Diploma thesis

Identification and strain typing of *Candida albicans* isolates from clinical samples

submitted by

Katharina Heidrich

Date of birth: 16 March 1987

In partial fulfillment of the requirements for the degree of

Doktorin der gesamten Heilkunde / Doctor of medicine

(Dr. med. univ. / MD)

at the

Medical University of Graz

Institute of Hygiene, Microbiology and Environmental Medicine

Supervisors

Ass.-Prof. Priv.-Doz. Ing. Mag. Dr. rer. nat. Walter Buzina

Univ.-Prof. Dr. med. univ. Robert Krause

Graz, 20 January 2014 Katharina Heidrich

Affidavit

Herewith I, Katharina Heidrich, declare that I authored the present diploma thesis fully on my own and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of the thesis other than those indicated in the thesis itself.

Graz, January 2014

Acknowledgements

I would like to express my utmost gratitude to my supervisor Prof. Walter Buzina for his excellent support, motivation, advice, and proofreading during the thesis.

I would like to thank Prof. Robert Krause sincerely for the interesting topic, his supervision, advice and proofreading. I would also like to thank Prof. Krause and his team for the collection and the providing of the *Candida* isolates.

I am heartily grateful to Bettina Kölli for her excellent and friendly guidance and support concerning all laboratory issues and her warm and motivating words when the laboratory work got frustrating.

Furthermore I would like to thank Monika Keimel for the realization of the MALDI-TOF investigations and Prof. Cornelia Lass-Flörl and her team of the Medical University of Innsbruck for re-testing the susceptibility of our resistant isolate.

I would like to thank the staff of the Institute of Hygiene, Microbiology and Environmental Medicine for the warm reception in their team.

I thank the curator of the MLST database, Dr. Marie-Elisabeth Bougnoux at the Fungal Biology and Pathogenicity Unit, Institut Pasteur, France, for assigning the new alleles and STs from our strains and adding them to the MLST consensus database of *C. albicans*.

Very warm thanks go to Johannes for his contagious motivation throughout our studies and for countless hours of listening, discussing, and encouraging regarding the present thesis.

Last but certainly not least, I would like to thank my parents and my sister for their unwavering encouragement and support throughout my studies.

Abstract

Introduction Invasive fungal infections are live-threatening diseases that occur particularly in immunodeficient and multimorbid patients. The facultative pathogenic yeast *Candida albicans* colonizes the mucosal surfaces and the skin of healthy individuals. Even though, it constitutes the most important fungal pathogen. The mortality of blood stream infections due to *Candida* species is about 40%. Therefore, fast detection and proper treatment are crucial for the outcome of those affected.

Material and Methods For this study 181 clinical yeast isolates were classified to species level by means of a mass spectrometric technique (MALDI-TOF MS). Susceptibility testing for 8 antifungal agents was performed with all *Candida albicans* isolates. A panel of 44 invasive and colonizing isolates was chosen for Multi Locus Sequence Typing (MLST) to investigate the phylogenetic relationships. An echinocandin resistant isolate was analyzed regarding mutations in the *FKS1*-gene, known as a hotspot for mutations that lead to reduced echinocandin susceptibility.

Results Of 181 isolates, 140 were *C. albicans* (77%). All isolates were susceptible to the tested antifungals, except for two. An echinocandin resistant isolate showed 2 nucleotide point mutations in *FKS1*, while a susceptible isolate obtained some weeks prior from the same patient did not show these mutations. In our PCR investigations we found numerous new MLST types. These types predominantly clustered in clades 1, 2 and 3. No statistically relevant differences in the distribution of invasive and colonizing isolates within clonal clusters were found.

Discussion The early detection of echinocandin resistance and the development of alternative treatment strategies are of great clinical importance. Nevertheless, *FKS1* mutations are infrequent in *Candida albicans*. Despite some limitations, MLST is currently seen as the reference method for phylogenetic typing. Our cluster distributions as determined by MLST were similar to previous investigations. Samples from the same patients, obtained from different sites, showed the same MLST profile, suggesting endogenous transmission. Patients most likely acquire candidal infections from their own colonizing flora and thus colonization is a risk factor for disseminated candidiasis in the susceptible host.

Keywords *Candida albicans* • Multilocus Sequence Typing • Candidemia • Echinocandins • Fungal Drug Resistance

Zusammenfassung

Einleitung Bei invasiven Pilzinfektionen handelt es sich um lebensbedrohliche Erkrankungen, die in erster Linie immunkompromittierte und multimorbide Patienten und Patientinnen betreffen. Der Hefepilz *Candida albicans* kolonisiert symptomlos die Haut und Schleimhäute von Gesunden. Dennoch ist er der wichtigste humanpathogene Hefepilz – die Candidämie hat eine Mortalität von etwa 40%. Eine schnelle Diagnose und adäquate Therapie sind entscheidend für das Überleben der Betroffenen.

Material und Methoden Für diese Studie wurden 181 klinische Hefepilz-Isolate mittels des massenspektrometrischen Verfahrens MALDI-TOF MS bis zur Artebene bestimmt**.** Bei allen *Candida albicans* Stämmen wurden Resistenztestungen durchgeführt. Mit einer Auswahl von 44 Isolaten wurden Multi Locus Sequence Typing Polymerase-Kettenreaktionen (MLST PCR) durchgeführt. Bei einem Echinocandin-resistenten Isolat wurde mittels PCR und Sequenzierung das *FKS1*-Gen, ein bekannter Hotspot für Echinocandin-Resistenz, untersucht.

Ergebnisse 140 von 181 Pilzkulturen wurden als *C. albicans* identifiziert. Bis auf zwei Isolate waren alle Proben sensibel auf die getesteten Antimykotika. Ein gegen Echinocandine resistentes Isolat wies zwei Punktmutationen im *FKS1*-Gen auf. Bei den PCR-Untersuchungen konnte eine Vielzahl neuer MLST-Typen gefunden werden. Diese stammten in den meisten Fällen aus den großen Stammfamilien 1-3. Bei der Zuordnung zu den Stammfamilien wurden keine statistisch relevanten Unterschiede zwischen kolonisierenden und invasiven *Candida*-Isolaten gefunden.

Diskussion Die frühzeitige Entdeckung von Echinocandinresistenzen und die Entwicklung alternativer Behandlungsstrategien sind von größter Bedeutung bei Patienten mit invasiver Candidose. Allerdings sind *FKS1*-Mutationen bei *C. albicans* selten. Trotz gewisser Einschränkungen ist MLST derzeit die Referenzmethode für phylogenetische Analysen. Ähnlich wie in anderen Studien beinhalteten die MLST-Cluster 1-3 die meisten Stämme. Mehrere Isolate vom gleichen Patienten, zu verschiedenen Zeitpunkten und an unterschiedlichen Stellen entnommen, hatten ein identes MLST Profil. Dies spricht für einen endogenen Infektionsweg. Dies unterstreicht die Annahme, dass Patientinnen und Patienten *Candida* Infektionen aus ihrer eigenen kolonisierenden Flora entwickeln und diese daher einen Risikofaktor für Immungeschwächte darstellt.

Table of content

Glossary and Abbreviations

AIDS – acquired immune deficiency syndrome
am – anamorph

BAL – bronchoalveolar lavage
BC – blood culture
BP – base pairs

C. – *Candida*
CFU – colony forming units
CLRs – C-type lectin receptors
CLSI – clinical and laboratory standards institute
CVC – central venous catheter

dNTP – dioxynucleotidetriphosphate
DST – diploid sequence type

EDTA – ethylenediaminetetraacetic acid
ESCMID – European Society of Clinical Microbiology and Infectious Diseases
EUCAST – European Committee on Antimicrobial Susceptibility Testing

GM-CSF – granulocyte macrophage colony-stimulating factor

HRM – high resolution DNA melting

IL – Interleukin
IUPAC – International Union of Pure and Applied Chemistry

LOH – loss of heterozygosity

MALDI -TOF – matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
MIC – minimal inhibitory concentration
MLP – microsatellite length polymorphism
MR – mannose receptor
MTL – Mating-Type-Like locus

NJ – neighbor joining

PAMPs – pathogen-associated molecular patterns
PRRs – pathogen recognition receptor

SAB – Sabouraud Agar
SNP – single nucleotide polymorphism
SSLP – simple sequence length polymorphism
ST – sequence type

TLRs – toll-like receptors
tm – teleomorph

UPGMA – Unweighted Pair Group Method with Arithmetic averages

List of Figures

List of Tables

1 INTRODUCTION

In the following pages, some general information about fungi from a medical point of view is summed up. Detailed information concerning the fungal pathogen *Candida (C.) albicans* and invasive candidiasis is given. Finally, the focus is on the molecular typing of *C. albicans* by Multi Locus Sequence Typing-Polymerase Chain Reaction (MLST-PCR).

1.1 The kingdom of fungi

The earth houses approximately 8.7 million eukaryotic species [1](reviewed in [2]). Of these, according to recently published data, 611,000 are fungi [1]. To date, 43,271 fungal species are catalogued [1]. However, only a few hundred have the potential to cause disease in humans [3]; Brown et al. estimated the count of fungi potentially infecting humans to 600 [4]. Fungal diseases range from mild, superficial skin affections, as caused by *Malassezia* species (spp.) up to severe, life threatening systemic diseases by *Candida* spp., *Aspergillus fumigatus,* and others [2]. The latter are emerging and come up as opportunistic infections in the majority of causative agents [3]. The main reason for this development is the increase of multimorbid, immune-compromised patients due to the progress in medical treatments and immunosuppressive ailments, such as AIDS [3,4]. Some fungi, for instance *Aspergillus* spp. or *Alternaria alternata*, constitute potent antigens, able to cause hypersensitive reactions [5].

In former times, fungi were assigned to the plant kingdom [3]. Because of their physiologic and genetic properties, we nowadays consider them as an independent kingdom of eukaryotic organisms [3,6]. Just like animals – and in contrast to plants – fungi do not produce chlorophyll; they are heterotrophic organisms [3]. Fungi feed by excretion of digestional enzymes into the substrate and ensuing adsorption of the digested substances through the cell wall [3]. The fungal cell wall contains primarily chitin and glucans and in lower quantities other components like cellulose and chitosan [3].

1.1.1 *Candida* species

The genus *Candida*, derived from the Latin word candidus (white, snowy, lucent) comprises a heterogeneous group of fungi. Their unifying trait is that they grow as yeasts (see Figure 2A) [7]. Formerly, the lack of a sexual stage was defined as a characteristic of *Candida* as well [7]. However, the teleomorph (tm = sexual) stages of some species could be assigned to its anamorph (am = asexual) counterpart, e.g., *C. krusei* (am) – *Issatchenkia orientalis* or since recently *Pichia kudriavzevii* (tm) [8], *C. guilliermondii* (am) – *Pichia guilliermondii* (tm), *C. kefyr* (am.) – *Kluyveromyces marxianus* [7]. New *Candida* species are described frequently [9-14], but only about 10 species are involved in human disease [7].

Although colonizing healthy mucosae as a harmless commensal [3,15], *Candida* spp. may cause local infections, like mucocutaneous candidosis, in case of local or slight general immunological deficiency [3]. The most frequent local affections include thrush and vulvo-vaginitis [3]. Onychomycosis and intertrigo are also commonly caused by this yeast [3]. Invasive infections with *Candida* spp. as causative agent have been emerging in the past three decades [16] and are still a serious problem leading to high mortality rates from 19 up to 71%, depending on the causative pathogen and the patient's underlying condition (reviewed in [17]). Figure 1 illustrates the most common affections caused by *Candida* spp.

By far the most important fungal pathogen is ***Candida albicans*** (Robin) Berkhout, primarily described in 1923. This yeast belongs to the fungal class of Hemiascomycetes, order Saccharomycetales, family Endomycetaceae [3,18]. In contrast to many other fungi, e.g., molds, *C. albicans* has never been isolated terrestrially, meaning outside an organism [19,20]. It seems that *C. albicans* is perfectly adapted to live in an, especially mammalian, host [20].

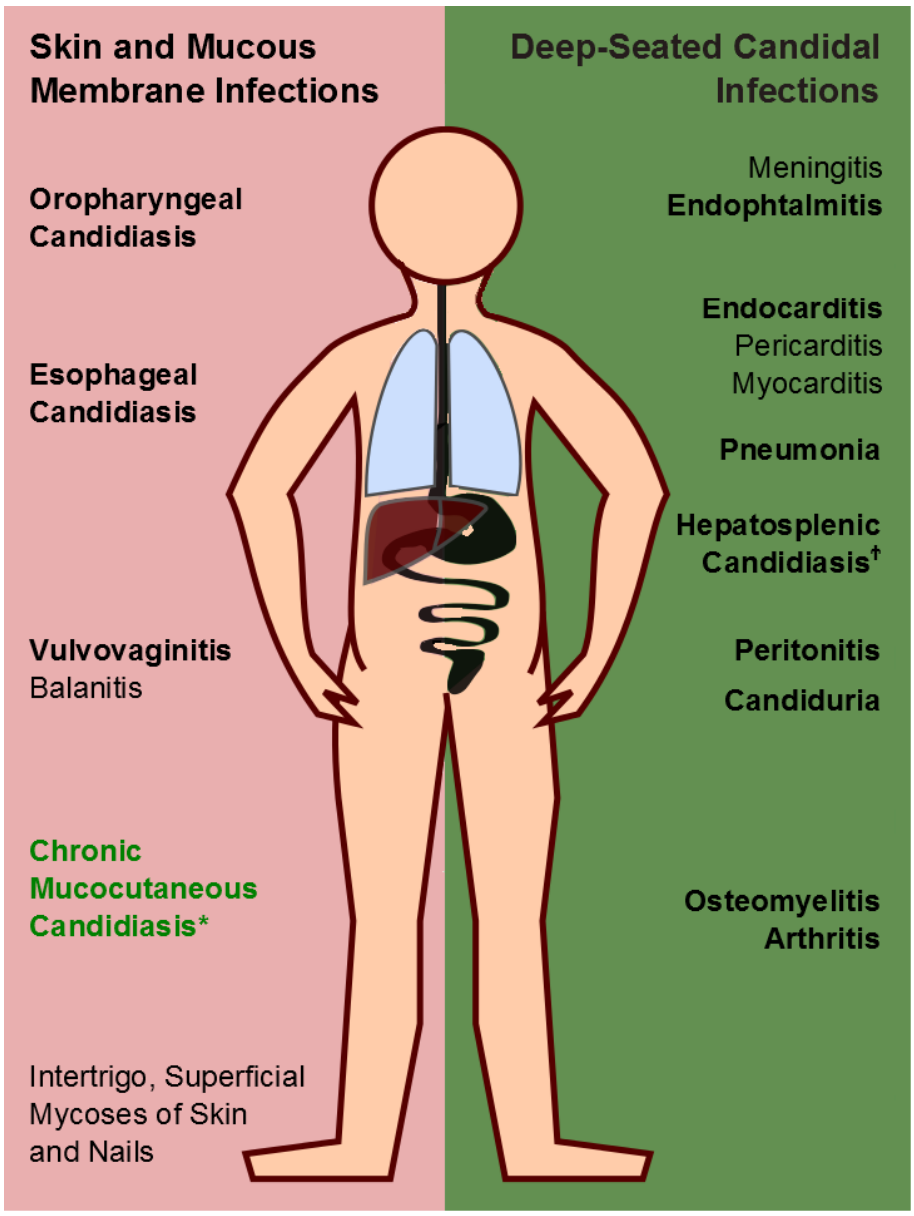


Figure 1: Conditions caused by *Candida* spp. apart from candidemia [7]

† see 1.3 for more information about candidemia and invasive Candidiasis
*chronic and/or recurrent candidal infections of the skin, nails and mucous membranes, frequently hereditary [7,21]

1.2 *Candida albicans*

1.2.1 Culture

C. albicans cultures appear cream-colored and smooth on Sabouraud (SAB) agar dishes [3]. Cultures can sometimes look corrugated [7]. Cultures on chromogenic agar show different colors, for example, greenish-blue or occasionally pink [22] on CHROMAgar® *Candida* medium (Becton Dickinson, Heidelberg, Germany) and blue on ChromID® *Candida* agar (bioMérieux, Marcy l'Etoile, France). *Candida* colonies emit a typical yeast smell.

