspend in 50 μL TE buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA) containing 20 mg/mL RNase A. Incubate for 5–30 min at 37°C to dissolve DNA. Adapted from Gerrits vd Ende and de Hoog.[67]
2. Fastprep extraction. Collect 1 cm^2 of mycelium from plate culture with a disposable loop into 1 mL of TE buffer, pH 8.0. Add about 0.5 mL volume of glass beads (0.5 mM Zirconia/Silica beads, BioSpec Products, Inc., Bartlesville, OK). Disrupt the mycelium using FastPrep instrument (QBiogene), set at number 4, for 30 s. Isolate DNA from the lysate using QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions. Adapted from Manitchotpisit et al.[13]
3. FTA® filter extraction. Collect and suspend fungal mycelium by scraping from culture plate to 0.2–1 mL of sterile water yielding a mixture of fungal cell mass and water in proportion of ca. 1:10–20 v/v and vortex briefly to suspend. Transfer 125 μL of the solution directly to a FTA Microcard (Whatman International Ltd.). Place the damp cards open in a microwave and subject to two cycles of heating at 800 W for 30 s, with a pause of at least 30 s between cycles. Place a pyrex beaker containing 50 mL of sterile water to dissipate excess heat. Store the treated filters in sterile plastic bags containing desiccant. For use in PCR, remove a 2 mm punch from FTA filter with Harris micro-punch (Whatman International Ltd.) into a PCR reaction tube and wash the filter twice for 1 min with 100 μL of Whatman FTA wash reagent and twice for 1 min with 100 μL of TE buffer. Dry tubes with filters for 5 min at 55°C on a dry heat block. Add PCR reaction mixture directly to the washed and dried filter punch. The punches can be reused at least five times in a new PCR reaction with same or different primers without carryover of primers or products from the first reaction, after washing and drying the filter as described before. Adapted from Borman et al.[68]

3.2.2 Detection Procedures

3.2.2.1 Morphological Identification

The colonies are cream or pink and then later becoming brown or black (see Section 3.1.1.2). The microscopical morphology of *Aureobasidium*, particularly the synchronous blastoconidial formation, is best studied utilizing the Dalmau plate method for demonstrating chlamydospores in *Candida albicans*. *Aureobasidium* is differentiated from *Hormonema*

dematiodes by synchronous blastoconidia produced only from hyaline hyphae. However, Yurlova et al. have noted that it is often difficult to distinguish between these modes of conidiogenesis and they suggested differences in the number of conidiogenesis loci and physiological tests as methods for differentiation.[40] These differences, however, are also small and variable. *A. pullulans* and *H. dematioides* are easily distinguishable by genotype, for example, by means of DNA sequencing or RFLP analysis.[6]

3.2.2.2 PCR Amplification of rDNA

Perform amplification of ribosomal DNA target in 50 µL reaction volume in 250 µL reaction tubes or plates. Prepare a PCR reaction mastermix for samples and negative control reaction, containing 1 U of DNA polymerase (e.g., DyNAzyme™ II DNA polymerase, Finnzymes, Espoo, Finland), 1× solution of matching reaction buffer, 250 µM of each dNTP, and 50 pmol of each primer per reaction. Divide into 49 µL aliquots and add 1 µL of genomic DNA extract for template. Use following thermal conditions: initial denaturation for 2 min in 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing at T_{ann} for 45 s and elongation at 72°C for 1 min, followed by a final elongation in 72°C for 10 min in thermocycler apparatus with heated lid. For amplification of ITS region, use primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)[69] and T_{ann} of 50°C. For the D1/D2 region of lsu rDNA gene, use primers NL 1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL 4 (5′-GGTCCGTGTTTCAAGACGG-3′)[61] and T_{ann} of 52°C. For PCR-RFLP analysis, perform PCR protocol with primers 5.8SR (5′-TCGATGAAGAACGCAGCG-3′) and LR7 (5′-TACTACCACCAAGATCT-3′).[51] Use elongation time of 2 min instead of 1 min at 72°C and T_{ann} of 50°C. Visualize the products by electrophoresis in 1% agarose gel with ethidium bromide staining (0.5 µg/mL). The expected products sizes are 581–582 bp for ITS1–ITS4, 614 bp for NL1–NL4, and ~1900 bp for 5.8SR-LR7. The primers are universal fungal, that is, amplify the target region from all fungal species. Purify the products for sequencing and RFLP application with, for example, QIAquick PCR purification kit (Qiagen). In case of failed amplification, try diluting the template DNA in 1:100 and 1:1000 in sterile water, use more efficient DNA polymerase (e.g., Phusion® high fidelity DNA polymerase, Finnzymes) or repeat DNA extraction with different protocol.

3.2.2.3 DNA Sequencing

Comparison of ITS region sequence is suitable for species identification of putative *A. pullulans*. Varieties are most reliably resolved using the variable D1/D2 region of lsu rRNA gene or ELO gene.[7] The most popular method for sequencing is enzymatic Sanger sequencing, using, for example, BigDye™ 3.1 chemistry of Applied Biosystems (Life Technologies, Carlsbad, CA). Sequencing is available as commercial service. The obtained PCR products can be sequenced using one of the primers used in PCR, that is, ITS4 for ITS region and NL4 for lsu D1/D2, or with both forward and reverse primers to get sequence from both DNA strands, for best fidelity.

3.2.2.4 Phylogenetic Analysis of Obtained Sequences

Compare the obtained sequences with published DNA sequences using online Blast or Fasta alignment tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi and http://www.ebi.ac.uk/Tools/fasta33/nucleotide.html) against GenBank or EMBL DNA database. Note that Blast alignment easily "drops out" the perimeters of the alignment if lower similarity occurs on the area, thus giving a partial alignment with high percentage identity. Fasta is slower compared to Blast but performs full alignment for the target sequence. High similarity percentages (>99%) with reference sequences from authenticated strains should be emphasized in interpretation of the results. See Figure 3.2 for reference strains. An ITS sequence from *A. pullulans* is supposed to have a similarity of 97%–100% with preferably two or more independent *A. pullulans* database sequences. The D1/D2 lsu rRNA sequences of isolates belonging to same variety generally show 99%–100% similarity, whereas the distance between varieties is generally 2%–3%. For ITS-sequencing-based identification of non-*A. pullulans* strains, guidelines given by Ciardo et al.[58] are recommended to be followed.

3.2.2.5 RFLP Analysis of PCR Products

Digest the obtained 5.8SR-LR7 PCR products (10 µL per digestion) with *DdeI* and optionally with *HhaI*, *MspI*, and *RsaI* restriction endonucleases according to manufacturer's instructions. Amplify and digest corresponding fragment in parallel from representative, authenticated reference strains. Separate the digests in 2% agarose gel with appropriate size standard. Stain with ethidium bromide. *DdeI* produces unique digestion pattern for *A. pullulans*, whereas other enzymes distinguish between *Hormonema* sp. and other relatives of *Aureobasidium*.[4]

3.3 CONCLUSIONS AND FUTURE PERSPECTIVES

With increasing survival of immunocompromised patients, the significance of rare and weakly pathogenic fungi becomes more obvious. During past years, several species with some pathogenicity to humans have been affiliated to the genus *Aureobasidium*, but apart from *A. pullulans*, these have later been shown to be more closely related to other genera. *Aureobasidium pullulans* represents an opportunistic fungus that only occasionally causes disease, but it has been reported to be involved in serious conditions including pneumonia, meningitis, and systemic fungemia. The diagnosis of *Aureobasidium* traditionally relies on observing characteristic macro- and micromorphology, yet these characteristics vary by strains and *Aureobasidium* is often mixed up with other black yeast-like fungi or even yeasts. *A. pullulans* is of commercial interest due to its biocontrol abilities and production of various metabolites. In this

perspective, the fungus is relatively well-characterized and the taxonomy, identification, and subspecific typing of agricultural, biotechnological, and environmental strains have been assessed in numerous studies. In contrast, there are few studies that involve both clinical aspects, and phylogeny or development of molecular identification methods. No commercial products are presently available for specific detection of *A. pullulans*. Molecular methods like probing techniques, qPCR, and DNA sequence analysis are nevertheless very well suited also for the identification of *Aureobasidium* in clinical laboratory, but most methods still need further validation.

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4 *Bipolaris* and *Drechslera*

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4.1 INTRODUCTION

The term "dematiaceous fungi" refers to a heterogeneous group of darkly pigmented fungal organisms, which derive their coloration from melanin in the cell walls, and produce brown yeast-like cells, pseudohyphae, and irregular true hyphae in tissues. Being commonly present in the soil, plants, and other environments, dematiaceous fungi are introduced into human hosts through inhalation, or after injury and other traumatic events, causing a range of superficial and deep infections that are collectively known as chromoblastomycosis, eumycetoma, and phaeohyphomycosis.