1.2.2 Microscopy & growth forms

In light microscopy budding cells or blastoconidia can be observed. These spherical cells have a size of 3–8 x 2–8 µm [3]. Pseudomycelium can be seen as well, forming aciniform groups with the blastoconidia [3]. Pseudomycelium or pseudohyphae are chains of elongated budding cells that stay attached to each other after the budding event [19]. *C. albicans* colonies produce chlamydospores in nutrient-poor, carbohydrate-rich media, such as Tween 80 agar [3]. Chlamydospores are larger yeast cells with thickened cell wall [7]. Germ tubes can be observed in contact with human serum [3]. The expression of chlamydospores and the germ tube outgrowth constitute diagnostic criteria for the identification of *C. albicans* [3].

C. albicans is considered to be polymorphic due to its ability to produce pseudohyphae as well as true hyphae [7,19]. Hyphae are tubular filaments without constrictions at the septae [23]. The hyphae form plays an important role in the pathogenesis of invasive disease [7]. It seems to be more capable in both crossing tissue surfaces by endocytosis as well as direct tissue invasion due to epithelial damage (reviewed in [19]). Mutants unable to form normal hyphae are avirulent in a mouse model [24].

A multitude of factors was discovered to promote true hyphae growth. Besides the presence of serum already mentioned above, these factors are a high pH value, physiologic temperature and starvation (reviewed in [2]). Genotoxic stress, that means DNA damage or inhibition of DNA replication, also stimulate hyphae growth [19]. Hydrogen peroxide (H_2O_2), ultraviolet radiation, the presence of hydroxyurea can constitute genotoxic stress factors [19]. On contact with a surface, *C. albicans* yeast cells can switch to hyphae growth as well. This process is called contact sensing [25]. In special surface conditions, thigmotropism can appear. This term describes a Ca^{2+}-regulated orientative growth of the mycelium [26](reviewed in [2]). Contact sensing and thigmotropism both contribute to the formation of biofilms [2].

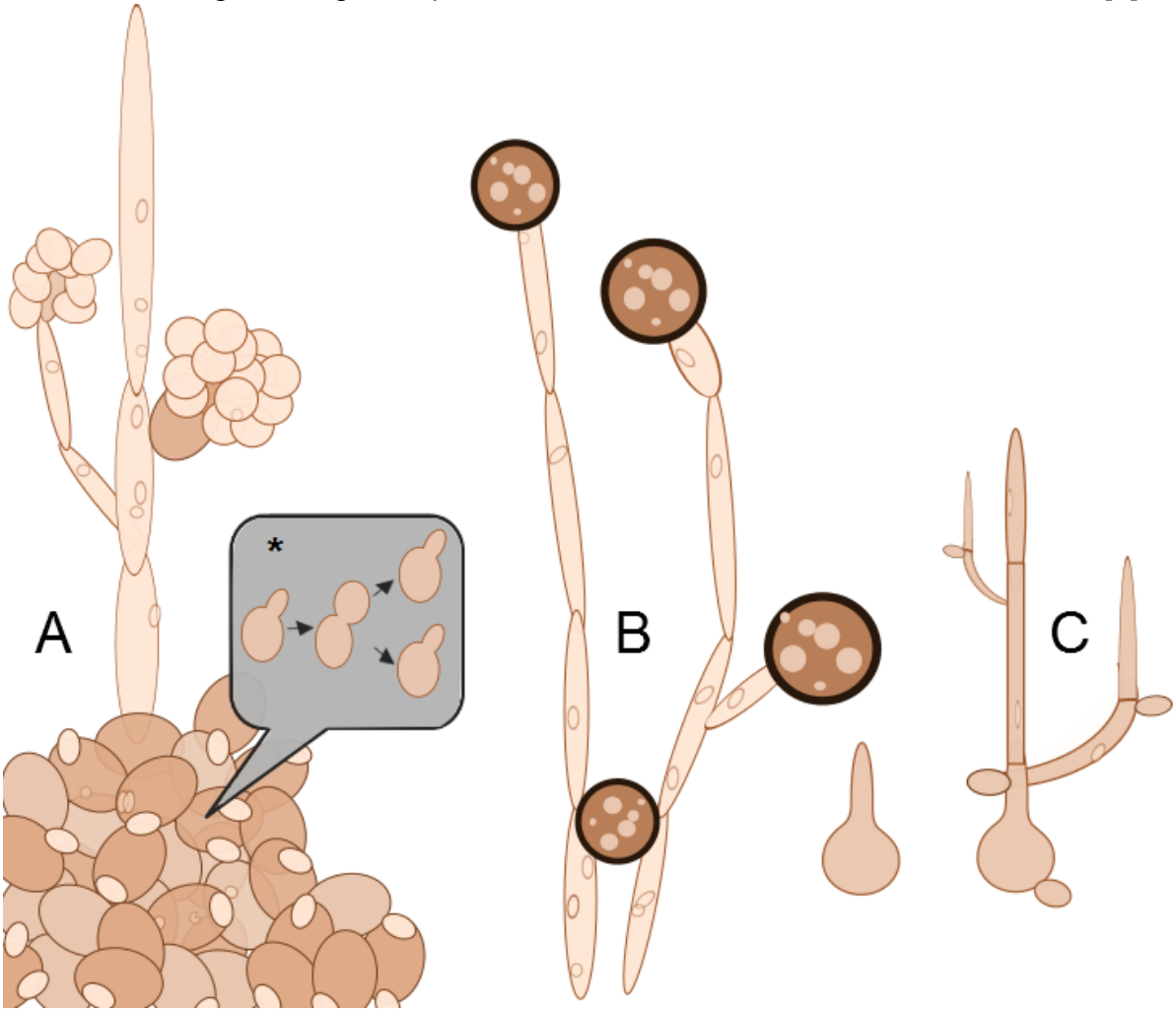


Figure 2: *C. albicans* morphology, adapted from[3,7]

A: pseudohyphae emerging from cell clumps, blastoconidia, *budding procedure
B: chlamydospores C: germ tube outgrowth, true hyphae

Another change of morphology is the so-called white-opaque switching. This term, which was primarily described in 1987 [27], denominates the change of a white budding cell to an elongated opaque cell [28]. In this form, *C. albicans* is mating-competent [28,29]**.** Only cells with homozygous Mating-Type-Like locus (MTL a/a or α/α, see 1.2.4) can undergo white-opaque switching [29].

1.2.3 Virulence factors

Arendrup et al. identified *C. albicans* and *C. tropicalis* as the most virulent *Candida* species in an animal model [30]. Although the size of the inoculum in the cited study was considerably major to the dose that produces disease in a susceptible host, this finding may explain why *C. albicans* is the most commonly found fungal species involved in human disease [30]. The yeast disposes of various capabilities that increase its virulence and empower it to inhabit various host niches [2]. An important virulence factor is the phenotypic polymorphism already mentioned above. Furthermore, *C. albicans* possesses proteins called adhesins and invasins. The adhesins, especially the agglutinin-like sequence proteins, favor the adhesion of *C. albicans* among themselves and to biotic and abiotic surfaces (reviewed in [2]). Some of them are only expressed in the hyphae form, while others are expressed morphologically independent (reviewed in [2]). Invasins bind to host ligands, which leads to passive incorporation of the yeast into the host cell (reviewed in [2]). Moreover, *C. albicans* exhibits another possibility of invasion: hyphae can emit hydrolases. These lead to cellular damage and active penetration of the host cell (reviewed in [2]).

The formation of biofilms is also an important virulence factor [2]. Biofilms are colonies of the pathogen, formed on devices, e.g., catheters or denture, and surrounded by an extracellular matrix [31]. Either the hyphae as well as the yeast form are required for the formation of biofilms [31]. Apart from the continuous release of fungus cells, biofilms show a reduced susceptibility against antimycotic drugs in comparison with sole yeast cells [31].

C. albicans has evolved several mechanisms to adapt to a changing metabolic environment. During blood stream invasion and in organs with high glucose levels, for example brain or liver, its nutrition is dominated by glycolysis [20](reviewed in [2]). However, the yeast can switch to lipolysis and gluconeogenesis in nutrient-poor environments, for example after phagocytosis by a macrophage [20](reviewed in [2]). Apart from the nutritional conditions, *C. albicans* is able to adapt to various environmental changes, such as the pH-value. In humans, the pH ranges from 2 (gastric acid) and 4 (vagina) to 7.4 (bloodstream) [2]. The yeast is even capable to modulate the pH: in a nutrient-poor environment, it is able to alkalinize its environment to auto-induce hyphal growth (reviewed in [2]).

1.2.4 Reproduction and genetics

Despite of its diploidity, *C. albicans* does not appear to undergo meiosis [32,33]. The mode of reproduction is widely clonal [32](reviewed in [33]). Nevertheless, *C. albicans* has evolved several mechanisms to maintain high genetic diversity, such as loss and gain of heterozygosity [34], chromosomal alterations and mutations [32] and a parasexual cycle [33,35]. Because of these mechanisms, the yeast can adapt rapidly to microenviromental changes and for example, develop antifungal resistance [32,36]. The parasexual cycle is outlined in Figure 3. This epigenetic process requires a loss of heterozygosity in the MTL locus (a/α → α/α or a/a), followed by transition into the phenotypic opaque state and mating of two MTL-contrary cells [33]. The newly formed tetraploids return to their diploid state by chromosomal loss [35]. The parasexual cycle is fundamentally regulated by Wor1 (white-opaque regulator 1) (reviewed in [29]). It is suppressed in the heterozygous form (reviewed in [29]). In homozygous white cells, it is no longer suppressed, but expressed at low levels (reviewed in [29]). In the opaque form, its expression increases (reviewed in [29]). Mating seems to occur proportionally rare [33], but it may be promoted through certain environmental triggers (see Figure 3) [29].

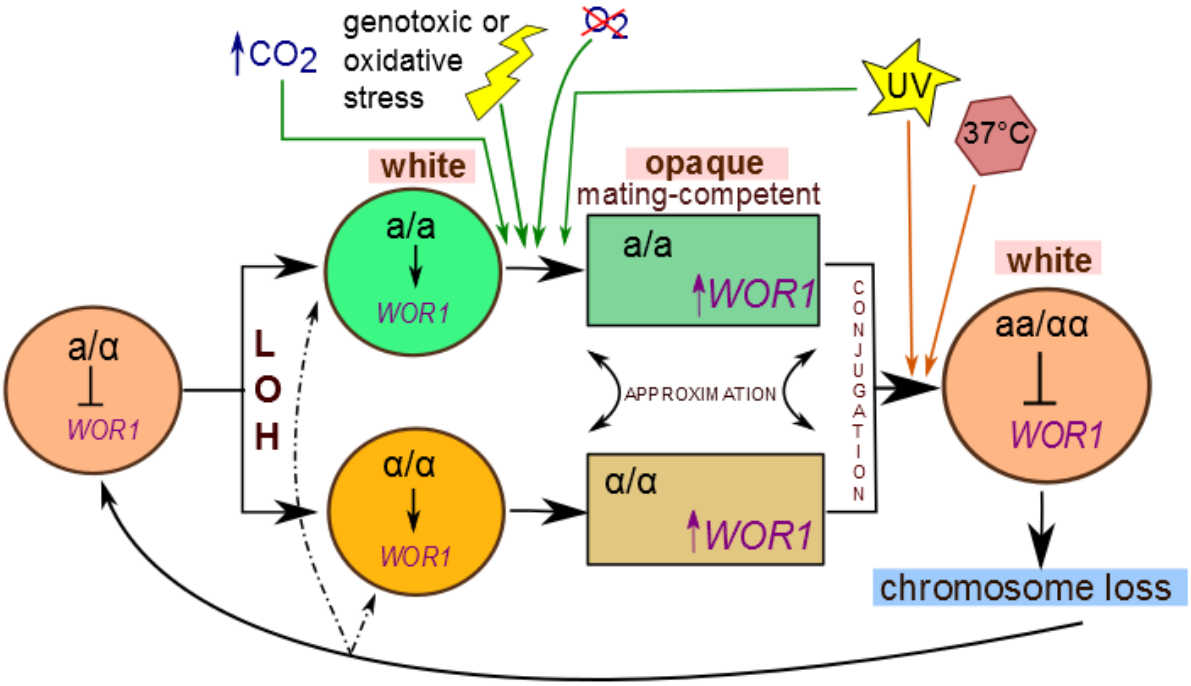


Figure 3: the parasexual cycle, generated according to [28,29]

LOH: Loss of heterozygosity

1.2.5 Epidemiology

Although infections with other *Candida* species like *C. glabrata*, *C. krusei* or *C. tropicalis* have been increasing in the past few decades, *C. albicans* still remains the most important pathogen *Candida* species. The findings in a longitudinal Swiss study indicate that the mean frequency of *C. albicans* is 66% of all *Candida* isolates in a 10-year-period [37]. Referring to Ghannoum et al., *C. albicans* is isolated from the oral cavities of 40% (*Candida* spp.: 75%) of healthy individuals even though among other fungi, for example *Fusarium*, *Aspergillus*, *Cryptococcus* and non-culturable fungi [15]. As Krause et al. showed in a study at University Hospital of Graz in 2001, *Candida* spp. are found in the feces of more than 50% of asymptomatic healthy individuals. *C. albicans* was again the most commonly isolated species [38].