Currently, over 130 dematiaceous fungal species belonging to 70 genera have been implicated in human infections. While both chromoblastomycosis and eumycetoma are associated with a relatively small group of dark-walled fungi, the former is characterized by the formation of sclerotic bodies in tissue and is usually seen in tropical areas, and the latter is a deep tissue infection characterized by the presence of mycotic granules, usually of the lower extremities. On the other hand, phaeohyphomycosis is generally reserved for the remainder of clinical syndromes (ranging from superficial infections, allergic disease, pneumonia, cerebral infection to disseminated disease) caused by a large number of dematiaceous fungi including members of *Bipolaris* and *Drechslera* genera.

4.1.1 Classification and Morphology

The genus *Bipolaris* belongs to the mitosporic *Cochliobolus* group, family *Pleosporaceae*, order Pleosporales, class Dothideomycetes, subphylum Pezizomycotina, phylum Ascomycota, and kingdom Fungi. The family *Pleosporaceae* covers the genera of *Brachycladium*, *Cochliobolus*, *Crivellia*, *Decorospora*, *Edenia*, *Lewia*, *Macrospora*, *Macroventuria*, mitosporic *Cochliobolus*, mitosporic *Pleosporaceae*, *Pleospora*, *Pyrenophora*, *Setosphaeria*, and some unclassified *Pleosporaceae*; the mitosporic *Cochliobolus* group is made up of the genera of *Bipolaris* and *Curvularia*. The genus *Bipolaris* (teleomorph: *Cochliobolus*) is further divided into 33 recognized species and 36 unassigned species [1].

Bipolaris spp. are primarily plant pathogens; however, several (i.e., *Bipolaris spicifera* [obsolete synonyms: *Curvularia spicifera*, *Brachycladium spiciferum*, *Drechslera spicifera*, and *Helminthosporium spiciferum*], *Bipolaris hawaiiensis* [obsolete synonyms: *Drechslera hawaiiensis* and *Helminthosporium hawaiiensis*] and *Bipolaris australiensis* [obsolete synonyms: *Drechslera australiensis* and *Helminthosporium australiensis*]) have been implicated in human infections [2].

Bipolaris colonies grow moderately fast and are effuse, grey to blackish brown, suede-like to floccose with a black reverse. Pale brown pigmented, pseudoseptate conidia are produced through pores (poroconidia) in a sympodially elongating geniculate (or zigzag) conidiophore and are straight, fusiform to ellipsoidal, rounded at both ends, smooth to finely roughened, and germinating only from the ends (bipolar) [3].

At the species level, *B. spicifera* conidia are cylindrical with a broadly rounded base, have three septa and a distinctly raised hilum, which shows a zone of dark

pigmentation. *B. australiensis* conidia are ellipsoidal with a more or less tapering base, which becomes truncated because of the hilum, and 20% of its conidia are with four or five septa. In addition, *B. spicifera* conidia are slightly broader than those of *B. australiensis*, and *B. spicifera* isolates have a small hyaline area just above the hilum, which is absent in *B. australiensis*. *B. hawaiiensis* conidia are approximately 6.8 by 23.3 µm and have predominantly four or five septa [2]. Moreover, *B. spicifera* is differentiated from *B. australiensis* by its thinner cell wall, from *B. hawaiiensis* by its production of swollen conidia with 3 to 7 septa, and from *B. papendorfii* by its being curved and broadest at the second cell.

The genus *Drechslera* belongs to the mitosporic *Pleosporaceae* group in the family Pleosoraceae, and contains the genera *Alternaria*, *Dendryphiella*, *Dendryphion*, *Drechslera*, *Embellisia*, *Exserohilum*, *Nimbya*, *Pithomyces*, *Pyrenochaeta*, *Stagonospora*, *Stemphylium*, *Ulocladium*, and *Unifilum*. At present, there are 20 recognized species and 11 unassigned species within the genus *Drechslera* (teleomorph: *Pyrenophora*) [1].

Drechslera spp. are generally present in soil and plants although one of its members, i.e., *Drechslera biseptata*, has been reported recently from a brain abscess. Many previous *Drechslera* or *Helminthosporium* isolates from human and animal cases have been shown to actually belong to the genera *Bipolaris* or *Exserohilum* [2].

Drechslera colonies grow rapidly and are suede-like to downy, brown to blackish brown with a black reverse. Conidia are pale to dark brown, usually cylindrical or subcylindrical, straight, smooth walled, and are formed apically through a pore (poroconidia) in a sympodially elongating geniculate conidiophore. Conidia are transversely septate (phragmoconidia), and the hilum is not protuberant [3].

As closely related members of the mitosporic *Cochliobolus* group and the mitosporic *Pleosporaceae* group within the family *Pleosporaceae*, *Bipolaris* (teleomorph: *Cochliobolus*), *Curvularia* (teleomorph: *Cochliobolus*), *Drechslera* (teleomorph: *Pyrenophora*), and *Exserohilum* (teleomorph: *Setoshaeria*) are differentiated through a combination of characters such as conidial shape, the presence or absence of a protruding hilum, the contour of the basal portion of the conidium and its hilum, the point at which the germ tube originates from the basal cell, and the sequence and location of the first three conidial septa.

Specifically, *Bipolaris* conidia are fusiform ellipsoidal and central cells are not much darker and broader than the distal ones; hilum is not protuberant and germination is bipolar. *Drechslera* conidia are cylindrical, germinating from any cell, and hilum is not protuberant. *Curvularia* conidia have two to three broader and darker central cells, often curved, with or without a prominent hilum; germination is bipolar. *Exserohilum* conidia are fusiform cylindrical to obclavate, with a protuberant hilum; germination is bipolar [4].

Furthermore, in common with *Curvularia*, some *Bipolaris* spp. also produce curved conidia with hyaline apical cells. The curvature in *Bipolaris* conidia involves a slight change in the central cell, whereas those in *Curvularia* conidia are due to the enlargement of the central cell. Thus, *Bipolaris* is distinguishable from *Curvularia* by the absence of an enlarged cell in its curved conidia.

4.1.2 Clinical Features and Pathogenesis

Bipolaris and *Drechslera* are dematiaceous (dark-walled) fungi that are occasionally involved in human infections including cutaneous infections, sinusitis, keratitis, peritonitis, allergic bronchopulmonary disease, brain abscess, meningitis, fatal fungal endarteritis, fungemia, and disseminated fungal disease [5–29].

Bipolaris spicifera is the most commonly reported human pathogenic *Bipolaris* species, especially in subtropical and tropical regions (e.g., Texas, South Carolina, Arizona, and Georgia in the United States; Brisbane, Australia; Pakistan; and India). Other less common *Bipolaris* spp. associated with human disease are *Bipolaris hawaiiensis* and *Bipolaris australiensis*.

4.1.2.1 Cutaneous infections

Bipolaris spicifera has been reported as the causative agent of cutaneous infections in both immunocompetent and immunocompromised individuals [30–34]. Bilu et al. [35] documented a case of *Bipolaris spicifera*-related cutaneous fungal infection on the left cheek of a 5-year-old boy with B-precursor-cell acute lymphoblastic leukemia.

4.1.2.2 Mycotic keratitis

Bipolaris spp. have also been shown to cause mycotic keratits [36–43]. Bashir et al. [44] described a *Bipolaris hawaiiensis*-related corneal ulcer with abscess and hypopyon in an immunocompetent male following trauma with rice stalk to the left eye. Direct examination of the corneal scrapings showed septate, branched fungal hyphae. Culture of the scrapings on blood agar and Sabouraud dextrose agar yielded *Bipolaris hawaiiensis*.

4.1.2.3 Rhinosinusitis

Allergic fungal sinusitis (AFS) is a noninvasive form of fungal rhinosinusitis accounting for 6%–9% of all rhinosinusitis requiring surgery. Patients with AFS typically show chronic rhinosinusitis with nasal polyps, inhalant atopy, elevated total serum immunoglobulin E (IgE), and sinus-obstructing inspissates of a characteristic extramucosal "peanut buttery," viscoelastic, eosinophil-rich, hyphae-containing material called "allergic mucin." Sinus computer tomography (CT) shows findings of chronic rhinosinusitis that often include central areas of increased contrast ("hyperattenuation") within abnormal paranasal sinuses due to the presence of fungal-containing allergic mucin. AFS allergic mucin cultures are often positive for either dematiaceous fungi such as *Bipolaris spicifera* or *Curvularia lunata*, or *Aspergillus* species such as *A. fumigatus*, *A. flavus* or *A. niger* [45,46]. Indeed, *Bipolaris* spp. are increasingly recognized as the

cause of fungal sinusitis in humans with the sphenoid and posterior ethmoid sinuses being most often involved followed by the anterior ethmoid sinus, frontal sinus, and maxillary sinus involvement [47–58].