Invasive fungal infections have been increasing significantly over the years [39]. While the rate of infection with *Aspergillus* spp. and less frequent fungi remains fairly constant, the increment is accountable to *Candida*, and again *C. albicans* depicts the most frequently isolated species [39]. The worldwide incidence of candidemia ranges from 1.2–25 cases per 100,000 (reviewed in [40]). It may be added that these differences are partially in consequence of unequal study designs and varying patient groups involved into the studies [40]. In a recent study performed in 5 major general hospitals in the United Kingdom, the crude mortality of candidemia was determined to be 40%, with half of the cases caused by *C. albicans* [41].

1.3 Invasive Candidiasis

1.3.1 Definition

This term describes *Candida* infections apart from those of the skin and mucosa. It includes candidemia, deep organ infection and acute and chronic disseminated candidiasis [7]. The presence of *Candida* in at least one blood culture, whether the patient has symptoms or not, defines the term candidemia [7]. Symptoms are largely unspecific. Patients usually show persistent fever [7]. Signs of systemic inflammatory response syndrome, such as hypotension, tachycardia and tachypnea, can occur as well [7]. In 10–35% of the concerned subjects, dependent on the reviewed literature, characteristic skin lesions can be observed [7,42]. These lesions can be erythematous or purpuric, they may show erythroid nodules or even plaques [42]. The lesions can be particularly on the extremities or disseminated throughout the integument [42].

Eye involvement is diagnostic for dissemination [7]. It occurs in about 16% of patients with invasive candidiasis [43]. Endophtalmitis is a grave affection, more likely to be found in patients with invasive *Candida* infection that developed unnoticed and consequently untreated (reviewed in [43]). Chorioretinitis is an affection that may occur in a less advanced stage [43]. However, as the vitreous body is not involved and it is asymptomatic, the early diagnosis of chorioretinitis is challenging [43].

The gastro-intestinal tract, the lung, the liver and the spleen are the organs most often involved in acute and chronic dissemination [39]. Hepatosplenic candidiasis or chronic disseminated candidosis is a severe affection solely seen in neutropenic patients [7]. It usually occurs after recovery from chemotherapy-induced neutropenia [44]. The liver and the spleen are the most commonly affected organs with clinical signs, like broad-spectrum-antibiotics resistant fever, abdominal tenderness and pain, hepatosplenomegaly, and elevation of alkaline phosphatase and inflammatory markers [45]. Diagnosis and treatment of this affection remain challenging [45].

1.3.2 Risk Factors

A multitude of factors influence the risk to acquire an invasive fungal infection, especially candidiasis. Very young (<1 year, preterm or small-for-date newborns) and elderly patients (>65 years) have a notably higher risk to suffer from an invasive candidal infection (reviewed in [40]). Critically ill patients, for example, patients with acute severe pancreatitis [46] or septic patients [47], and especially those with long-term hospitalization in intensive care units, belong to the risk population (reviewed in [7,40,47]).

The presences of indwelling catheters, total parenteral nutrition, hemofiltration and hemodialysis or mechanical ventilation are independent risk factors (reviewed in [47]). Surgical patients, particularly major abdominal surgery and abdominal trauma patients are at risk as well (reviewed in [7,40,47]). Individuals who suffer from hematologic malignancy and patients who underwent transplantations are also at major risk for the development of an invasive fungal infection [7]. This fact was confirmed by a post mortem study conducted at a university hospital in Germany, in which 2072 autopsies were analyzed [39]. *Candida* spp. and *Aspergillus* spp. were the major pathogens in this study [39].

Disorganization of the normal microbial flora, e.g., through long-term antibiotic treatments, also facilitates candidiasis [7,19]. The influence of *Candida* colonization to development of invasive candidiasis has been discussed controversially [48]. Data from 2006 suggest that subjects with colonization of rectum or ostomy or both, urinary tract or respiratory tract have got a higher risk to develop invasive candidiasis [48]. However, patients with oropharyngeal and gastric colonization are not at higher risk in accordance with this study [48].

1.3.3 Pathophysiology

Epithelial cells of the mucosa and the skin play an outstanding role in the control of *C. albicans* invasion (reviewed in [49]). The high rates of mucosal colonization within the healthy population reinforce this finding (reviewed in [49]). Epithelial cells express interleukin-8 (IL 8) and granulocyte macrophage colony-stimulating factor (GM-CSF) in case of *C. albicans* invasion [49]. These chemokines induce a recruitment of immune cells, like neutrophils, lymphocytes, and monocytes (reviewed in [50]). A malfunctioning mucosal barrier, for example, in consequence of surgery, trauma, chemotherapy, or other mucosa-disruptive factors, promotes *C. albicans* invasion [49]. In combination with neutropenia, mucosal disruption can lead to the development of invasive candidiasis [49].

In case of contact to a fungal antigen, the innate immune system is the “vanguard” that initiates an inflammatory response [49]. The specific immune response, mediated by T-lymphocytes or B-lymphocytes, follows somewhat delayed [49]. The innate or non-specific immune system is not as unspecific as its name predicts, it is rather in disposal of several mechanisms for fast detection of invading agents [49].

Amongst others, the toll-like receptors (TLRs), above all, TLR 2 and 4, the C-type lectin receptors (CLRs), mainly Dectin-1 and Dectin-2, and the mannose receptors (MRs) constitute important human pattern-recognition receptors (PRRs) [49,51]. These receptors recognize fungal pathogen-associated molecular patterns (PAMPs) [49] and facilitate phagocytic intervention [51]. Cell wall components, like mannan, galactomannan, or ß-1,3 glucan, constitute the majority of PAMPs due to the fact that the carbohydrates and cell-wall proteins of the *C. albicans* cell wall are absent in men (reviewed in [19]). The PAMPs can vary notably among different morphologic *C. albicans* forms [51](reviewed in [49]) For example, Dectin-1 can recognize its ligand ß-1,3 glucan in blastoconidia, as ß-1,3 glucan is presented in the so-called bud-scars, meaning zones where the daughter cell separated from its mother cell [52]. In the filamentous form, either true or pseudohyphae, ß-1,3 glucan is not presented and hence cannot be recognized by Dectin-1 [52].

The way how PRRs induce an immune response has not yet been totally understood [51], however, investigations have shown that the PRRs promote phagocytosis by mediation of the linkage between fungus and phagocytes [51]. The PRRs also activate intracellular pathways, finally leading to chemokine and cytokine production, phagocytosis and antimicrobial effector functions [51].

As stated previously, the most important actors of the second defense line against invasive fungal infections are the phagocytic cells [51]. These include macrophages, neutrophils, monocytes and dendritic cells [51]. After activation through cytokines and chemokines or through the PRRs, *Candida* cells are taken up into the phagocytes [51]. After lysis of the ingested fungus and maturation, the phagocytic cell presents fungal antigens at its surface, hereby inducing the adaptive immune system [51]. Macrophages and monocytes express several pro-inflammatory cytokines, most notably TNFα, IL-1 and IL-6, leading to neutrophil cell attraction and response of the adaptive immune system [49].

It has to be mentioned that the fungus developed a strategy to elude phagocytes. The hostile environment within the phagocytic cell can lead to a stress reaction within the *Candida* cell, inducing hyphae growth (see 1.2.3). Hyphae may escape the phagocytes through perforation of the cell membrane [53](reviewed in [2]). However, some phagocytes, such as neutrophils, are capable to avoid hyphal escape (reviewed in [51]). This fact may explain the notably higher susceptibility of neutropenic patients to invasive fungal infections (see 1.3.2). Induction of phagocyte apoptosis and inhibition of the maturation process are further mechanisms of *C. albicans* to escape from the host's immune response (reviewed in [51]).

The dendritic cells span the gap between innate and adaptive immune system through priming of naïve T-cells [54]. Depending on the expressed cytokines, Th-1, Th-2, Th-17 and regulatory T-cell responses are differentiated [54]. Th-1 cells and their prototypic cytokine IFNγ play an outstanding role in the immune response to invasive candidiasis (reviewed in [49]). IFNγ is important for the specific immunoglobulin expression against *Candida*, among other functions (reviewed in [49]).

As to the function of Th17-cells, conflictive findings have been figured out, depending on the site of infection [54]. Studies either showed that these cells, through segregation of IL 17, stimulate the immune response, or degrade the immune reaction with consequently higher fungal burden (reviewed in [54,55]).

In a nutshell, pathogenesis of invasive Candidosis is a network of complex biologic interactions, not only involving immune cells, but also epithelial cells and a multitude of biologic messengers. The delicate balance between colonizing *Candida* cells and host may be disturbed, promoting candidal invasion, when the mucosal barrier does not work properly (see 1.3.2) (reviewed in [49]). In case that the fungus disposes of a direct bloodstream entry, such as biofilm formation on indwelling catheters, a tight immune response is required to parry the infection successfully. By nature of their conditions, this is particularly demanding in the *Candida*-susceptible patient population [49].

1.3.4 Diagnosis

Because of the high mortality, which yet increases with delay of adequate treatment, fast and exact detection methods are crucial [56]. Naturally, a positive blood culture (BC) is diagnostic for candidemia (reviewed in [56]). In suspected and proven candidemia, BCs should be obtained daily to monitor the fungal clearance [56]. The cultures should be incubated not less than 5 days [56]. In suspected disseminated candidosis, BCs are essential as well, with additional swabs and/or punctions and/or fluids from the possibly affected body sites [56]. These samples should be cultured and also examined with direct microscopy and histopathology [56]. Particularly because of their different resistance characteristics, it is obligatory to identify the isolates to the species level [56].

These methods have some limitations – cultural methods need at least 24 hours of incubation time. Cultures on chromogenic agar allow the discrimination of *C. albicans* and other yeasts (see 1.2.1). Direct microscopic examination and histopathology can be challenging, depending on the abundance of the fungus and the investigator's experience [56]. Susceptibility testing should be performed in all cases of invasive candidiasis to ascertain the most appropriate antifungal agent [56].

To detect fungal biomarkers in blood, a ß-1,3-glucan test is available, but it is not specific for *C. albicans* [56]. Nevertheless this test is faster than cultural detection [56], in case it shows a positive result, an empiric antifungal treatment can be initiated and adapted afterwards. Another tool is the combinatory mannan/antimannan test, which is specific for *Candida* spp., with the highest species sensitivity for *C. albicans* [57] (reviewed in [56]). Further biomarker tests are raring to go, but not yet recommended because of lacking or unclear clinical data [56]. PCR investigations are also available but not yet recommended for the same reasons and also because of high inter-laboratory differences [56]; however, PCR techniques can be useful to identify the species from a culture in case of ambiguity. Measurement of therapeutic drug levels should be performed in case of lacking therapeutic success, adverse effects, and in patients with impaired renal or liver function or both, or with extra-corporeal membrane oxygenation [56]. Drug monitoring should be performed in patients with 5-flucytosine therapy and it is also recommended in patients who receive posaconazole or voriconazole [56].

1.3.5 Treatment

All patients with positive blood culture, whether they suffer from signs and symptoms of invasive infection or not, should be treated [58]. Echinocandins are the first-line therapy for invasive candidiasis due to *C. albicans* [59-61]. Until lately fluconazole could be used as well; despite some limitations, e.g., breakthrough-infection under prophylactic fluconazole therapy and critically ill and/or neutropenic patients [60-62]. As fluconazole showed inferior results in the treatment of candidemia in comparison to echinocandins (reviewed in [59]), the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) no longer recommends fluconazole as a first choice [59]. The ESCMID recommends echinocandins as the first line treatment and liposomal amphotericin B or voriconazole as a second choice [59]. Intravasal catheters should be removed whenever possible [59]. In case of impossibility, echinocandins or liposomal amphotericin B should be administered as these drugs showed superior effects in biofilms (reviewed in [59]). Neonates with invasive candidiasis can be treated with amphotericin B deoxycholate and its liposomal preparations, micafungin and fluconazole [63,64]. Intravasal catheters have to be removed [63].

drug	dosage	evidence
Anidulafungin	200/100 mg	A-I
Caspofungin	70/50 mg	A-I
Micafungin	100 mg	A-I
lip. Amphotericin B	3 mg/kg	B-I
Voriconazole	6/3 mg/kg/day	B-I
Fluconazole	400–800 mg	C-I

Table 1: ESCMID recommendations for the treatment of invasive candidosis

adapted from [59]

It has to be mentioned that echinocandins do not penetrate sufficiently into the eye [65]. In case of eye involvement another drug, e.g., fluconazole, and local treatment has to be administered additionally [65]. However, data from recently published studies suggest that micafungin does penetrate the eye satisfactory after intravenous administration [66,67]. Despite that, the authors of these studies still recommend vitrectomy and additional intravitreal application in severe cases of *Candida* endophtalmitis [66,67].

1.3.6 Prophylaxis

Prophylaxis is recommended in patients that undergo allogeneic hematopoietic stem cell transplantation [44]. The first-line drug in the state of neutropenia and during the first 100 days of neutrophil recovery (without graft-versus-host disease) is fluconazole (400 mg/day) [44]. Voriconazole (2 x 200 mg/day) and micafungin (50 mg/day) can be considered alternatively [44]. In patients within a risk population else then allogeneic hematopoietic stem cell transplantation, an antifungal prophylaxis may be considered, but there is no general recommendation [44,59]. In patients receiving an antifungal prophylaxis, surveillance should be as close as in other patients since breakthrough-infections, for example, by species with intrinsic or acquired resistance, are possible [44]. Within the pediatric population, preterm neonates with low and very low birth weight have a higher risk to develop invasive candidiasis (reviewed in [63]). Prophylactic treatment with fluconazole, 3 mg/kg intravenously twice a week is hence recommended in infants with birth weight below 1000 g and/or infants in 27th gestational week or younger [68]. Nystatin can be administered as well, but it has got some limitations: On the one hand, it has to be given enterally, and on the other hand, the doses and the frequency of administration are superior to fluconazole [68].

1.3.7 Susceptibility

Resistance to echinocandins is seen rarely in *C. albicans* (0.0–0.6%) and other non-*albicans Candida* spp. like *C. tropicalis*, *C. krusei* and *C. parapsilosis* with 0.0%, 0.0% and 0.0–1.2%, respectively [69]. The *FKS1* gene, encoding the glucan synthase, which is a target of echinocandins, was identified as a hotspot for mutations that lead to reduced susceptibility to this drug class [70-72]. However, until now only cases of reduced susceptibility with prior echinocandin therapy were reported [40].

With regard to the triazoles, the resistance rate is also low in *C. albicans* (0.0–0.6%) except for posaconazole, which showed higher resistance rates in Europe (8.3%) and Latin America (6.5%) [69]. Non-*albicans Candida* species like *C. glabrata* show notably higher resistance rates [37,69].

1.4 Molecular typing of C. albicans

A multitude of techniques has been used to investigate *C. albicans* to understand the biology, population structures, epidemiology and transmission of the yeast [16]. A distinction is made between non-DNA-based methods, such as Multilocus Enzyme Electrophoresis, and DNA-based methods. The latter detect polymorphisms directly in the genome [16]. DNA-based methods are subdivided into conventional and exact DNA-based typing methods [16]. The latter are characterized through a high intra- and inter-laboratory reproducibility and the production of unambiguous data [16]. These characteristics are crucial for global surveys as they allow the comparison and joint analysis of data obtained in laboratories all over the world. The discriminatory power is a numerical index defined as the capability of a method to discriminate two unrelated strains [73]. The closer the number approximates to 1, the higher the discriminatory power [73].

1.4.1 Microsatellite Length Polymorphism analysis (MLP)

Microsatellites or simple sequence length polymorphisms (SSLP) are non-coding, repetitive DNA-fragments of different length, containing 1–6 base pairs [16,74]. These fragments can be investigated through qualitative PCR. The Primer pairs used for this method bind in specific microsatellite regions [16,74]. Naturally, the DNA products vary in size, depending on the number of microsatellite repetitions [16,74]. These differences can be visualized with gel electrophoresis [16]. The discriminatory power differs within the microsatellite markers (reviewed in [16]). A high discriminatory power of 0.999 can be obtained with a set of 11 microsatellite markers [75]. To date, no generally available internet database has been provided due to technical challenges [16].

1.4.2 High resolution DNA melting (HRM)

HRM is a PCR technique in which polymorphic DNA regions are amplified in the presence of fluorescence markers, which only bind double-stranded DNA (reviewed in [74]). After amplification, the fluorescent DNA products are heated so that the double strands separate (“melting”) and the fluorescent markers dispense (reviewed in [74]). The brightness of fluorescence consequently increases. The melting temperature can be measured, the shape of the melting curve can be illustrated. The melting temperature and the shape of the curve are specific for the sequences of each DNA fragment (reviewed in [74]). They primarily depend on the guanine/cytosine content and the number of base pairs [74]. With 0.77, the discriminatory power is lower than in MLP and MLST; however, this technique may be a useful epidemiologic tool as it is cost-effective, quick, and comparatively easy to perform [76] (reviewed in [74]). As HRM only requires real-time PCR-equipment, it can be applied in standard clinical laboratories [76].

1.4.3 Multi Locus Sequence Typing PCR

MLST was developed by Maiden et al. in 1998 [77]. Primarily implemented for molecular typing of the haploid organism *Neisseria meningitidis*, the method has been expanded to various species, bacteria as well as fungi, e.g., *Borrelia burgdorferi*, *Campylobacter jejuni*, *Escherichia coli*, *Streptococcus pyogenes,* and various *Candida* spp. MLST is based on DNA sequence analysis of housekeeping gene fragments [77]. This method distinguishes different isolates unambiguously by discovery of polymorphic sites. In diploid organisms like *C. albicans*, there are 10 possibilities of nucleotide combinations (homozygous: A/A, C/C, G/G, T/T, heterozygous: A/T, A/C, A/G, C/T, C/G, G/T) at each site [78-80]. These polymorphic sites in each gene fragment determine distinct alleles. The summary of the alleles or sequence types (ST) of all loci identify their allelic profile or diploid sequence type (DST). The STs and DSTs with their corresponding nucleotide sequences, clinical data, geographical and anatomical origin and susceptibility to antimicrobial agents, are collected in an internet database [81] (http://www.mlst.net, http://calbicans.mlst.net). This portability of the method through the internet is a striking advantage; it facilitates global and local studies [77-79].

Above all, MLST is highly discriminating, objective, and it holds a high inter-laboratory comparability as the produced sequence data are unambiguous [78,80,82]. The discriminatory power index for the actual MLST scheme for *C. albicans* is 0.99996 [82]. The reproducibility is 99.72% [82]. Due to these conveniences, MLST is currently regarded as the reference method for molecular typing in *C. albicans* [75]. A disadvantage of the method is that it does not contain gene fragments of chromosomes 3, 5 and 7 [16,32]. Another drawback of the method is that it is quite time-consuming, expensive and requires specialized personnel.

1.5 Objectives

In this thesis we utilized MLST to type clinical *C. albicans* samples. The aims of our study were (1) to identify which strains generally exist in Graz, but also in Austria, as this was the first MLST investigation in the country. We also wanted to know whether and in what extent these strains differ from other countries (see Figure 4).

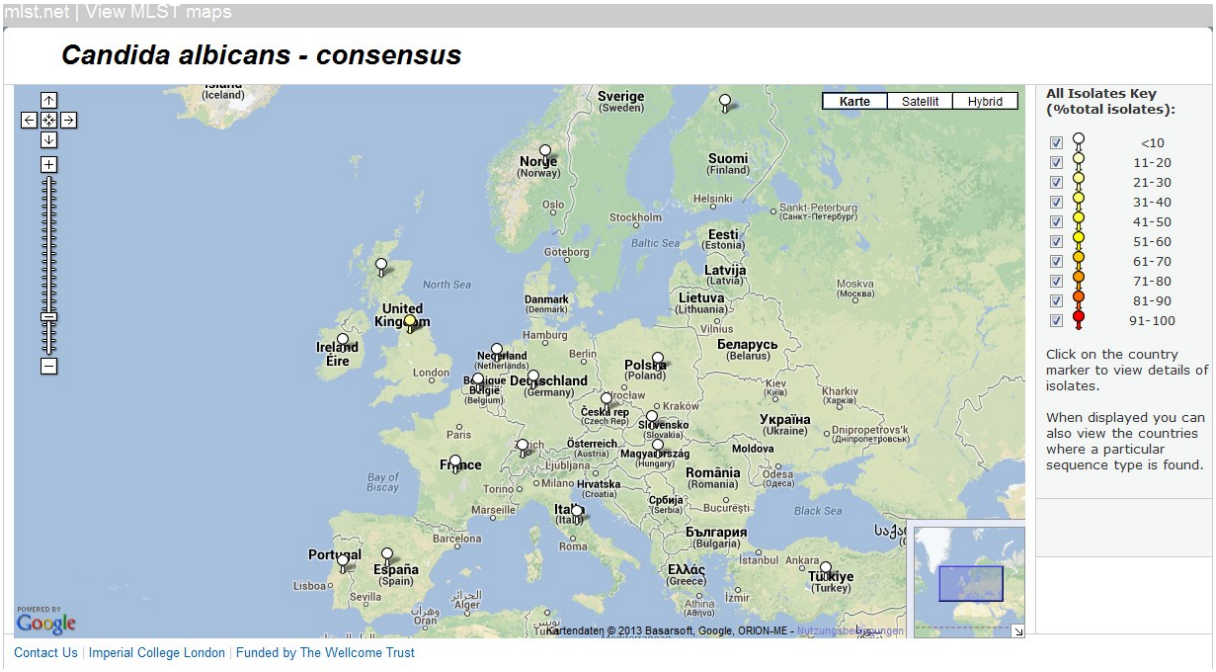


Figure 4: C. albicans MLST map as of June 2013.

By this time, no data from Austria and only few data from adjacent countries have been added.

Candida infections may either be acquired from an exogenous source, for example, from the parents or siblings to a child, or healthy personnel to a patient; or be transmitted endogenously from the patient's own colonizing strain/strains (reviewed in [16]). The elucidation of these transmission pathways, or at least which of them is more common, will be useful in the decision whether prophylactic or therapeutic antifungal treatment would make sense or not. It was therefore also an aim of our study to (2) identify the mode of transmission in our isolates.

We compared strains of commensal with strains of invasive isolates in question of (3) pathogenicity differences. We additionally wanted to find out whether there were (4) any differences in susceptibility regarding the different strains.

The investigations conducted in this thesis were made in the framework of the project "Host and pathogen characteristics in patients with *Candida* spp. in the lower respiratory tract compared to patients with candidemia and healthy individuals". The Ethics Committee of the Medical University of Graz approved this study by the vote 19-322 ex 07/08.

2 MATERIAL AND METHODS

2.1 Isolates

For this study 181 yeast isolates were obtained from University Hospital of Graz, either Department of Internal Medicine or Division of Plastic, Esthetic and Reconstructive Surgery. The samples were delivered on CHROMAgar *Candida* Medium (Becton Dickinson). The samples were collected between 2008 and 2012 and divided in subgroups, either healthy individuals without antibiotic treatment, healthy individuals with antibiotic treatment, or intubated and mechanically ventilated patients without pneumonia and without antibiotic treatment, or intubated and mechanically ventilated patients without pneumonia but with antibiotic treatment, and patients with candidemia. Isolates were cultured from blood, oral and perianal swabs, catheter insertion sites, urine and ascites aspirates. All isolates were added to the culture collection of the Institute of Hygiene, Microbiology and Environmental Medicine, mycology laboratory; associated with the Biobank at the Medical University of Graz (strain numbers 052.13–232.13).

2.2 Culture

To perform Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and preservation techniques, the isolates were inoculated on Sabouraud (SAB) agar plates and incubated at 36°C for 24 hours.
SAB was produced in house in the Institute of Hygiene, Microbiology and Environmental Medicine. Therefore 1 L of distilled water and 65 g SAB dextrose agar (Oxoid CM0041) were heated by continuous stirring until the solution was clear. After autoclaving at 121°C and cooling down to 50–60°C, 20 mL each of the solution was poured in sterile petri dishes. After solidifying of the agar the SAB plates were stored in a refrigerator at 4–8°C.

2.3 MALDI-TOF MS

All isolates were tested with MALDI-TOF mass spectrometry to verify them as *C. albicans* isolates. MALDI-TOF MS allows the determination of fungi by their specific mass spectrometry profile of (mostly) ribosomal proteins.
As indicated by the manufacturer, some colonies of each sample were mixed with a special matrix directly on the sample tray. The further steps succeeded automatically by means of the automated microbial identification system VITEK® MS (bioMérieux).

2.4 Preservation

To store the isolates, we used the VIABANK vial system (VIABANK medical wire & equipment, Corsham, United Kingdom). Preservation with this system is suitable for organisms which are able to produce permanent forms. The VIABANK vials contain a sterile cryopreservative broth and 20 ceramic beads. With the help of an inoculation loop, some colonies from a fresh subculture were mixed with the broth. After closing the vial, it was shaken carefully to spread the fungus substrate through all beads. Then the remaining fluid was drawn off with a sterile pipette and, after replacing the cap, the vial was frozen at -70°C. To recover the fungus isolate, one bead was taken with the help of an inoculation loop and rolled over a SAB plate. Afterwards the plate was incubated at 36°C for 24 hours.

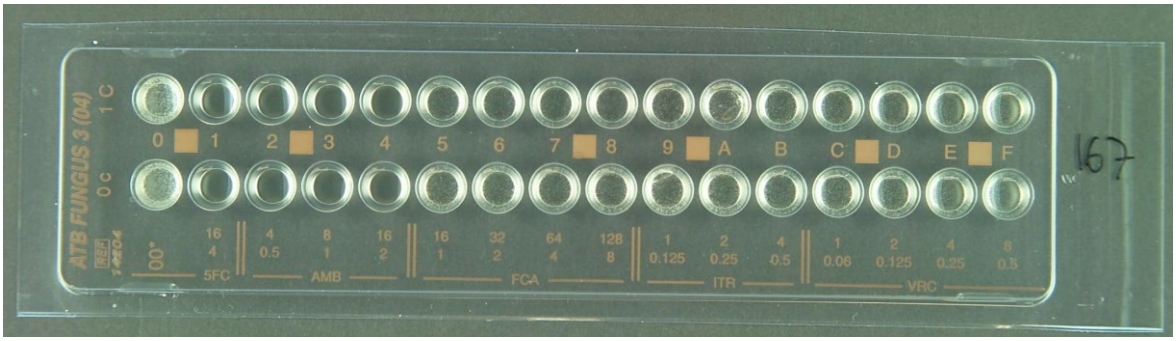


Figure 5: ATB® Fungus 3 testing stripe after 24h incubation

2.5 Susceptibility testing

All *C. albicans* isolates were tested for their susceptibility to flucytosine, amphotericin B, fluconazole, itraconazole and voriconazole using the ATB® FUNGUS 3 Kit (bioMérieux). For these purposes, some colonies of each isolate were suspended in physiological sodium chloride solution to obtain a suspension with a turbidity corresponding to a McFarland 2 standard, which corresponds approximately to $6x10^8$ colony forming units (CFU) per mL. Then 20 µL of the McFarland 2 suspension was mixed with the ATB broth and 135 µL of the mixture was dropped into each notch of the ATB Fungus strip. After closing the strips with the incubation lids, they were incubated in a humid chamber at 36°C. After 24 hours they were read (see Figure 5: ATB® Fungus 3 testing stripe after 24h incubation). Flucytosine and Amphotericin B have to inhibit the fungus completely in the given concentrations, as they are fungicidal, while azoles must only lead to a partial inhibition (i.e. 80%), they are fungistatic.