Buzina et al. [59] presented a detailed description of *Bipolaris spicifera*-associated rhinitis in a 19-year-old immunocompetent man after the patient presented with restricted nasal breathing. A CT scan showed massive sinusitis with significant decalcification and destruction of the bone at skull base. An endoscopic examination revealed a total obstruction of both nostrils with glassy polyps. The immunological examination of the patient's blood demonstrated a highly elevated level of total IgE. Histological examinations of formalin-fixed and paraffin-embedded tissue and mucus samples resected/collected from sinus showed inflammatory sinonasal polyps and clusters of eosinophilic granulocytes within the mucus. Gomori's methenamine silver (GMS) staining uncovered septate fungal hyphae in the fungal masses (fungus balls) as well as within the mucus, although a fungal invasion of the tissue was not observed. Culture of the mucus on Sabouraud dextrose agar at 25°C showed fast-growing colonies, reaching a diameter of 4 cm in 7 days. Colonies were velvety, brownish, and flat. Microscopic observation showed septate, pigmented hyphae and unbranched, zigzagged conidiophores with thick-walled, darkly pigmented, cylindrical conidia (predominantly with three septate). The fungus was identified as *Bipolaris spicifera*, which was further confirmed by sequencing analysis of the internal transcribed spacer (ITS) region of the ribosomal gene cluster using fungus-specific primers. Indeed, the resulting 575 bp amplicon demonstrated a 100% identity with *Cochliobolus spiciferus*, which is the teleomorph of *Bipolaris spicifera*. In a separate study, Castelnuovo et al. [53] described a phaeohyphomycotic sinusitis due to *Bipolaris hawaiiensis* in an immunologically competent patient. The patient was successfully treated by surgical drainage and amphotericin B.

Dyer et al. [60] reported a *Bipolaris australiensis*–associated allergic bronchopulmonary disease in a 40-year-old white male with past history of allergic rhinitis and asthma. The patient presented with back pain and cough. CT scan of the chest showed a mass in the lower lobe of the right lung. Culture of the bronchial biopsy and lavage yielded a pure isolate of *Bipolaris australiensis* that was confirmed by DNA technique.

4.1.2.4 Other Infections

Kobayashi et al. [61] described a *Bipolaris spicifera*-related case of disseminated infection in an immunocompetent male. Histopathological studies of lymph node, lung, and liver biopsy specimens revealed a dark pigmented, granular fungal structure inside the granuloma. Culture of lymph node specimen grew a fungal isolate that was identified as *Bipolaris spicifera* on the basis of morphology and molecular analysis. The patient responded to combination therapy with intravenous amphotericin B and voriconazole, but the infection reoccurred during oral itraconazole.

Gadallah et al. [14] documented a case of *Bipolaris hawaiiensis* peritonitis in a 73-year-old female on continuous cyclic peritoneal dialysis (CCPD) with a nonfunctioning peritoneal catheter. The catheter harbored characteristic dark gray particles consisting of a fungal ball within the lumen of the catheter. Microscopic examination confirmed the organism attached to the inner wall of the catheter. The patient was treated by removing the catheter and the administration of a 2 week course of oral itraconazole 100 mg twice daily (without using amphotericin B or ketoconazole).

In combination with surgical debridement *Bipolaris* infections are often treated with amphotericin B, which is the antifungal drug of choice for chronic invasive sinusitis [7]. Ketoconazole, itraconazole, voriconazole, and natamycin may also be utilized [8,23,41]. Itraconazole is a highly effective antifungal agent for chromoblastomycosis and subcutaneous phaeohyphomycosis; ketoconazole is useful for mycetoma. Postoperative oral corticosteroids and antiallergic inflammation therapy may also be prescribed for AFS [62,63].

4.1.3 Laboratory Diagnosis

Dematiaceous (brown- or dark-pigmented) fungi are a heterogeneous group of molds that are classified in the genera *Alternaria*, *Bipolaris*, *Cladophialophora*, *Curvularia*, *Drechslera*, *Exserohilum*, *Exophiala* (*Wangiella*), *Fonsecaea*, *Madurella*, *Phialophora*, *Scedosporium*, and *Scytalidium*. These fungi are widespread in soil, wood, and decomposing plant debris and are opportunistic human pathogens causing phaeohyphomycosis, chromoblastomycosis, and eumycotic mycetoma.

Traditionally, dematiaceous fungi are identified on the basis of morphological characteristics such as the presence of annellides (*Phaeoannellomyces*, *Exophiala*), phialides (*Phialophora*, *Wangiella*), or adelophialides (*Phialemonium* without collarettes, *Lecythophora* with collarettes); differentiation of conidiophores (*Xylohypha*, *Cladosporium*) and conidial hilum, septation and germination (*Bipolaris*, *Drechslera*, *Exserohilum*) [2]. Fontana-Masson stain is helpful for distinguishing the pigmented dematiaceous organisms from other septated fungal forms [45]. For histological examination, GMS and hematoxylin and eosin (H&E) stains may be employed. Enhancement of sporulation by growth of species of *Bipolaris*, *Curvularia*, *Drechslera*, and *Exserohilum* on cellulose substrates may facilitate their identification in culture and production of spores at relatively high concentrations [64].

Biochemical tests (e.g., 12% gelatin test, nitrate assimilation) and determination of growth temperature (e.g., 37°C for *Exophiala jeanselmei*, 40°C for *Exophiala* [*Wangiella*] *dermatitidis* and *Bipolaris spicifera*, 42°C for *Xylohypha bantiana*, and 45°C for *Dactylaria constricta* var. *gallopava* and *Scedosporium inflatum*) may also be useful for their differentiation [65]. Fluorescent halogen immunoassay (fHIA) may be utilized for examination of mycoaerosols from individuals with exposure and sensitization to fungal allergens in indoor environments. Indeed, *Bipolaris* conidia were commonly

detected from these individuals by this immunostaining procedure [66].

In recent years, molecular techniques have been applied for identification of dematiaceous fungi including *Bipolaris* and *Drechslera* [58,67]. Polymerase chain reaction (PCR) amplification and sequencing analysis of rRNA internal transcribed spacer (ITS) regions allow rapid and precise diagnosis of fungal infections in humans [67–69].

4.2 METHODS

4.2.1 Sample Preparation

Specimens may be stained with lactophenol cotton blue stain or periodic acid-Schiff (PAS) stain and examined under the microscope for mycotic elements. Portions of samples are grown on inhibitory mold agar, modified Sabouraud agars, or potato dextrose agar at 25°C and 35°C.

After growth of 1–7 days on potato dextrose agar slants, mycelia are collected by scraping the slant with a sterile stick in 1 mL of sterile water. The material is transferred to a 2 mL screw-cap tube. The tubes are centrifuged for 1 min at 6000 × *g*. If the mycelia do not pellet, the material is contained with a pediatric blood serum filter (Porex Corp). The supernatant is removed and the material is resuspended in 200 μL of IDI sample buffer and transferred to the lysis tube containing glass beads. The lysis tubes are vortexed on the highest setting for 5 min. The tubes are then placed in a boiling water bath for 15 min. The tubes are centrifuged for 5 min at 16000 × *g*. The supernatant is stored at −20°C until amplification [70].

Alternatively, about 1 cm^2 of fungal material is transferred to a 2 mL Eppendorf tube containing a 2:1 (wt/wt) mixture of silica gel and Celite (silica gel H, Merck 7736/Kieselguhr Celite 545; Machery) and 300 μL of TES buffer (2 g Tris [hydroxymethyl]-aminomethane, 0.38 g Na-EDTA [ethylenediaminetetraacetic acid], and 2 g sodium dodecyl sulfate in 80 mL of ultrapure water [pH 8]). The fungal material is ground with a micropestle for 1–2 min. The volume is adjusted by adding 200 μL of TES buffer. After vigorous shaking and the addition of 10 μL of a 10 mg/mL concentration of proteinase K to the tube, the mixture is incubated at 65°C for 10 min. With the addition of 140 μL of 5 M NaCl solution, the mixture is combined with 1/10 volume (~65 μL) of 10% CTAB (cetyltrimethylammonium bromide) buffer, followed by incubation for another 30 min at 65°C. One volume (~700 μL) of chloroform-isoamyl alcohol (vol/vol = 24/L) is added and mixed by inversion. After incubation for 30 min at 0°C (on ice water) and centrifugation at 14,000 rpm at 4°C for 10 min, the top layer is transferred to a clean Eppendorf tube. The sample is added with 225 μL of 5 M NH_4-acetate and incubated for at least 30 min (on ice water) and centrifuged. The supernatant is transferred to a clean sterile Eppendorf tube and mixed with a 0.55 volume (~510 μL) of ice-cold isopropanol. After centrifugation for 7 min at 14,000 rpm and 4°C (or room temperature), the supernatant is decanted. The pellet is washed with ice-cold 70% ethanol twice and dried by using a vacuum dryer. The powder is resuspended in 48.5 μL of Tris-EDTA buffer with 1.5 μL of 10 mg of RNase/mL, incubated at 37°C for 15–30 min, and stored at −20°C until use [71].

4.2.2 Detection Procedures

4.2.2.1 Sequence Analysis of ITS Regions

Pounder et al. [70] described a real-time PCR with SYBR green DNA-binding dye and amplicon melting temperature analysis for fungal detection using pan-fungal primers ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′) [72]. The identity of the fungi is verified by subsequent sequencing analysis.

Procedure

1. PCR mixture is composed of 1× Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science) (containing deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM $MgCl_2$, additional $MgCl_2$ is added to a final concentration of 4.6 mM), 0.4 μM each of ITS1 forward and ITS4 reverse primers, 1× SYBR green (Molecular Probes), and 3 μL template DNA.
2. Thermal cycling parameters include 95°C for 10 min; 50 cycles of 95°C for 5 s, 60°C for 20 s, and 76°C for 30 s; and a final extension at 72°C for 2 min.
3. The quality of the amplicon is determined using the derivative of the melt analysis curve (55°C–99°C, 45 s hold at 55°C, 5 s/°C) using the RotorGene 3000 (Corbett Robotics, Inc).
4. The amplified product is purified for bidirectional sequencing using ExoSAP-IT (USB Corp). Five microliters of Big Dye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems) is added to 4 μL of each primer (0.8 pmol/μL) and 3 μL of purified PCR product. Cycle sequencing is performed with a 9700 thermal cycler (ABI), using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reaction products are passed through a Sephadex G-50 fine column to remove unincorporated dye terminators. Purified sequencing reaction products are run on an ABI Prism 3100 Genetic Analyzer with a 50 cm capillary array.
5. Sequences are analyzed with the SmartGene Integrated Database Network software version 3.2.3 vr. SmartGene is a web-based software and database system with reference sequences derived from the National Center for Biological Information (NCBI) GenBank repository.

Note. In case that real time PCR instrument is not available, standard PCR may be performed with primers ITS1 and ITS4, and the resulting amplicon is sequenced with the

same primers. Sequence-based identifications are defined by percent identity: species, ≥99%; genus, 93%–99%; and inconclusive, ≤93%. For strains producing discrepant identification between the methods based on phenotypic characteristics and ITS sequence analysis, the D1–D2 region of the large-subunit RNA gene is amplified with primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) and sequenced for species clarification [73].

4.2.2.2 Sequence Analysis of D1–D2 Region of 28S rRNA

Kobayashi et al. [61] utilized primers NL-1 and NL-4 in the first-round PCR and primers Saga-F (5′-TTGCGCTAGTAACGGCGAGTGAAG-3′) and Saga-R (5′-CCATTACGCCAGCATCCTAGCAGA-3′) in the second-round PCR to amplify the D1–D2 region of fungal 28S rRNA gene. The resulting PCR product (of 614 bp in length) is sequenced directly by using the primers Saga-F and Saga-R. The sequence from the fungal isolate is then compared to those of dematiaceous fungi in GenBank via nucleotide–nucleotide BLAST analysis.

4.3 CONCLUSION

Bipolaris and *Drechslera* are dematiaceous (dark-walled) fungi that are widely distributed in soil, plants, and air. Several *Bipolaris* and *Drechslera* species are occasionally implicated in human phaeohyphomycosis including cutaneous and subcutaneous infections, keratitis, allergic fungal sinusitis, allergic bronchopulmonary mycosis, disseminated disease, and brain abscess, sometimes with fatal consequence [74].

Conventional diagnosis of phaeohyphomycosis relies on pathological investigation of clinical specimens with the help of Fontana–Masson stain, which is specific for melanin, along with gross and microscopic examination of cultures. As *Bipolaris* and *Drechslera* demonstrate close morphological similarity, the only distinction between them is that *Bipolaris* germinates only from polar cells and *Drechslera* spores germinate from any cell (of the spore).

Considering the difficulty in identification and differentiation of *Bipolaris* and *Drechslera* from other dematiaceous fungi based on phenotypic characterization, application of molecular techniques such as PCR amplification and sequence analysis of ribosomal internal transcribed spacer (ITS) regions is essential for their correct determination, which in turn aids the selection of appropriate control and prevention strategies against these pathogens.

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5 *Botryomyces*

Dongyou Liu and R.R.M. Paterson

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5.1 INTRODUCTION

'Dematiaceous fungi' is a colloquial, nontaxonomic term used to describe a heterogeneous group of fungal organisms that have melanized cell walls. The presence of melanins in these fungi not only accounts for their dark-green, brown, or black color, but also enhances their survival under stressful conditions. Further morphological, physiological, and biological adaption has endowed these organisms with the ability to tolerate desiccation, temperature, and osmolarity changes. Because of their distinct morphological and biological features, dematiaceous fungi are separated into black yeasts, meristematic fungi, microcolonial fungi, and other darkly pigmented fungi, with each of these categories encompassing multiple fungal taxa of its own.

Black yeasts are characterized by the production of daughter cells through yeast-like multilateral or polar budding. Many black yeasts also demonstrate mycelial growth and generate conidia (either unseptated or containing three transversal septa) from phialides (ranging from simple phialides, phialides with collarettes, annelated phialides, to rhachides) or undifferentiated conidiogenous cells.

Meristematic fungi form aggregates of thick-walled, densely melanized cells and grow by isodiametric cell wall expansion and division. Propagules are released by breaking apart of aggregates or by endogenous conidiogenesis that subsequently disrupts the mother cell wall. Some may form blastic conidia from yeast-like budding cells and thus may be regarded as black yeasts. Meristematic fungi are found on the exposed surfaces of desert rock, outdoor statues, leathery plant leaves, and Antarctic rock, as well as in hypersaline coastal ponds. They have the potential to erode and destroy marble, sandstone, and glass.

Microcolonial fungi refer to the in situ growth pattern of the meristematic fungi and some black yeasts on mineral substrates (e.g., rock, glass, or metal), which is characterized by the formation of black cauliflower-like colonies consisting of densely aggregated thick-walled cells. Although colonies of microcolonial fungi belonging to the different taxa are indistinguishable morphologically, upon isolation on suitable growth media, microcolonial fungi readily develop into various morphologies, allowing genus- and species-specific identification.

5.1.1 Classification and Morphology

The genus *Botryomyces* (Botrys, Greek for bunch of grapes, + mykes, Greek for fungus) is a meristematic black fungus belonging to the mitosporic Dothideomycetes group, class Dothideomycetes, subphylum Pezizomycotina, phylum *Ascomycota*, and kingdom Fungi [1]. The mitosporic Dothideomycetes group is divided into nine genera: *Asteromella*, *Botryomyces*, *Cenococcum*, *Cryomyces*, *Cyclothyrium*, *Cystocoleus*, *Racodium*, *Sclerostagonospora*, and *Seifertia*. The genus *Botryomyces* contains a single species *Botryomyces caespitosus*, which was first described from human skin lesions [2]; *Botryomyces angioformans* is a doubtful species from an unconfirmed disease of which no material has been preserved [3].

B. caespitosus colonies are pink when young and form restricted, meristematic cell clumps that disarticulate irregularly. Meristematic growth is characterized by the production of swollen isodiametric cells with thick cell walls, in which melanin is deposited. The fungus produces multicelled, irregularly septate, thick-walled spores, which may be regarded by some to be vegetative structures [2].

The genus *Botryomyces* is one of the 25 genera of meristematic black fungi that inhibit cracks in marble and rock surfaces in the Mediterranean Basin (Italy, Spain) and Ukraine. They are associated with biodeterioration of monuments, sculptures, and archaeological objects. Because meristematic black fungi present characteristics comparable to those

of fungi isolated from deserts rocks, they are occasionally referred to as "microcolonial fungi." In addition, the term "black yeast" is sometimes used to describe black fungi that have yeast-like stages of reproduction and a meristematic growth pattern.

Meristematic black fungi are classified under four families within the *Ascomycota*: (i) *Herpotrichellaceae* (order Chaetothyriales) covers *Exophiala* and *Sarcynomices petricola*; (ii) *Dothideaceae* (order Dothideales) consists of some epiphytic species occasionally isolated from rocks such as *Trimmatostroma abietis*, *Aureobasidium pullulans*, and *Hortaea werneckii*; (iii) *Capnodiaceae* (order Capnodiales) includes *C. renispora*, which was isolated from a tile; and (iv) *Pleosporaceae* (order Pleosporales) includes *B. caespitosus*, which is closely related to *Alternaria* (which is also frequently found on stones) based on internal transcribed spacer (ITS) sequence analysis [4,5].

Stone-inhabiting meristematic black fungi tend to show intercrystalline growth by colonizing the weakest parts along marble crystals, leading to the detachments of crystals. They also grow preferentially in cavities and in already-formed cracks and fissures, often producing a deepening of the fissures.

5.1.2 Clinical Features

Meristematic black fungi such as *B. caespitosus* are recognized agents of phaeohyphomycosis [6] as distinct from chromoblastomycosis. Readers should be aware of the term "botryomycosis," which, surprisingly, refers to a bacterial infection (affecting the skin, and sometimes the viscera due to *Staphylococcus aureus* and several other bacteria) [7] and should not be confused with the current disease. Phaeohyphomycosis is cosmopolitan although patients are usually adults and approximately half are immunocompromised; however, this figure has probably increased substantially until the present time. Lesions may occur on almost any part of the body, often on exposed areas, with the upper arm lesions being most prevalent. The most typical and common lesions are cutaneous or subcutaneous abscesses or cysts. Primary lesion is a single, discrete, asymptomatic small nodule and this evolves gradually to an encapsulated, fluctuant abscess with a liquefied center. However, the overlying epidermis is hardly affected. Occasionally, a slightly elevated, granulomatous plaque appears when the main site of the lesion is in the dermis and epidermis. Infrequently, it is observed as a small verrucous nodule or a verrucous plaque comprising a coalescent nodule, which actually resembles chromoblastomycosis. Phaeohyphomycosis may involve the central nervous system or other internal organs (e.g., liver, lungs, and pancreas) and may appear as a hematogenous metastasis from cutaneous or subcutaneous infections or with no visible lesions.