	MIC breakpoint (mg/L)		
Antifungal agent		S ≤	R >
	Amphotericin B	1	1
	Anidulafungin	0.03	0.03
	Caspofungin	NE*	NE*
	Fluconazole	2	4
	Itraconazole	NE*	NE*
	Micafungin	0.016	0.016
	Posaconazole	0.06	0.06
	Voriconazole	0.12	0.12

Table 2: EUCAST MIC breakpoints [56]

*not established

Furthermore the antifungals caspofungin, anidulafungin, micafungin and posaconazole were tested with the ETEST system (bioMérieux). The Etest or Epsilometer test allows a direct determination of the minimal inhibitory concentration (MIC) of an antifungal. For this purpose, the Etest strip is impregnated with the antifungal in descending order of concentration. Therefore a concentration gradient arises after adding it to an inoculated agar plate.

An ellipsoid zone of inhibition appears after 24 hours of incubation at 36°C. The MIC is read at the point where the ellipse crosses the strip (see Figure 6). In accordance with the producer's manual, RPMI Glucose agar dishes (bioMérieux) were inoculated with a suspension of each isolate in physiological sodium chloride solution with a McFarland 0.5, corresponding to approximately $1.5x10^8$ CFU/mL. Reading was performed after 24 hours of incubation in a damp chamber at 36°C. To quantify susceptibility, EUCAST [83] guidelines were used, if existing.

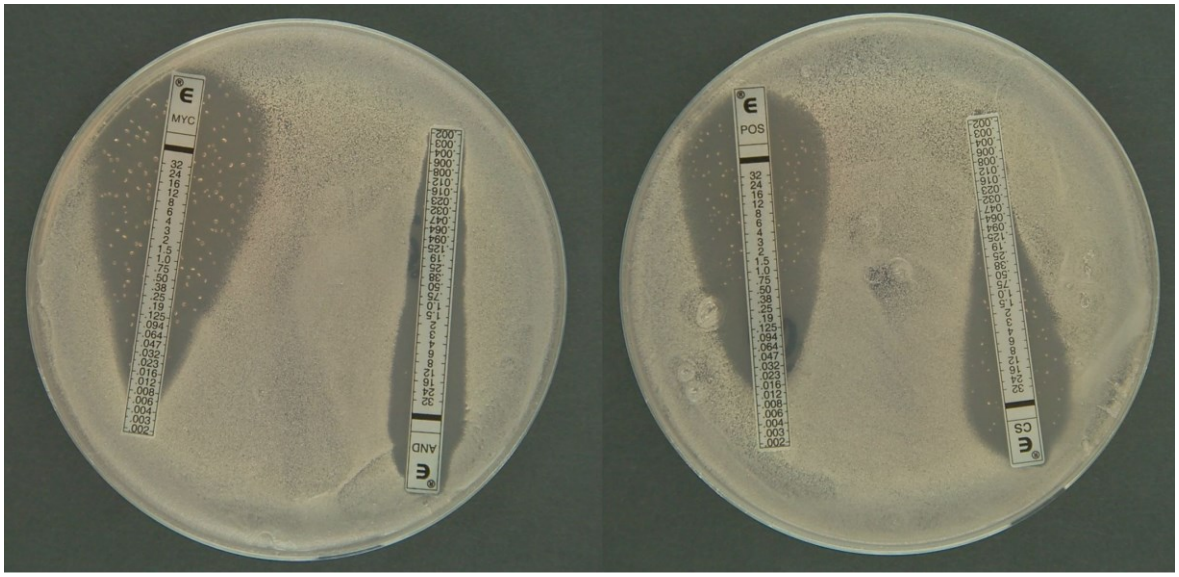


Figure 6: ETEST stripes on RPMI Agar after 24h incubation

MYC: micafungin, AND: anidulafungin, POS: posaconazole, CS: caspofungin

2.6 PCR

A panel of 44 isolates was chosen for MLST-PCR and recultured on SAB agar. In Table 3 these isolates are listed with their source (BC = blood culture, CVC = central venous catheter), year of isolation, diagnosis and some further information. Samples 109 and 110 were obtained from the same patient, but different sites. BC Isolate 181 was obtained a few months prior to the oral sample 104 from the same patient.

2.6.1 DNA isolation

DNA extraction was carried out by means of Applied Biosystems PrepMan® Ultra Sample Preparation Reagent (life technologies, U.S.A.). 200 µL of PrepMan Buffer and 1 loop from a pure culture of each sample were mixed thoroughly in 1.5-mL tubes and heated in boiling water for 12 minutes. After a 3 minutes centrifugation step at 16,100 *g*, the DNA-containing supernatant was decanted and transferred into a new tube.

Table 3: clinical samples for MLST

* healthy control patients who received an antibiotic prophylaxis in the context of their surgical intervention

+ Biobank Graz accession numbers

Strain	Number +	Region	Source	Year	Diagnosis	Age	Sex	Other information
25	067.13	Graz	oral	2009	healthy	57	m	antibiotic therapy
27	068.13	Graz	oral	2009	healthy	46	m	antibiotic therapy
28	069.13	Graz	oral	2009	healthy	54	m	antibiotic therapy
29	070.13	Graz	oral	2009	healthy	68	f	antibiotic therapy
30	071.13	Graz	unknown	2009	healthy	50	f	antibiotic therapy
32	072.13	Graz	oral	2008	healthy	77	f	antibiotic therapy
33	073.13	Graz	oral	2009	healthy	55	f	
34	074.13	Graz	oral	2009	healthy	67	m	
35	075.13	Graz	oral	2009	healthy	58	m	
37	076.13	Graz	oral	2009	healthy	47	f	antibiotic therapy
38	077.13	Graz	tracheal	2009	healthy	72	f	
40	079.13	Graz	oral	2009	healthy	55	f	
42	081.13	Graz	tracheal	2009	healthy	68	m	antibiotic therapy
43	082.13	Graz	oral	2009	healthy	42	f	
44	083.13	Graz	oral	2009	healthy	57	m	
47	084.13	Graz	oral	2009	healthy	68	m	antibiotic therapy
48	085.13	Graz	oral	2009	healthy	52	f	
49	086.13	Graz	oral	2008	healthy	44	f	
50	087.13	Graz	oral	2009	healthy	62	f	
51	088.13	Graz	oral	2008	healthy	44	f	
104	132.13	Graz	oral	2009	candidemia	42	f	
106	133.13	Graz	BC	2010	candidemia	83	m	
108	135.13	Graz	BC	2010	candidemia	64	f	
109	136.13	Graz	oral	2010	candidemia	74	m	
110	137.13	Graz	ascites punction	2010	candidemia	74	m	
111	138.13	Graz	cyst	2010	candidemia	63	f	pancreatic cyst
112	139.13	Graz	CVC	2010	candidemia	69	m	
113	140.13	Graz	BC	2010	candidemia	58	m	
114	141.13	Graz	BC	2010	candidemia	81	m	
115	142.13	Graz	BC	2010	candidemia	78	m	
116	143.13	Graz	BC	2010	candidemia	81	m	
117	144.13	Graz	BC	2010	candidemia	53	m	
118	145.13	Graz	BC	2010	candidemia	46	f	
120	146.13	Graz	BC	2011	candidemia	56	m	
121	147.13	Graz	BC	2011	candidemia	80	m	
122	148.13	Graz	BC	2011	candidemia	63	m	
124	149.13	Graz	BC	2012	candidemia	70	f	
127	152.13	Graz	BC	2012	candidemia	64	f	
128	153.13	Graz	BC	2012	candidemia	58	f	
129	154.13	Graz	BC	2012	candidemia	70	m	
136	161.13	Graz	CVC swab	2012	candidemia	57	m	
173	186.13	Graz	BC	2012	candidemia	66	f	
178	189.13	Graz	BC	2012	candidemia	58	f	
181	191.13	Graz	BC	2009	candidemia	42	f	

2.6.2 MLST-PCR

2.6.2.1 Primer design

The seven primer pairs were chosen as recommended on the *C. albicans* MLST website [79]. All primers (see Table 4) are specific for DNA sites that encode housekeeping genes. Referring to previous studies, this scheme is holding the highest discriminatory power of all *C. albicans* MLST schemes [78,84]. Primers were commercially produced by Invitrogen™ Custom DNA Oligos (life technologies). The primer stock solution was diluted with distilled water to obtain a concentration of 10 µMol/L.

PCR was carried out with total reaction volumes of 50 µL, containing 37 µL water, 5 µL 10x Taq Buffer*, 1 µL dNTP Mix*, 1 µL Taq DNA Polymerase* (*5prime, Germany), 2 µL of the forward and reverse primer (10 µMolar) and 2 µL DNA template. PCR was performed on an Applied Biotechnologies 2720 Thermal Cycler (life technologies) as proposed by Bougnoux et al.:
An initial step at 93°C for 5 minutes was succeeded by 30 cycles of 93°C for 30 seconds, 55°C and 72°C for one min each and a final step at 72°C for 4 min [80].

Locus	Gene	Fragment size	Chromo-some	Primer
***AAT1*a**	Aspartate aminotransferase	373	2	Fwd 5'-ACTCAAGCTAGATTTTTGGC- 3' Rev 5'-CAGCAACATGATTAGCCC- 3'
ACC1	Acetyl-coenzyme A carboxylase	407	R	Fwd 5'-GCAAGAGAAATTTTAATTCAATG- 3' Rev 5'-TTCATCAACATCATCCAAGTG- 3'
ADP1	ATP-dependent permease	443	1	Fwd 5'-GAGCCAAGTATGAATGATTTG- 3' Rev 5'-TTGATCAACAAACCCGATAAT- 3'
***PMI1*b**[a]	Mannose phosphate isomerase	375	2	Fwd 5'-ACCAGAAATGGCCATTGC- 3' Rev 5'-GCAGCCATGCATTCAATTAT- 3'
SYA1	Alanyl-RNA synthetase	391	6	Fwd 5'-AGAAGAATTGTTGCTGTTACTG- 3' Rev 5'-GTTACCTTTACCACCAGCTTT- 3'
VPS13	Vacuolar protein sorting protein	403	4	Fwd 5'-TCGTTGAGAGATATTCGACTT- 3' Rev 5'-ACGGATGGATCTCCAGTCC- 3'
***ZWF1*b**	Glucose-6-phosphate dehydrogenase	491	1	Fwd 5'-GTTTCATTTGATCCTGAAGC- 3' Rev 5'-GCCATTGATAAGTACCTGGAT- 3'

Table 4: Genes and Primers, adapted from [78,79,84]

[a]Formerly *MPI1*b [85]

2.6.3 Resistance genes PCR

One sample, isolated from the oral mucosa in a candidemic patient, showed a reduced susceptibility to echinocandins. The relevant isolate as well as a blood culture isolate from the same patient, obtained some weeks prior to the oral one, where therefore investigated with an additional PCR. This PCR amplified the ß-1,3-glucan synthase catalytic subunit 1 (*FKS1*) gene (see Table 5), which is known as a hotspot for mutations that lead to reduced susceptibility to echinocandins [86]. Primers were commercially produced by Invitrogen™ Custom DNA Oligos (life technologies). The primer stock solution was diluted with distilled water to obtain a concentration of 10 µMol/ L.

Locus	Gene	Fragment size	Primer
GSC1	ß-1,3-glucan synthase catalytic subunit 1	450	Fwd 5'-GAAATCGGCATATGCTGTGTCC- 3' Rev 5'-AATGAACGACCAATGGAGAAG- 3'

Table 5: *FKS1* - hotspot primer

PCR was carried out with total reaction volumes of 50 µL, containing 37 µL water, 5 µL 10x Taq Buffer*, 1 µL dNTP Mix*, 1 µL Taq DNA Polymerase* (*5prime), 2 µL of the forward and reverse primer (10 µMolar) and 2 µL DNA template. PCR was performed on an Applied Biotechnologies 2720 Thermal Cycler (life technologies). An initial step at 94°C for 2 minutes was followed by 30 cycles of 94°C for 45 s, 48°C for another 45 s and 68°C for 90 s. These cycles were followed by 5 min at 68°C and a final step at 4°C for 60 min. To check the quality of the PCR-products, agarose gel controls were performed at random (see Figure 18 in the supplementary material section).

2.6.4 Purification

The PCR products were purified using the MSB® Spin PCRapace purification system (STRATEC molecular GmbH, Germany). Each PCR sample was mixed with 250 µL binding buffer and transferred onto the spin filter (part of the kit). After 3 minutes centrifugation at 13,400 *g*, the filter was removed and transferred into a new tube. 50 µL elution buffer were added prior to a further centrifugation step for 1 minute at 9,200 *g*. The filter was then discarded, the supernatant in the tube representing the purified PCR product.

2.6.5 Cycle sequencing

Cycle sequencing was arranged using the BigDye® Terminator v3.1 Cycle Sequencing Kit (life technologies). The concentrations used for cycle sequencing are listed in Table 6. The primer stock solution was diluted with distilled water to obtain a concentration of 1.6 µMol/ L. Some amplicons, most notably *SYA1* products, had low chromatogram signals or a high noise signal. In this case, agarose gel electrophoresis was performed (see Figure 18, supplementary material section). Whether gel electrophoresis revealed narrow bands, the amount of template was raised.

Cycle sequencing was carried out on an Applied Biosystems 2720 Thermal Cycler (life technologies). An initial step of 94°C for 18 s was followed by 25 cycles of 94°C for 15 s, 50°C for 10 s and 60°C for 2 min with a final extension step at 4°C for 60 min.

Primer(1,6 µM)	Conc. [ng/µL]	A. d. [µL]	5x Buffer [µL]	Big Dye-Mix [µL]	Primer [µL]	Template [µL]	total [µL]
*AAT1*a Fwd	60	2.5	2	1.8	1	2.7	10
*AAT1*a Rev	60	2.5	2	1.8	1	2.7	10
ACC1 Fwd	60	2.5	2	1.8	1	2.7	10
ACC1 Rev	60	2.5	2	1.8	1	2.7	10
ADP1 Fwd	60	2.5	2	1.8	1	2.7	10
ADP1 Rev	60	2.5	2	1.8	1	2.7	10
*PMI1*b Fwd	60	2.5	2	1.8	1	2.7	10
*PMI1*b Rev	60	2.5	2	1.8	1	2.7	10
SYA1 Fwd	60	1.3	2	1.8	1	3.9	10
SYA1 Rev	60	1.3	2	1.8	1	3.9	10
VPS13 Fwd	60	2.5	2	1.8	1	2.7	10
VPS13 Rev	60	2.5	2	1.8	1	2.7	10
*ZWF1*b Fwd	60	2.5	2	1.8	1	2.7	10
*ZWF1*b Rev	60	2.5	2	1.8	1	2.7	10
GCS1 Fwd	60	2.5	2	1.8	1	2.7	10
GCS1 Rev	60	2.5	2	1.8	1	2.7	10

Table 6: cycle sequencing scheme

To remove remaining dNTPs, primers and other material that may disturb the sequencing analysis, further purification steps followed the cycle sequencing. The cycle sequencing product was vortexed in a 1.5-mL tube with 25 µL 98% ethanol and 2.5 µL 125 µM EDTA and then centrifuged for 20 minutes at a temperature of 4°C at 16,100 *g*. After discarding the supernatant carefully, 200 µL of 70% ethanol were added and another centrifugation step for 15 minutes at a temperature of 4°C at 16,100 *g* succeeded. Thereafter the supernatant was removed and the pellet was dried in an incubator at 37°C for at least 1 hour. Then the pellet was resuspended in 15 µL Applied Biosystems® Hi-Di™ Formamide (life technologies) and pipetted onto a 96-well plate. Analysis was performed on the Applied Biosystems® 3130 Genetic Analyzer (life technologies).