Specifically, *B. caespitosus* may gain entry into human hosts by traumatic inoculation, through prolonged contact with domestic animals, and presumably via airborne dissemination of propagules. The fungus is responsible occasionally for chromoblastomycosis-like subcutaneous infections after trauma. Clinical symptoms may range from dermatomycosis (mycoses), cutaneous phaeohyphomycosis to mycotic granuloma. Skin lesions appear on arms and legs, usually in immuno compromised patients or in patients with chronic renal failure, transplants, and immunosuppressive therapy [8,9]. Human infection of tonsils with *B. caespitosus* may exhibit recurrent tonsillitis, sore throat, dysphasia, high temperature, and enlarged tonsils. Upon examination, the tonsils may show "grains" in the crypts [10].

5.1.3 Diagnosis

As meristematic fungi lack pronounced diagnostic features, species-specific identification on the basis of microscopic morphology and reproductive structures (e.g., conidiophores, conidia, and conidial ontogeny) is often difficult. This is further exacerbated by the fact that many meristematic fungal species are highly pleomorphic, with anamorph life cycles and widely divergent methods of propagation. Some species display meristematic growth as the only type of reproduction, consisting of isodiametrically dividing cells and endoconidiation, which do not allow delimitation of taxa [11]. Thus, morphology gives only a presumptive identification at genus level, and the use of physiological characteristics (e.g., nitrogen and carbohydrate assimilation tests, growth at different temperatures and proteolytic activity) are helpful for their identification.

B. caespitosus may be detected from surfaces by tape lifts or tease mounts from bulk samples. The laboratory diagnosis of chromoblastomycosis is performed by the demonstration of sclerotic bodies upon direct microscopical examination of wet KOH mounts of aspirated pus, skin scrapings, or biopsy material. However, *B. caespitosus* causes occasionally a chromoblastomycosis-like infection; it is in fact a phaeohyphomycosis.

Colonies on malt extract agar are restricted, cauliflower-like, heaped, pale brown initially becoming brown-black with age. Microscopy reveals that hyphae and budding cells are absent. The thallus is composed of clumps of irregularly septate, thick-walled cells, which are subhyaline, becoming dark brown with age. These disarticulate into smaller cell packets. Blastic conidia are in fact occasionally present. A series of approximately 50 physiological tests (e.g., growth on glucose, arabinose, salicin) are also available [3]. Hence, some useful characters are present in the case of this species at least. *B. caespitosus* differs from *Sarcinomyces phaeomuriformis* by young colonies being pink.

Molecular techniques have been applied for the identification of meristematic black fungi including *B. caespitosus*. Polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and analyses of amplified small (SSU, 18S rRNA) and large (LSU, 5.8S rRNA and internal transcribed spacers ITS1, ITS2) subunit ribosomal genes are employed [4,11–14].

5.2 METHODS

5.2.1 Sample Preparation

Molds from a specimen are grown on inhibitory mold agar, modified Sabouraud agars, or potato dextrose agar. Microscopic structures are observed on tease or tape preparations and slide cultures for up to 21 days.

After growth for 1–7 days on potato dextrose agar slants, lysates are prepared from approximately 1 cm^2 of mycelia with IDI lysis kits (GeneOhm Sciences, San Diego, CA). Briefly, in a biological safety cabinet, mycelia are collected by scraping the slant with a sterile stick in 1 mL of sterile, molecular-grade H_2O. The material is transferred to a 2 mL screw-cap tube. The tubes are centrifuged for 1 min at 6000 × *g*. If the mycelia does not pellet, the material is contained using a pediatric blood serum filter (Porex Corp., Fairburn, GA). The supernatant is removed. The material is resuspended in 200 µL of IDI sample buffer and transferred to the lysis tube, which contains glass beads. Lysis tubes are vortexed on the highest setting for 5 min. The tubes are placed in a boiling water bath for 15 min and centrifuged for 5 min at 16,000 × *g*. The supernatant is stored at −20°C until amplification [13].

5.2.2 Detection Procedures

Pounder et al. [13] described a real-time PCR with SYBR green DNA-binding dye and amplicon melting temperature analysis for fungal detection using pan-fungal primers ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′) that cover the internal transcribed spacer 1 (ITS1)–5.8S–ITS2 rRNA gene cluster. The identities of the fungi are verified by subsequent sequencing analyses.

Procedure

1. PCR mixture is composed of 1 × Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science) (containing deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM $MgCl_2$, additional $MgCl_2$ is added to a final concentration of 4.6 mM), 0.4 µM each of ITS1 forward and ITS4 reverse primers, 1 × SYBR green (Molecular Probes), and 3 µL template DNA.
2. Thermal cycling parameters include 95°C for 10 min; 50 cycles of 95°C for 5 s, 60°C for 20 s, and 76°C for 30 s; and a final extension at 72°C for 2 min.
3. The quality of the amplicon is determined using the derivative of the melt analysis curve (55°C–99°C, 45 s hold at 55°C, 5 s/°C) using the RotorGene 3000 (Corbett Robotics, Inc).
4. The amplified product is purified for bidirectional sequencing using ExoSAP-IT (USB Corp). Five µL of Big Dye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems) is added to 4 µL of each primer (0.8 pmol/µL) and 3 µL of purified PCR product. Cycle sequencing is performed with a 9700 thermal cycler (ABI), using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reaction products are passed through a Sephadex G-50 fine column to remove unincorporated dye terminators. Purified sequencing reaction products are run on an ABI Prism 3100 Genetic Analyzer with a 50 cm capillary array.
5. Sequences are analyzed with the SmartGene Integrated Database Network software version 3.2.3 vr. SmartGene is a web-based software and database system with reference sequences derived from the National Center for Biological Information (NCBI) GenBank repository.

Note. In case that real time PCR instrument is unavailable, standard PCR may be performed with primers ITS1 and ITS4, and the resulting amplicon is sequenced with the same primers. Sequence-based identifications are defined by percent identity: species, ≥99%; genus, 93%–99%; and inconclusive, ≤93%. For strains producing discrepant identification between the methods based on phenotypic characteristics and ITS sequence analysis, the D1–D2 region of the large-subunit RNA gene is amplified with primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) and sequenced for species clarification [15].

5.3 CONCLUSIONS

B. caespitosus is a black fungus in the mitosporic Dothideomycetes group that is responsible for a phaeohyphomycosis in humans. The organism is also associated with biodeterioration of monuments. Given the close morphological similarity among black fungi, use of molecular techniques such as PCR and sequencing is critical for accurate and specific identification of the fungus.

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6 *Botryosphaeria* and *Lasiodiplodia*

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6.1 INTRODUCTION

The genus *Botryosphaeria* consists of a large number of cosmopolitan, plant-infecting fungi that are commonly associated with dieback and cankers of woody hosts in tropical and subtropical regions. Of these, *Botryosphaeria rhodina* and its anamorph *Lasiodiplodia theobromae* are occasionally involved in human phaeohyphomycosis, producing clinical diseases of varying severity (e.g., subcutaneous abscess, keratitis, pneumonia, and death).

6.1.1 Classification and Morphology

The genus *Botryosphaeria* belongs to the family *Botryosphaeriaceae*, order Botryosphaeriales, class Dothideomycetes, subphylum Pezizomycotina, phylum Ascomycota, and kingdom Fungi. The family *Botryosphaeriaceae* covers 11 recognized genera: *Barriopsis*, *Botryosphaeria*, *Guignardia*, *Melanops*, mitosporic *Botryosphaeriaceae*, *Neodeightonia*, *Otthia*, *Phaeobotryon*, *Phaeobotryosphaeria*, *Saccharata*, and *Spencermartinsia* as well as some unclassified *Botryosphaeriaceae* [1].

The genus *Botryosphaeria* consists of 16 recognized and 58 unassigned species, of which *Botryosphaeria rhodina* (synonym *Botryodiplodia theobromae*) is a teleomorph of *Lasiodiplodia theobromae* [1]. *Botryosphaeria rhodina/Lasiodiplodia theobromae* is a common endophyte and opportunistic pathogen affecting >500 tree species in the tropics and subtropics, leading to stem-end rot and dieback as well as rot in fruits in most species it infects [21].

Botryosphaeria spp. tend to form uni- to multilocular ascomata with multi-layered walls, occurring singly or in clusters, and intermixed with pycnidial conidiomata. Asci are bitunicate, with a thick endotunica, stalked or sessile, clavate, with a well-developed apical chamber, forming in a basal hymenial layer, intermixed among hyaline pseudoparaphyses that are constricted at the septa. Ascospores are hyaline, aseptate, fusoid to ellipsoid or ovoid, bi- to triseriate, without a mucoid sheath or appendages; ascospores turn brown and become septate and slightly verruculose upon germination.