2.6.6 Data analysis

2.6.6.1 MLST Data

Analysis of the raw sequencing data succeeded with Applied Biosystems DNA Sequencing Analysis Software (life technologies). After analysis and post-processing, each forward and reverse sequence pair was assembled with the help of Lasergene® SeqMan Pro software (DNAStar, U.S.A.). By means of this program, so-called contigs were produced. A contig is the overlapping consensus sequence data of a forward and reverse DNA strain. Any contig was searched automatically and manually for single nucleotide polymorphisms (SNPs). The SNPs were badged using the IUPAC nucleotide code (see Table) as illustrated in Figure 7 [87]. Each consensus sequence was compared with the database on the MLST website to obtain the allele number (ST – sequence type) and the MLST profile number (DST – diploid sequence type). When the ST was not found, the sequence was reviewed and an alignment of all alleles in the database together with the putative new allele was conducted. This approach should assure that the allele is really new and no analysis fault. New alleles and DSTs were sent to the curator of the MLST website to obtain the allelic and DST numbers. The 7 consensus sequences of each isolate were concatenated and afterwards aligned by means of muscle. With the help of this alignment all polymorphic sites were identified.

Symbol	Mnemonic	Translation
A		adenine
C		cytosine
G		guanine
T		thymine
R	PU**R**INES	A or G
Y	P**Y**RIMIDINES	C or T
M	A**M**INO GROUP	A or C
K	**K**ETO GROUP	G or T
S	**S**TRONG INTERACTION	C or G
W	**W**EAK INTERACTION	A or T
N	A**N**Y	A, T, C or G
-		gap

Table 7: The IUPAC nucleotide code, adapted from [87]

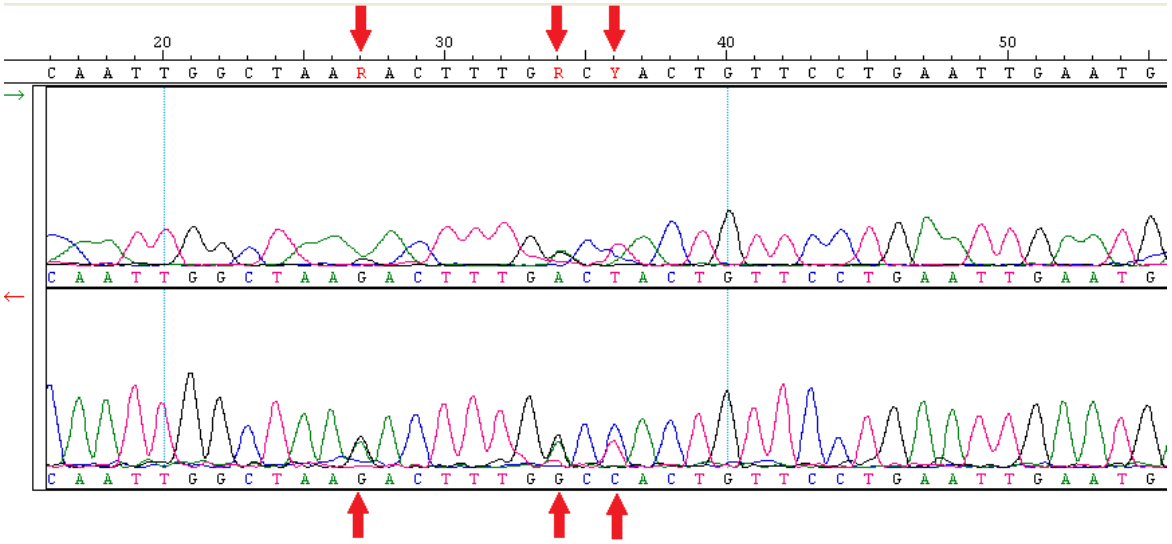


Figure 7: contig with SNPs

Statistical analysis was performed with MEGA 5. As the software is not suitable for heterozygous sequence data, the polymorphic sites were rewritten twice in case of homozygosity and once each in case of heterozygosity (see Figure 8). This strategy was described previously by other research groups [82,88]. As the evaluation by the MLST website's curator needed a considerable amount of time, a different approach was used to get information about the relationships of the clades. With the help of eBURST(http://eburst.mlst.net) all DST that were available to date (August 2013) the clonal clusters with their predicted founder were obtained (see 6). The sequences of the predicted founders of the largest 20 clonal clusters were downloaded from the website and added to the polymorphic-site alignment explained above. With this data and the help of MEGA 5 phylogenetic analysis software the Unweighted Pair Group Method with Arithmetic averages (UPGMA) was used to create a dendrogram, with a gamma distribution to model the rate variation [89]. Neighbor-joining analysis was performed additionally. The evolutionary distances were calculated by means of the Maximum Composite Likelihood method [90]. Bootstrapping with 1000 replications proofed the significance of the nodes [33]. To distinguish the clonal clusters, a cut-off value of p=0.04 was utilized as proposed by Odds et al [88]. Sequences that appeared as identical in UPGMA analysis were aligned by means of clustal omega to verify their identity. After assignation of the isolates to their clusters, a Chi-squared test was performed to test the statistical significance.

Figure 8: Adjustment of polymorphic sequence data

2.6.6.2 *FKS*1 – data

Sequence data of isolate 104 and 108, forward and reverse each, were assembled by means of Lasergene® SeqMan Pro software as described above for the MLST data. The consensus sequences were aligned assisted by clustal omega and translated to their corresponding amino acid sequences by means of emboss transeq. The amino acid sequences were aligned as well to discriminate silent from amino-acid changing mutations. Additionally, an NCBI nucleotide and protein blast query was performed in order to check out whether the sequence data have already been published.

2.6.6.3 eBURST analysis

By means of eBURST, we could not only obtain the reference strains, but analyze the MLST database regarding to different strain properties, for example, addressing potential pathogenicity differences.[91] For this purpose, the *C. albicans* database was searched for strains with the source “blood”. These strains were analyzed with eBURST and compared with all strains in the database.

At the end of our study, all strains were added to the MLST database by the curators and we were able to co-analyze our strains with all strains in the database by means of eBURST. Our strains are now available on the MLST database using the following headings: id=2275–2315 or Sequencer=Buzina Walter.

3 RESULTS

3.1 MALDI-TOF MS

Altogether 181 isolates were analyzed by means of MALDI-TOF MS. Of them, 140 were *C. albicans* (77%), 27 *Candida glabrata* (15%), 4 *Candida dubliniensis* and *Candida parapsilosis* (2% each), 2 *Candida krusei* (1%) and 1 isolate each were *Candida boidinii, Candida kefyr, C. tropicalis* and *Saccharomyces cerevisiae* (0.6%), respectively.

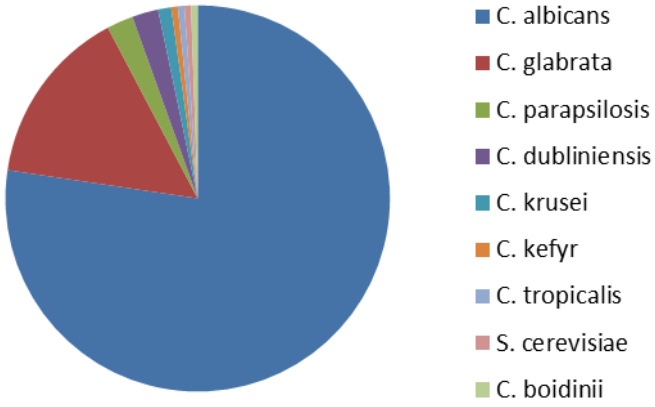


Figure 9: *Candida* species distribution

3.2 Susceptibility testing

The MICs of the susceptibility testing with Etests are shown in Figure 10, Figure 11 and Figure 12. In 140 *C. albicans* isolates, only 2 showed a reduced susceptibility. Isolate 43 showed a MIC of >16 for flucytosine, considered as resistant according to CLSI guidelines [92]. Isolate 104 had MICs of 1.5, 1 and 0.5 for caspofungin, anidulafungin and micafungin, respectively. Because of high inter-laboratory differences, there are no established cutoff-values for caspofungin and micafungin. However, they can be considered as “reduced susceptible” referring to a recent US-American study that defined resistance in the case of *C. albicans* as a MIC of >0.5 µg/mL for caspofungin, micafungin and anidulafungin using the CLSI broth dilution method [69]. Thus, 0.7% of the *C. albicans* isolates in this study showed reduced susceptibility to flucytosine and echinocandins, respectively. In Table 13 in the supplementary material section all susceptibility testing results are listed.

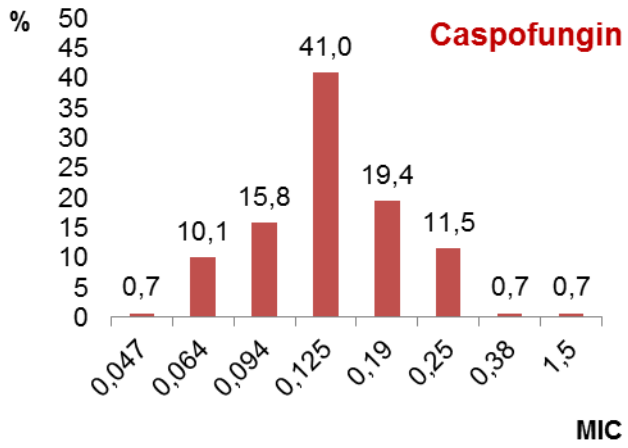


Figure 10: MIC distribution for caspofungin

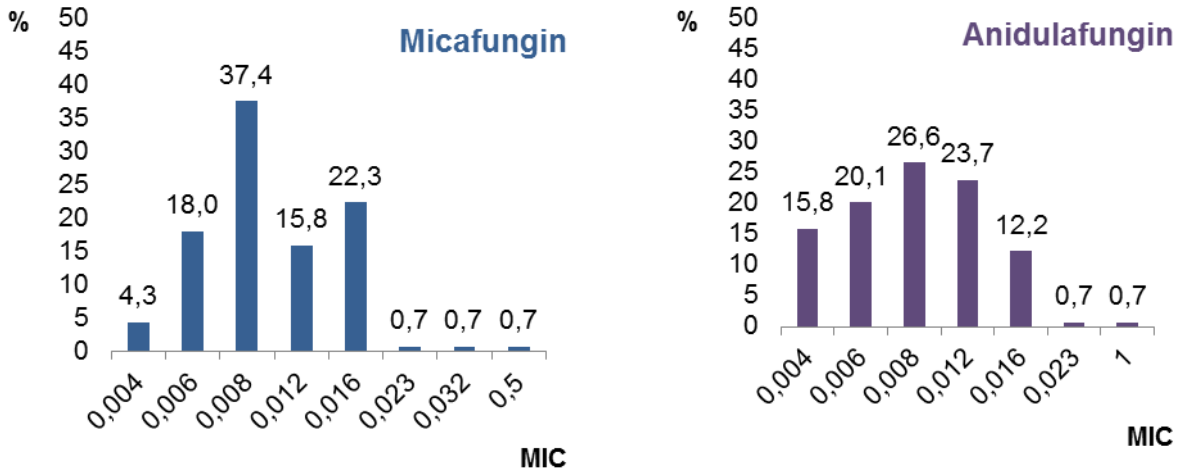


Figure 11: MIC distribution for micafungin and anidulafungin

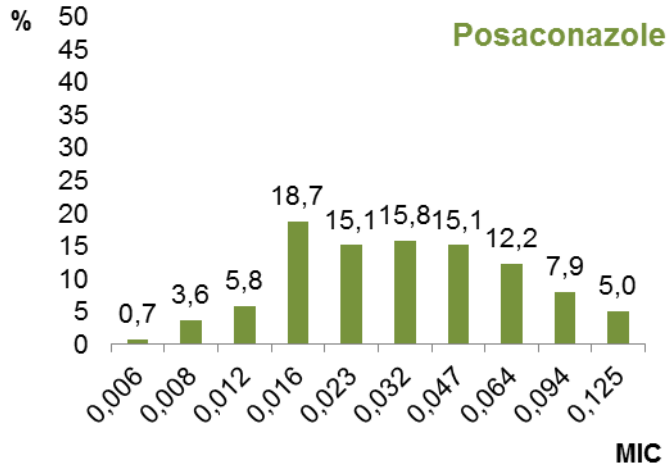


Figure 12: MIC distribution for posaconazole

3.3 MLST

MLST was performed with 44 isolates; 20 oral control isolates and 22 invasive isolates obtained from naturally sterile sites (blood culture or punction) as well as 2 oral samples from patients with candidemia. The sequence data were submitted to GenBank and assigned accession numbers (KF780174–478).

In three samples the acquisition of sequence data for any of the 7 loci failed despite of multiple repetitions of PCR, cycle sequencing and adaption of reagents. Hence these three samples (25 - oral; 122, 136 - invasive) were excluded from the study. Alignment of sequence data of the seven loci resulted in the following findings: 11 (2.9%) polymorphic sites in locus *AAT*1a, 5 (1.2%) in *ACC*1, 15 (3.4%) in *ADP*1, 12 (3.2%) in *PMI1*b, 11 (2.8%) in *SYA*1, 14 (3.5%) in *VPS*13 and 12 (2.4%) in *ZWF*1b.

Sample	DST	CC	AAT1a	ACC1	ADP1	PMI1b	SYA1	VPS13	ZWF1b
34	828	**3**	35	7	4	4	4	117	4
37	95	**2**	8	14	8	4	7	3	8
40	124	**2**	8	14	8	4	7	10	8
113	572	**1**	8	5	5	2	2	6	5
118	598	**13**	21	3	14	18	72	102	84
120	721	**S**	8	2	5	4	2	24	5
124	142	**2**	8	14	8	4	7	10	22
127	124	**2**	8	14	8	4	7	10	8
178	124	**2**	8	14	8	4	7	10	8

Table 8: Samples with DSTs already on the MLST database

CC = Clonal Cluster (as determined by eBURST analysis)

The MLST profiles of merely 9 samples were already in the MLST database. DST 124, the putative founder of clade 2, was shared by 3 samples (40, 127, 178; see Table 8 for details). Some clonal clusters, determined by eBURST, are available in the supplementary material section.

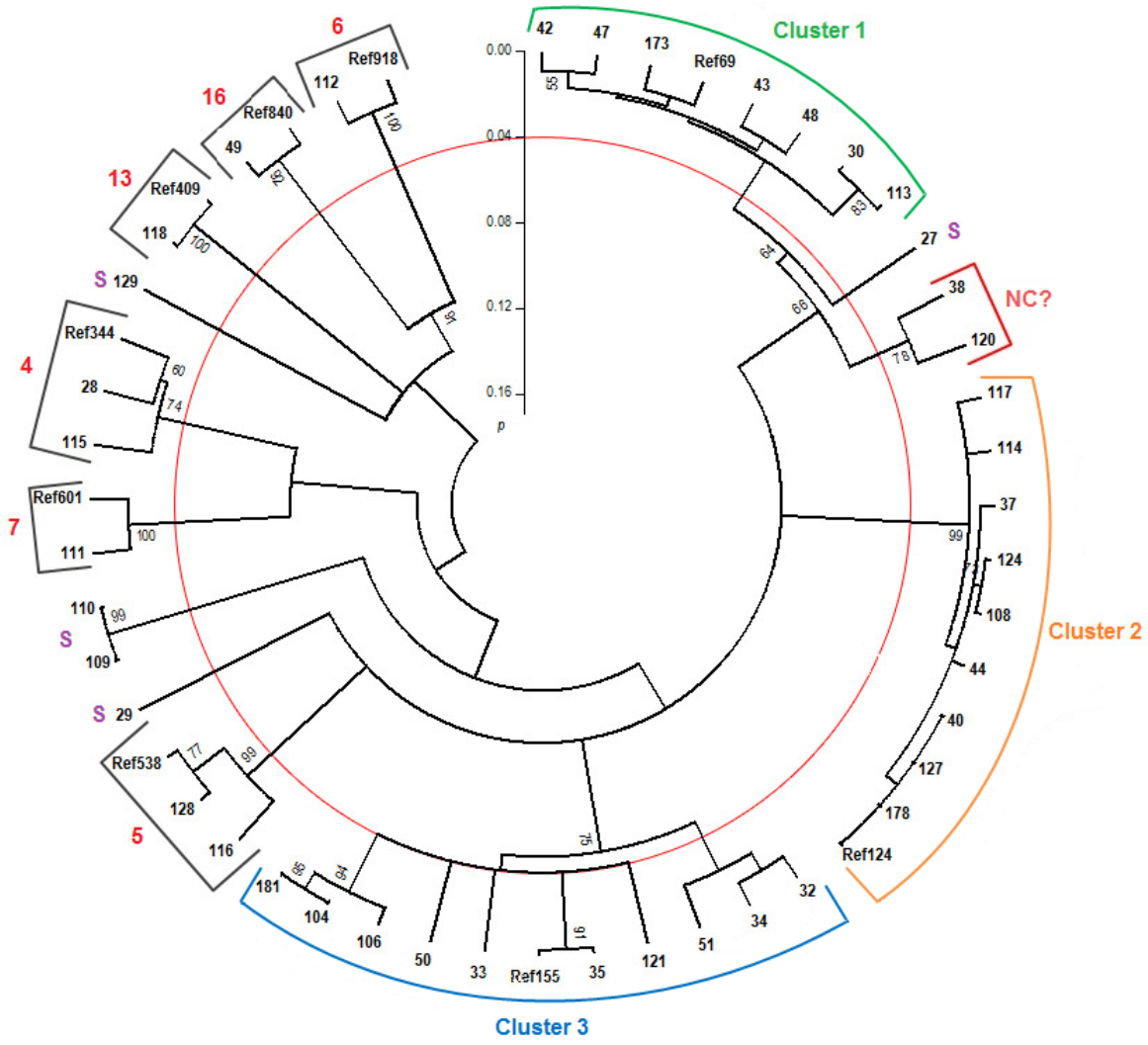


Figure 13: Neighbor-joining tree

the red line indicates the cutoff value of p=0.04
NC? = putative new cluster S = putative singleton sequences

As explained above, with only 7 DST already in the database, we had to use a different approach to obtain reliable results of our data: Neighbor-joining analysis with the putative founding strains of the main clades as reference strains resulted in 7 clearly distinguishable clusters and 3 samples considered as singletons (see Figure 13 for details). Singletons are strains that could not be assigned to any clade.[93] However, in two cases the classification to clonal clusters was ambiguous: Samples 27, 38 and 120 could not be assigned to cluster 1 as these samples exceeded the cut-off value of *p*=0.04. As samples 38 and 120 appeared to be in the same cluster and sample 120 corresponded to DST 721, classified as a singleton (see Table 9), they were considered as a new cluster. Sample 27 was presumed to be a singleton.

The node of samples 32, 34 and 51 exceeded the cut-off value as well, but they were attached to cluster 3 for two reasons: On the one hand, sample 34 corresponds to DST 828, which clusters in clade 3 (see Table 9). On the other hand, the common node of clade 3 and these three samples had a bootstrap value of 75%; considered as reliable [94].

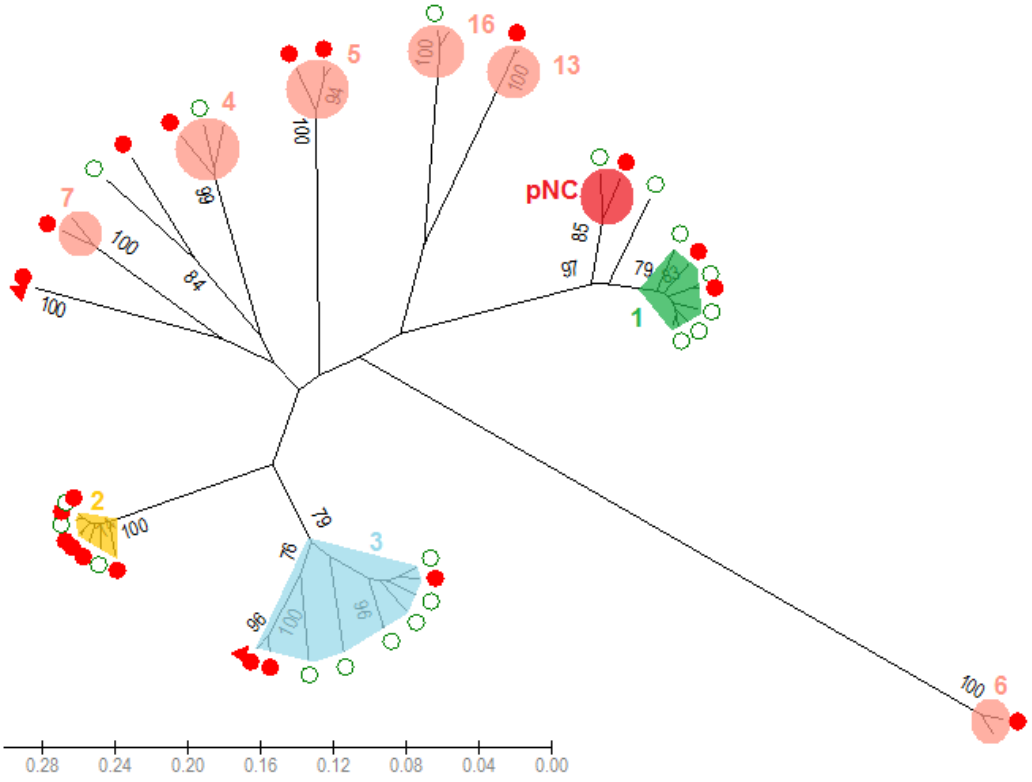


Figure 14: UPGMA tree with clonal clusters
green rings: control samples red filled circles: invasive samples
red triangles: oral samples of invasive candidiasis patients pNC: putative new clade

Invasive sample 181 and oral sample 104, obtained from the same patient, resulted to be identical strains in MLST analysis. The same is true for invasive sample 110 and oral sample 109. This was shown by neighbor-joining and UPGMA analysis. High bootstrap values of 95 and 99% in neighbor-joining, and 99 and 100% in UPGMA analysis, respectively (see Figure 13) verified this finding. The sequences were additionally aligned by means of clustal omega and showed analogousness. The sequence alignments are available in the supplementary material section.

Clade	No of isolates	invasive candidiasis*	commensalism	*p*
1	7	2	5	0,2568
2	9	6	3	0,3173
3	9	3	6	0,3173
4	2	1	1	1
5	2	2	0	0,1573
6	1	1	0	0,3173
7	1	1	0	0,3173
13	1	1	0	0,3173
16	1	0	1	0,3173
pNC	2	1	1	1
S	4	2	2	1
total	**39**	**20**	**19**	

Table 9: distribution of invasive and commensal isolates in different clades as determined by UPGMA and NJ analysis
*oral isolates (104, 109) from candidiasis patients were excluded
for the sake of completeness, p-values were computed for all clusters

3.4 eBURST Analysis

In Table 10 all DSTs are listed, and the MLST clusters as determined by UPGMA analysis and eBURST are shown. Figure 15 and Figure 16 show snapshots from all DSTs and DSTs which contained blood isolates from the MLST-database, respectively. Clade 1, evidently the largest in both snapshots, is the cluster in the middle, with its founder DST 69 in the center. The separate black dots are singletons.

Strain	ID	DST	AAT1a	ACC1	ADP1	MPIb	SYA1	VPS13	ZWF1b	eBurst clade	UPGMA clade
27	2301	2149	6	5	23	9	182	27	5	S	S
28	2275	2132	13	7	15	6	7	37	22	4	4
29	2276	2133	122	5	6	6	64	20	4	S	S
30	2277	2134	8	5	5	2	2	6	19	1	1
32	2302	2150	4	7	4	4	4	117	4	3	3
33	2278	2135	36	2	14	4	49	41	4	62	3
34	2279	828	35	7	4	4	4	117	4	3	3
35	2303	244	36	4	4	4	4	26	4	3	3
37	2280	95	8	14	8	4	7	3	8	2	2
38	2282	2136	2	5	5	4	2	24	16	1	NC?
40	2281	124	8	14	8	4	7	10	8	2	2
42	2283	2137	2	5	5	2	2	6	65	1	1
43	2284	2138	2	5	5	31	2	94	63	S	1
44	2304	2152	8	14	5	131	7	13	8	S	2
47	2285	2139	2	5	6	31	2	25	65	S	1
48	2286	2140	2	5	5	31	2	21	5	1	1
49	2287	2141	13	3	37	55	38	46	12	S	16
50	2305	2156	35	7	14	4	49	4	239	S	3
51	2288	2142	4	7	4	4	4	4	18	3	3
104	2306	2154	35	83	4	4	4	245	239	S	3
106	2307	2155	35	2	4	4	4	4	239	3	3
108	2289	2143	8	14	8	4	7	13	22	2	2
109	2308	2153	13	2	20	132	43	69	22	S	S
110	2309	2153	13	2	20	132	43	69	22	S	S
111	2310	2157	4	17	21	19	27	246	22	7	7
112	2311	2158	62	3	3	3	3	39	240	6	6
113	2290	572	8	5	5	2	2	6	5	1	1
114	2312	2151	8	3	124	4	7	3	8	2	2
115	2313	2159	13	7	15	6	7	247	15	4	4
116	2291	2144	107	12	21	1	6	59	4	5	5
117	2292	2145	8	14	6	4	42	3	8	2	2
118	2293	598	21	3	14	18	72	102	84	13	13
120	2294	721	8	2	5	4	2	24	5	S	NC?
121	2295	2146	35	29	4	4	4	4	72	3	3
124	2296	142	8	14	8	4	7	10	22	2	2
127	2297	124	8	14	8	4	7	10	8	2	2
128	2298	2147	62	58	21	5	6	30	4	5	5
129	2299	2148	4	29	6	18	64	53	15	40	S
173	2314	2160	2	5	2	2	2	248	5	1	1
178	2300	124	8	14	8	4	7	10	8	2	2
181	2315	2154	35	83	4	4	4	245	239	S	3

Table 10: IDs, DSTs and STs of all strains and comparison of MLST clades as determined by means of UPGMA and eBURST analysis

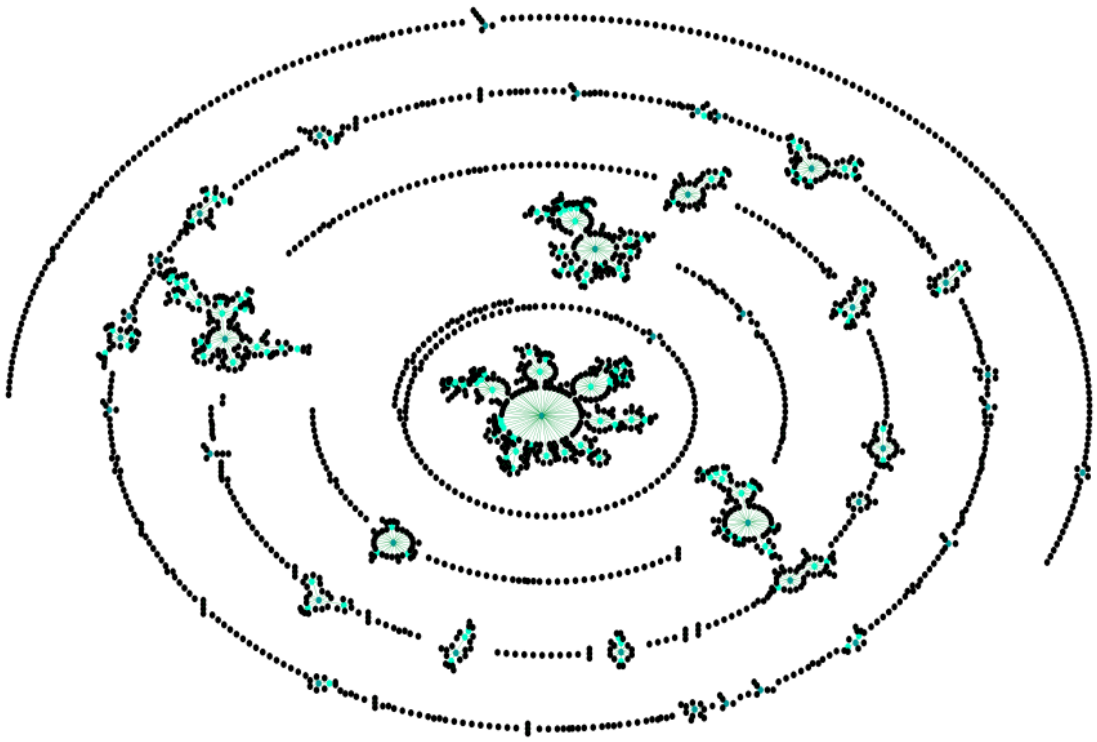


Figure 15: eBURST snapshot of clusters from 2131 DSTs, obtained from the MLST database in October 2013.