The genus *Lasiodiplodia* is classified in the mitosporic *Botryosphaeriaceae* group, family *Botryosphaeriaceae*. The mitosporic *Botryosphaeriaceae* group contains 17 species: *Aplosporella*, *Chaetoconis*, *Diplodia*, *Dothiorella*, *Endomelanconiopsis*, *Fusicoccum*, *Lasiodiplodia*, *Macrophomina*, *Microdiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Phyllosticta*, *Pseudofusicoccum*, *Sphaeropsis*, *Stenocarpella*, *Taeniolella*, and *Tiarosporella*. In turn, the genus *Lasiodiplodia* consists of nine recognized (*Lasiodiplodia crassispora*, *Lasiodiplodia gonubiensis*, *Lasiodiplodia margaritacea*, *Lasiodiplodia parva*, *Lasiodiplodia plurivora*, *Lasiodiplodia pseudotheobromae*, *Lasiodiplodia rubropurpurea*, *Lasiodiplodia theobromae*, and *Lasiodiplodia venezuelensis*) and eight unassigned species, of which anamorph *Lasiodiplodia theobromae* (obsolete synonyms *Botryodiplodia tubericola*, *Diplodia theobromae*, *Diplodia tubericola*, and *Lasiodiplodia tubericola*) has been associated with mycotic keratitis, lesions on nail and subcutaneous tissue in humans [2,3].

Lasiodiplodia theobromae colonies are grayish sepia to mouse grey to black, fluffy with abundant aerial mycelium, and fuscous black to black on reverse. Pycnidia (up to 5 mm in width) are simple or compound, often aggregated, stromatic, ostiolate, frequently setos. Conidiophores are hyaline, simple, sometimes septate, rarely branched cylindrical, arising from the inner layers of cells lining the pycnidial cavity. Conidiogenous cells are hyaline, simple, cylindrical to

subobpyriform, holoblastic, annellidic. Conidia are initially unicellular, hyaline, granulose, subovoid to ellipsoid oblong, thick walled, base truncate; mature conidia (of 20–30 × 10–15 μm) are two celled (with one septate), cinnamon to fawn or dark brown, often longitudinally striated. Paraphyses when present are hyaline, cylindrical, sometimes septate, up to 50 μm long [4].

6.1.2 Clinical Features

Botryosphaeria rhodina/Lasiodiplodia theobromae is a common plant pathogen in tropical Asia (India, Cambodia, Hong Kong, Japan, and the Philippines), Australia, Central and South America (Columbia, Guyana, Jamaica, and United States), where *Lasiodiplodia theobromae* has been shown to infect the eyes, skin, and soft tissues of humans, with clinical manifestations ranging from keratitis, corneal ulcers and abscess, onychomycosis, subcutaneous abscess and phaeohyphomycosis, endophthalmitis, sinusitis, pneumonia to death [5–18].

Summerbell et al. [15] documented a case of subcutaneous phaeohyphomycosis caused by *Lasiodiplodia theobromae* in a 50-year-old Canadian woman who stumbled on an outdoor wooden staircase and sustained an injury to the right leg while visiting Jamaica. Five weeks later, the patient presented with an ulcer at the injury site. An excisional biopsy revealed broad, septate, melanized fungal filaments penetrating into the tissue and its culture generated a nonsporulating melanized mycelium. After 16 weeks cultivation on modified Leonian's agar at 25°C, the fungus developed pycnidia characteristic of *Lasiodiplodia theobromae*. The ulcer was excised during the biopsy procedure and ultimately resolved after treatment with saline compresses.

Woo et al. [19] described a case of *Lasiodiplodia theobromae* pneumonia in a 45-year-old patient with hepatitis B virus-related hepatocellular carcinoma. Analysis of a direct KOH smear of the bronchoalveolar lavage (BAL) fluid revealed septate hyphae. Culture of BAL fluid specimens on Sabouraud dextrose agar (SDA) yielded a dematiaceous mold, which grew as cottony colonies that became dark gray within 7 days at 37°C. Microscopic examination of the mold after lactophenol cotton blue staining revealed septate brown hyphae of 6 μm in width. The mold was also grown on oatmeal agar in sunlight to stimulate sporulation. Colonies produced hairy, dark brown structures (pycnidia). Conidia released from the pycnidia were hyaline and nonseptate when young but were septate and brown, with longitudinal striations, when mature. Pycnidia were sometimes produced in aggregates as shown in periodic acid-Schiff-stained histological sections of the mold on oatmeal agar. Examination under higher magnification demonstrated a pycnidium with an obvious neck and conidiogenous cells and paraphyses (sterile filaments among conidia) lining the internal wall. The fungus was identified as *Lasiodiplodia theobromae* by microscopic morphology and elongation factor 1α (EF1α) gene sequencing. The patient died 14 days after cadaveric liver transplantation. Because the conidia of this fungus are borne inside pycnidia and are released in a slimy mass, it was speculated that the patient may have acquired the infection through consumption of longan (*Dimocarpus longan*), a very popular fruit in southern China during summertime, which is often infected with *L. theobromae*. Alternatively, the fungus may have been acquired from the cadaveric transplanted liver and may have invaded the lung via the bloodstream.

6.1.3 Laboratory Diagnosis

Traditionally, laboratory identification of molds is based on detection of fungal elements in clinical specimens and further examination of macroscopic and microscopic features of cultured isolates [20]. To augment sporulation specifically for identification, mold may be grown on oatmeal agar in sunlight. Despite being time consuming, phenotypic identification of molds and other fungi requires expertise for the recognition of characteristic microscopic features of various fungi.

More recently, molecular techniques such as polymerase chain reaction (PCR) and sequencing analysis of the internal transcribed spacer (ITS) regions have been employed for more precise and objective determination of fungi [21–24]. However, for accurate identification of *L. theobromae* to the species level, partial EF1α sequencing appears to be more reliable than ITS sequencing [19]. The ITS sequence of the patient isolate differed by six bases from that of another strain of *L. theobromae* but differed by only eight bases from that of a strain of *L. gonubiensis* [19]. In addition, the *Lasiodiplodia* spp. are clearly distinguishable from *Diplodia* spp. and *Dothiorella* spp. based on morphology and ITS and EF1α nucleotide sequences [2,3].

6.2 METHODS

6.2.1 Sample Preparation

Molds from a specimen are grown on inhibitory mold agar, modified Sabouraud agars, or potato dextrose agar. Microscopic structures are observed on tease or tape preparations and slide cultures for up to 21 days.

After growth for 1–7 days on potato dextrose agar slants, lysates are prepared from approximately 1 cm^2 of mycelia with IDI lysis kits (GeneOhm Sciences, San Diego, CA). Briefly, in a biological safety cabinet, mycelia are collected by scraping the slant with a sterile stick in 1 mL of sterile, molecular-grade H_2O. The material is transferred to a 2 mL screw-cap tube. The tubes are centrifuged for 1 min at 6000 × *g*. If the mycelia does not pellet, the material is contained with a pediatric blood serum filter (Porex Corp., Fairburn, GA). The supernatant is removed. The material is resuspended in 200 μL of IDI sample buffer and transferred to the lysis tube, which contains glass beads. Lysis tubes are vortexed on the highest setting for 5 min. The tubes are placed in a boiling water bath for 15 min and centrifuged for 5 min at 16,000 × *g*. The supernatant is stored at −20°C until amplification [22].

6.2.2 Detection Procedures

6.2.2.1 ITS1–5.8S–ITS2 rRNA Gene Cluster Sequencing Analysis

Pounder et al. [22] described a real-time PCR with SYBR green DNA-binding dye and amplicon melting temperature analysis for fungal detection using pan-fungal primers ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′) [24]. These primers generate a 500 bp fragment from the internal transcribed spacer 1 (ITS1)–5.8S–ITS2 rRNA gene cluster (ITS). Subsequent sequencing analysis of the amplicon helps verify the identity of the fungal isolates.

Procedure

1. PCR mixture is composed of 1 × Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science) (containing deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM $MgCl_2$, additional $MgCl_2$ to a final concentration of 4.6 mM), 0.4 μM each of ITS1 forward and ITS4 reverse primers, 1 × SYBR green (Molecular Probes), and 3 μL template DNA.
2. Thermal cycling parameters include 95°C for 10 min; 50 cycles of 95°C for 5 s, 60°C for 20 s, and 76°C for 30 s; and a final extension at 72°C for 2 min.
3. The quality of the amplicon is determined using the derivative of the melt analysis curve (55°C–99°C, 45 s hold at 55°C, 5 s/°C) using the RotorGene 3000 (Corbett Robotics, Inc).
4. The amplified product is purified for bidirectional sequencing using ExoSAP-IT (USB Corp). Five microliters of Big Dye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems) is added to 4 μL of each primer (0.8 pmol/μL) and 3 μL of purified PCR product. Cycle sequencing is performed with a 9700 thermal cycler (ABI), using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reaction products are passed through a Sephadex G-50 fine column to remove unincorporated dye terminators. Purified sequencing reaction products are run on an ABI Prism 3100 Genetic Analyzer with a 50 cm capillary array.
5. Sequences are analyzed with the SmartGene Integrated Database Network software version 3.2.3 vr. SmartGene is a web-based software and database system with reference sequences derived from the National Center for Biological Information (NCBI) GenBank repository.

Note: In case that real time PCR instrument is not available, standard PCR may be performed with primers ITS1 and ITS4, and the resulting amplicon is sequences with the same primers.

6.2.2.2 Partial EF1α Gene Sequencing Analysis

Woo et al. [19] utilized primers EF1–728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1–986R (5′-TACTTGAAGGAACCCTTACC-3′) for PCR amplification and DNA sequencing of a 289 bp fragment of the EF1α gene from *Lasiodiplodia theobromae* isolate. The sequence was compared with those of related species listed in the GenBank database by using ClustalX 1.83. Phylogenetic relationships were determined using the neighbor-joining method.

6.3 CONCLUSION

Botryosphaeria rhodina/Lasiodiplodia theobromae is a ubiquitous plant pathogen in tropical regions that may infect the eyes, skin, and soft tissues of humans, leading to keratitis, corneal ulcers and abscess, onychomycosis, subcutaneous abscess and phaeohyphomycosis, endophthalmitis, pneumonia, and death. As phenotypic techniques for mold identification are time consuming and variable, molecular methods are increasingly utilized. PCR and sequencing analysis of the ITS regions have proven valuable for determination of a variety of molds including *Lasiodiplodia theobromae*. Furthermore, examination of the EF1α gene sequence offers an additional tool for differentiation among *Lasiodiplodia* spp.

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7 Corynespora

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7.1 INTRODUCTION

Dematiaceous fungi are characterized by their production of dark-pigmented hyphae or yeast-like cells. Being widely distributed in the soil, plants and other environments, these dark-walled fungi have the ability to cause opportunistic infections in humans and animals, affecting the skin, subcutaneous tissues and internal organs. Among the 130 species of 70 genera of dematiaceous fungi that have been implicated in human infections, *Corynespora cassiicola* is a common pathogen of grasses and other herbaceous plants in the tropics, and has been confirmed as an etiologic agent of subcutaneous phaeohyphomycosis in humans.

7.1.1 Classification, Morphology, and Biology

The genus *Corynespora* is a mold belonging to the mitosporic Pleosporales group, order Pleosporales, subclass Pleosporomycetidae, class Dothideomycetes, subphylum Pezizomycotina, phylum Ascomycota, kingdom Fungi [1].

The mitosporic Pleosporales group encompasses 16 genera: *Ascochyta*, *Camarosporium*, *Chaetodiplodia*, *Chaetophoma*, *Chalastospora*, *Cheirosporium*, *Coniothyrium*, *Corynespora*, *Monascostroma*, *Ochrocladosporium*, *Phialophorophoma*, *Plenodomus*,*Pleurophoma*,*Pseudodiplodia*,*Pseudorobillarda*, and *Zeloasperisporium*. In turn, the genus *Corynespora* is separated into seven recognized species: *Corynespora cassiicola*, *Corynespora citricola*, *Corynespora olivacea*, *Corynespora melongenae*, *Corynespora proliferata*, *Corynespora sesamum*, and *Corynespora smithii*, in addition to an unassigned species *Corynespora* sp. SXZ-01 [1].

Within the *Corynespora* genus, *Corynespora cassiicola* is a well-known plant pathogen causing leaf spot disease in more than 70 host plant species including cowpea, cucumber, papaya, pepper, rubber, soybean, and tomato, primarily found in the tropics and subtropics [2–4]. Rubber trees infected with *C. cassiicola* develop necrotic lesions on leaves, as well as chlorosis and darkening of the veins, leading to defoliation and loss of crop [5]. *C. cassiicola* has also been associated occasionally with human infections [6,7] (Figure 7.1).

On potato dextrose agar (PDA), *C. cassiicola* colonies are effuse, gray or brown in the front and gray or black on reverse. Conidiophores (measuring 4–11 µm by 110–850 µm) are pale to light brown, 3–10 septate, cylindrical, straight or curved, unbranched, and smooth. Conidia (of 5–10 µm by 27–192 µm in size) develop at the conidiophore apex and appear smoky to olivaceous, smooth, obclavate to cylindrical, subhyaline to pale olivaceous brown, 4–20 pseudoseptate, singly or in acropetal chains, with a rounded apex and truncated base [8,9].

Phylogenetic analysis in the ribosomal DNA internal transcribed spacer (ITS) regions of ribosomal RNA (rRNA) gene, random hypervariable loci (caa5 and ga4), and the actin-encoding locus act1 identified six phylogenetic lineages in *C. cassiicola*, corresponding to host of origin, pathogenicity, and growth rate but not to geographic location [10]. In addition, restriction fragment length polymorphism (RFLP) analysis of ITS regions of rRNA and random amplified polymorphic DNA (RAPD) studies revealed significant genetic variation among *C. cassiicola* isolates collected from different host plants [2–5,10,11].

7.1.2 Clinical Features and Pathogenesis

Corynespora cassiicola is a pathogenic fungus associated with leaf spot disease in plants. The involvement of this fungus in human disease was first noted in 1969, when a case of

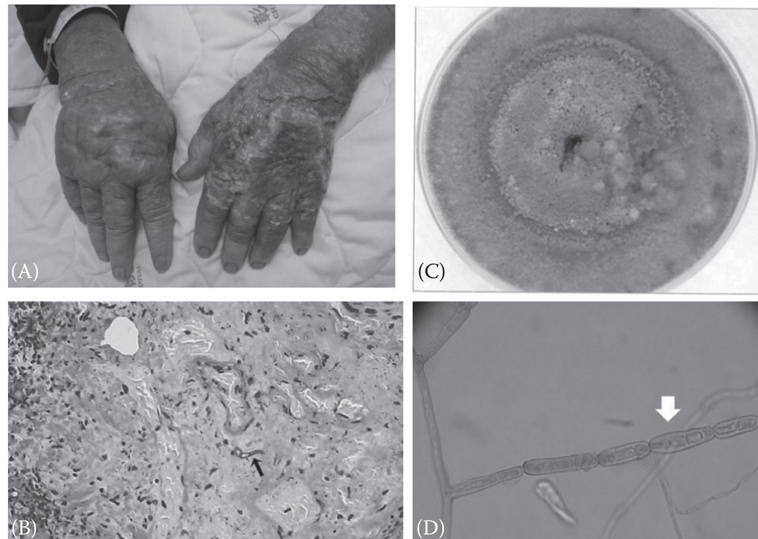

FIGURE 7.1 (A) Necrotic subcutaneous lesions over the dorsal aspects of both hands with purulent discharge in a patient with chronic subcutaneous infection caused by *Corynespora cassiicola*. (B) Hyphae with septa (black arrow) in the edematous papillary dermis (PAS stain, 400× magnification). (C) Confluent growth with velvety and gray–black colonies after 7 days of incubation on PDA at 28°C. (D) Straight and septate conidiophores and catenate conidia (white arrow) (lactophenol cotton blue stain, 400× magnification). (From Huang, H.K. et al., *J. Infect*., 60, 188, 2010.)

maduromycetoma of the foot was attributed to *C. cassiicola* [6]. Recently, *C. cassiicola* was shown to cause chronic subcutaneous infection in a 69-year-old female farmer with diabetes mellitus and on long-term oral antihyperglycemic therapy [7]. The patient presented erythematous lesions on the bilateral forearms and dorsal aspect of both hands with purulent discharge, which was accompanied by pain, heat, swelling, and tenderness. Multiple bullae developed on the erythematous lesions, which ruptured with milk-colored, pus-like discharge. The patient underwent debridement and culture of the debrided tissue and discharged pus on PDA (Becton Dickinson) formed velvety and gray-black colonies after 7 days of incubation at 28°C. Microscopically, the organism showed long, straight, septate conidiophores and elliptic, catenate conidia using lactophenol cotton blue stain. Histological examination of biopsy using periodic acid-Schiff (PAS) stain revealed tissue necrosis with the presence of septate hyphae in the edematous papillary dermis. Upon sequence analysis of the region comprising the ITS1 and ITS2 regions of the rRNA genes using fungus-specific universal primers ITS1 and ITS4, the fungus demonstrated a nucleotide identity of 98% (527/534) with the published *C. cassiicola* sequences in the GenBank (accession number FJ852715). The mold was thus identified as *C. cassiicola* according to the characteristic phenotypes and relevant molecular methods. The patient recovered fully after amphotericin B treatment [7].

The exposure to the fungus in plants such as betel nut and banana through trivial wounds (farmer) and the immunocompromised status (old age, diabetes mellitus, and adrenal insufficiency) might have contributed partly to this case of *C. cassiicola* infection [7]. The ability of *C. cassiicola* to cause cellular damages was demonstrated recently with the isolation and characterization of a 28 kDa, 27 residue *O*-glycosylated protein (known as cassiicolin) in this fungus [12,13]. Cassiicolin induces host-selective cellular damages resembling those observed in leaf fall disease of rubber trees with *C. cassiicola* infection [12,13].

7.1.3 Laboratory Diagnosis

Traditional methods for identification of fungi are based on examination of their characteristic macroscopic and microscopic features. Typically, clinical specimens (e.g., discharge and scrapings) are examined under microscope to identify hyphae and other mycotic elements, with the help of 10% KOH or lactophenol cotton blue stain. Biopsies are examined for mycotic elements and pathological changes using PAS stain or other specific fungal stains. Portions of clinical specimens are cultured on Sabouraud dextrose agar (SDA) or potato dextrose agar (PDA) at 25°C, 35°C, respectively. The resulting fungal isolates are further characterized on their colony morphology and microscopic features as well as biochemical properties.

Given the close morphological and biochemical similarity among many pathogenic fungal species, laboratory diagnosis of fungal infections using phenotypic criteria can be challenging and time consuming. With the development and application of nucleic acid amplification techniques, rapid and precise identification of fungal organisms has become possible. In particular, PCR amplification and sequencing analysis of the small subunit (SSU) and large subunit (LSU) as well as ITS1 and ITS2 regions of the rRNA genes provide a useful tool for confirmation of fungal identity including *C. cassiicola* [7,14–17].

7.2 METHODS

7.2.1 Sample Preparation

Specimens are stained with 10% KOH, lactophenol cotton blue stain or PAS stain and examined under microscope for mycotic elements. Portions of samples are grown on inhibitory mold agar, modified Sabouraud agars, or PDA at 25°C and 35°C.

After growth for 1–7 days on PDA slants, mycelia are collected by scraping the slant with a sterile stick in 1 mL of sterile water. The material is transferred to a 2 mL screw-cap tube. The tubes are centrifuged for 1 min at 6000 × *g*. If the mycelia do not pellet, the material is contained with a pediatric blood serum filter (Porex Corp). Supernatant is removed, and the material is resuspended in 200 µL of IDI sample buffer and transferred to the lysis tube, which contains glass beads. Lysis tubes are vortexed on the highest setting for 5 min. The tubes are placed in a boiling water bath for 15 min. Tubes are centrifuged for 5 min at 16,000 × *g*. The supernatant is stored at −20°C until amplification [15].

Alternatively, about 1 cm^2 of fungal material is transferred to a 2 mL Eppendorf tube containing a 2:1 (wt/wt) mixture of silica gel and Celite (silica gel H, Merck 7736/Kieselguhr Celite 545; Machery) and 300 µL of TES buffer (2 g Tris [hydroxymethyl]-aminomethane, 0.38 g Na-EDTA [ethylenediaminetetraacetic acid], and 2 g sodium dodecyl sulfate in 80 mL of ultrapure water, pH 8). The fungal material is ground with a micropestle for 1–2 min. The volume is adjusted by adding 200 µL of TES buffer. After vigorous shaking and the addition of 10 µL of a 10 mg/mL concentration of proteinase K to the tube, the mixture is incubated at 65°C for 10 min. With the addition of 140 µL of 5 M NaCl solution, the mixture is combined with 1/10 volume (~65 µL) of 10% cetyltrimethylammonium bromide (CTAB) buffer, followed by incubation for another 30 min at 65°C. One volume (~700 µL) of chloroform-isoamyl alcohol (vol/vol = 24/l) is added and mixed by inversion. After incubation for 30 min at 0°C (on ice water) and centrifugation at 14,000 rpm at 4°C for 10 min, the top layer is transferred to a clean Eppendorf tube. The sample is added with 225 µL of 5 M NH_4–acetate and incubated for at least 30 min (on ice water) and centrifuged. The supernatant is transferred to a clean sterile Eppendorf tube and mixed with a 0.55 volume (~510 µL) of ice-cold isopropanol. After centrifugation for 7 min at 14,000 rpm and 4°C (or room temperature), the supernatant is decanted. The pellet is washed with ice-cold 70% ethanol twice and dried by using a vacuum dryer. The powder is resuspended in 48.5 µL of Tris–EDTA buffer with 1.5 µL of 10 mg of RNase/mL, incubated at 37°C for 15–30 min, and stored at −20°C until use [16].

7.2.2 Detection Procedures

7.2.2.1 Pan-Fungal Real-Time PCR and Sequencing Analysis

Pounder et al. [15] described a real-time PCR with SYBR green DNA-binding dye and amplicon melting temperature analysis for fungal detection using pan-fungal primers ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′). The identity of the fungi is verified by subsequent sequencing analysis.

Procedure

1. PCR mixture is composed of 1× Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science) (containing deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM $MgCl_2$, additional $MgCl_2$ is added to a final concentration of 4.6 mM), 0.4 µM each of ITS1 forward and ITS4 reverse primers, 1× SYBR green (Molecular Probes), and 3 µL template DNA.
2. Thermal cycling parameters include 95°C for 10 min; 50 cycles of 95°C for 5 s, 60°C for 20 s, and 76°C for 30 s; and a final extension at 72°C for 2 min.
3. The quality of the amplicon is determined using the derivative of the melt analysis curve (55°C–99°C, 45 s hold at 55°C, 5 s/°C) using the RotorGene 3000 (Corbett Robotics, Inc).
4. The amplified product is purified for bidirectional sequencing using ExoSAP-IT (USB Corp). Five microliters of Big Dye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems) is added to 4 µL of each primer (0.8 pmol/µL) and 3 µL of purified PCR product. Cycle sequencing is performed with a 9700 thermal cycler (ABI), using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reaction products are passed through a Sephadex G-50 fine column to remove unincorporated dye terminators. Purified sequencing reaction products are run on an ABI Prism 3100 Genetic Analyzer with a 50 cm capillary array.
5. Sequences are analyzed with the SmartGene Integrated Database Network software version 3.2.3 vr. SmartGene is a web-based software and database system with reference sequences derived from the National Center for Biological Information (NCBI) GenBank repository.

Note. In case that real time PCR instrument is not available, standard PCR may be performed with primers ITS1 and ITS4, and the resulting amplicon is sequenced with the same primers [18]. Sequence-based identifications are defined by percent identity: species, ≥99%; genus, 93%–99%; and inconclusive, ≤93%.

7.2.2.2 Sequencing Analysis of ITS1 and ITS2

Leaw et al. [14] utilized fungus-specific universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCATCGATGAAGAACGCAGC-3′) to amplify the ITS1 region, and universal primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4

(5′-GCATATCAATAAGCGGAGGA-3′) to amplify the ITS2 region. Subsequent sequencing analysis of the resulting amplicons facilitated identification of a range of fungal pathogens.

Procedure

1. PCR mixture (50 µL) is made up of 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphosphates, 1.2 U of *Taq* DNA polymerase, 0.4 µM (each) of the ITS1 region primers (ITS1/ITS2) or the ITS2 region primers (ITS3/ITS4), 2 µL (1–5 ng) of DNA template. A negative control is included in each run by replacing the template DNA with sterile water in the PCR mixture.
2. Amplification is conducted at an initial 94°C for 3 min; 30 cycles of 94°C, 60°C, and 72°C for 1 min each; and a final 72°C for 3 min.
3. After checking the PCR products on 1.5% agarose gel, the amplicons are purified using a commercial PCR cleanup kit. The DNA fragments are sequenced using an ABI Prism 377 automated DNA sequencer (Applied Biosystems) with a BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems). All amplicons are sequenced on both strands using primers ITS1 and ITS2 for the ITS1 region and primers ITS3 and ITS4 for the ITS2 region.
4. After sequencing, portions of the 18S, 5.8S, and 26S rRNA gene sequences of the PCR products are removed to obtain the exact ITS1 and ITS2 sequences.
5. For all yeasts, the sequences of the 3′ ends of the 18S and 5.8S rRNA genes are GCGGAAGGA TCATTA and GTTTGAGCGTCATTT, respectively, and the sequences of the 5′ ends of the 5.8S and 26S rRNA genes are AAACTTTCAACAA and GACCTCAAATCAG, respectively.
6. The ITS1 or ITS2 sequence is compared with those available at the GenBank databases using the BLAST sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/) (blastn) with default settings except that sequences are not filtered for low complexity. Species identification is determined from the lowest expected value of the BLAST output.

Note. For strains producing discrepant identification between the methods based on phenotypic characteristics and ITS sequence analysis, the D1–D2 region of the LSU RNA gene is amplified with primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) and sequenced for species clarification.

7.3 CONCLUSION

The genus *Corynespora* is composed of eight mold species that are distributed ubiquitously in the environment of which *C. cassiicola* causes leaf spot disease in >70 plant species, and is occasionally associated with human infections [6,7]. As shown in a recent case of *C. cassiicola*-related chronic subcutaneous infection, the fungus may be acquired through exposure to plants and it has the ability to take advantage of the weakened host immune defense such as old age, diabetes mellitus, and adrenal insufficiency to establish in human hosts [7,18].

As conventional laboratory procedures for identification of *Corynespora* and other fungal pathogens are slow and demand specialized skills, molecular techniques are increasingly utilized for rapid and precise determination of these organisms. Indeed, PCR and sequencing analysis of the SSU and LSU as well as ITS1 and ITS2 regions of the rRNA genes offer a reliable supplemental approach for accurate speciation of fungal organisms including *C. cassiicola*.

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