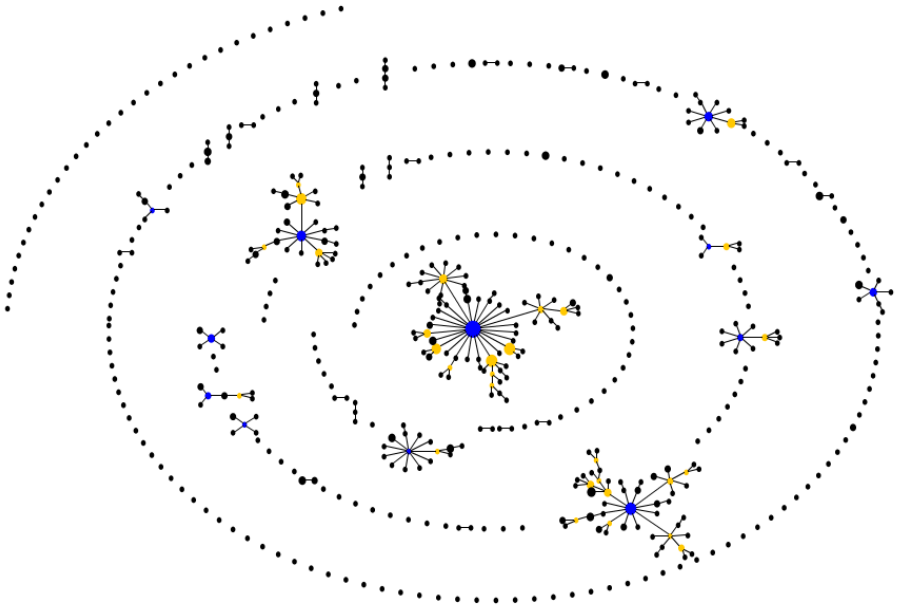


Figure 16: eBURST snapshot of clusters from all DSTs that contained blood samples, obtained from the MLST database in October 2013.

3.5 *FKS*1 data

Isolate 104, obtained from the same patient some weeks after isolate 181, showed 2 nucleotide point mutations in resistance hot spot *GSC1*. Both these mutations resulted in amino acid changes (see Figure 17). The MICs for all tested antifungal agents in these samples are listed in Table 11. The sequences were submitted to GenBank and assigned accession numbers (KF780479–480).

Strain	Source	5FC	AMB	FCA	ITR	VRC	CS	POS	AND	MYC
181	BC	4	0,5	1	0.125	0.06	0.38	0.012	0.032	0.032
104	oral	4	0,5	1	0.125	0.06	1.5	0.016	1.5	0.5

Table 11: comparison of susceptibility properties of isolates 181 and 104

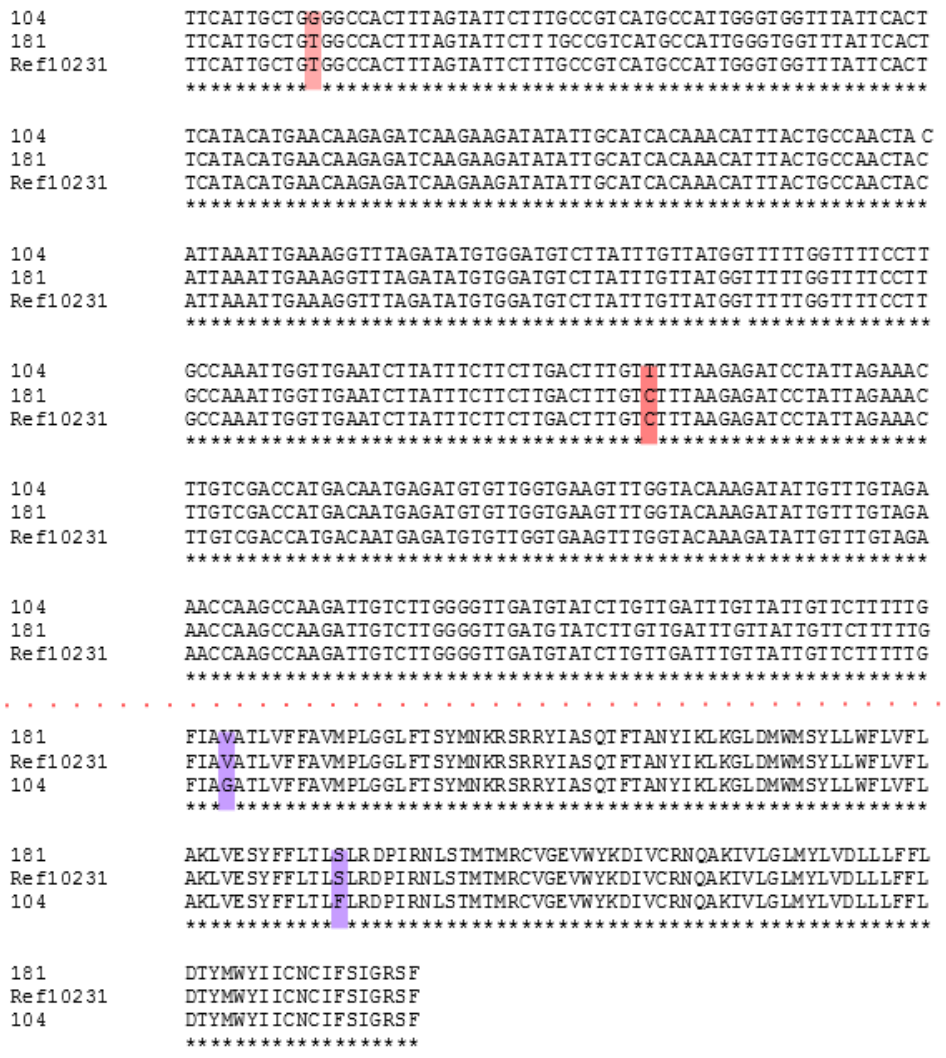


Figure 17: Nucleotide and corresponding protein alignment

Ref10231: NCBI blast query reference strain:
beta-1,3-glucan synthase catalytic subunit 1, partial [*Candida albicans*]

4 DISCUSSION

4.1 Species distribution as determined by MALDI-TOF MS

C. albicans was the most commonly isolated yeast in our study, at a considerable distance to other species. This finding is in concordance with previous studies. With 77%, the *C. albicans* ratio was higher than the percentage referred in the resistance report of the Institute of Hygiene, Microbiology and Environmental Medicine (Graz) from 2012 (69.7%), similar to the 2010 report (75.9%) but lower than in 2005 (82.5%) [95,96]. Taking into account that the isolates within our study were obtained between 2008 and 2012, our results highlight the general finding that the ratio of *C. albicans* is decreasing in favor to non-*albicans Candida* species [40]. The decrease might be caused by the increment of azole prophylaxis given to patients at a high risk (see 1.3.2), hence selecting species with reduced fluconazole susceptibility, e.g., *C. glabrata* and *C. krusei* [97,98]. *C. glabrata* was the second most common species in our study.

4.2 Susceptibility to antifungal agents

4.2.1 Flucytosine resistance

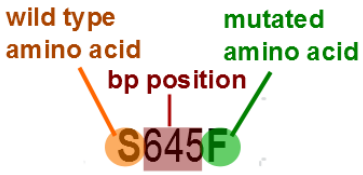
Fortunately, the resistance rate was low within our study. One oral isolate (number 43), obtained from a healthy control subject, showed reduced flucytosine susceptibility (MIC>16). The clinical importance of this finding is minor as flucytosine is administered rarely and only in combination with amphotericin B [59]. However, this result is interesting from an epidemiological point of view, as MLST clade-1 strains show flucytosine resistance at a notable percentage [99]. Isolate 43 affiliates with cluster 1 in UPGMA analysis, but not in eBURST analysis, the latter being the more reliable result [82]. Additionally, out of 7 putative clade-1 strains, it was the only isolate which showed flucytosine resistance. Concerning different *C. albicans* clades, no further susceptibility differences were found.

4.2.2 Echinocandin resistance and its consequences

Another oral *C. albicans* isolate, obtained from a female patient with recurrent candidemia, showed a reduced susceptibility to all echinocandins. This finding is of great clinical importance as the echinocandins constitute the first-line therapy in invasive candidiasis [59]. A BC isolate, taken only some weeks prior, did not show any resistance. We sent the resistant isolate to Professor Cornelia Lass-Flörl from the Division of Hygiene and Medical Microbiology of the Innsbruck Medical University who re-tested it with the MICRONAUT system for antimicrobial susceptibility testing (MERLIN, Bornheim-Hersel, Germany) and Etest. The results from Innsbruck confirmed our finding of a pan-echinocandin-resistant isolate.

The resistant oral and the susceptible BC isolate shared the same MLST profile. One could assume that two MLST-indistinguishable isolates are identical, but it has been shown that two MLST-identical isolates may differ in other typing methods and vice versa [100]. MLST and other typing methods were conceived to highlight differences between strains [100]. Only whole-genome sequencing could potentially recognize two identical isolates [100]. Nevertheless, we can assume that the two isolates are closely related. These findings suggest that the resistance might have been acquired during the treatment. Indeed, this patient was treated with echinocandins. Echinocandins are fungicidal to *Candida* species through inhibition of ß-D-glucan synthase [101]. This enzyme is responsible for the ß-1,3-glucan synthesis, which constitutes a major cell wall component [101]. So far, two mechanisms leading to reduced echinocandin susceptibility in *Candida* have been found. On the one hand, some *Candida* spp., including *C. albicans*, are capable to increase the chitin synthesis, which depicts another important cell wall component, in response to ß-1,3-glucan-depletion (reviewed in [101]). On the other hand, mutations in the *FKS*-genes, encoding a catalytic subunit of the glucan synthase, can also lead to a reduced echinocandin susceptibility [86] (reviewed in [101]). We hence investigated the resistant oral isolate (104) and its susceptible BC twin (181) with an additional PCR and sequencing step to amplify a part of the *FKS1* gene, although *FKS1* mutations are an uncommon finding in *C. albicans* [102].

Indeed, *FKS1* showed 2 point mutations in the resistant isolate. These mutations led subsequently to alterations in the amino-acid sequence. The susceptible BC isolate did not show these mutations. *FKS* mutations in *C. albicans* were found and discussed before (see Table 12). One of the mutations (serine [S] → phenylalanine [F] in position 645) in our isolate has already been described elsewhere [71,72,103,104]. The other mutation (valine [V] → glycine [G] in position 576) has not been published yet. Admittedly, the first hotspot region (HS1) was described to be a highly conserved region approximately between bp 640 and 650 [72]. The HS2 region lies within bp 1345 and 1365 [72]. Therefore, the V576G mutation is not within the formerly described hotspot regions, but near to HS1, which may hence be broader than previously assumed. To evaluate whether this mutation has an impact on the echinocandin susceptibility, further investigations would be necessary. Susceptibility testing with a hybrid mutant, exhibiting only the V576G mutation, could be used for further investigations. The possibility of development of pan-echinocandin-resistance should be kept in mind when antifungal prophylaxis or therapy is administered.



our study	**V576G**	**Laverdiere 2006**	**S645F**
	S645F	**Arendrup 2009**	**S645P**
		Singh 2009	**F641S**
Park 2005	**S645F**	**Castanheira 2010**	**F641Y**
Balashov 2006	**F641S**	**Nimi 2010**	**S645P**
	L644S		
	S645P	**Slater 2011**	**S645F**
	S645Y		**S645Y**
	S645F		**S645P**
		Wiederhold 2011	**F641S**
Katiyar 2006	**F641Y**		**S645P**

Table 12: reported mutations in *FKS1* hotspot 1 [71,72,102-106] (reviewed in [101])

4.3 MLST

4.3.1 Clusters and cluster stability

We found numerous new MLST types, but similar cluster distributions like in other countries, with some exceptions. As already shown in previous studies; clades 1–4 and 11 contained the highest numbers of strains [32]. This was true in our study for clades 1–3. By contrast, clade 4 contained only 2 isolates, similar to the other small clusters. This may be caused by general geographic differences between the clades. However, the possibility that sample numbers were too small to make a reliable statement regarding the cluster distribution cannot be excluded. Odds, for example, compared 1410 strains in one of his studies which addressed the clade distribution, phenotypic differences, pathogenicity differences and other variables [32].

Interestingly, isolate 118 was assigned to clade 13. This clade was previously described as the “africana” branch, as it contained predominantly isolates which were formerly assumed to be the separate species *Candida africana* (reviewed in [32]). A re-analysis with eBURST did not assign the “africana” strains to clade 13 but to clade 29, while clade 13 contained strains from blood, pus and other sites predominantly from Europe. This finding underlines the instability of smaller clusters determined by MLST [82]. It highlights once again that strain clustering is a random process [32].

The putative new clade (see Figure 13 and 14) could not be verified by means of eBURST, as it assigned isolate 38 to clade 1.

4.3.2 Pathogenicity differences

The opinions whether *C. albicans* clades differed in their pathogenicity or preference to an anatomical site varied in previous investigations [82,88]. However, the more recently performed studies showed no evidence of inter-strain pathogenicity differences, neither in a mouse model nor in analysis of the growing MLST database [88,107]. By contrast, there was evidence that cluster 1 may be especially well adapted to commensalism and superficial infections [88]. Our investigation highlighted the latter fact – in clade 1, five strains have been obtained from oral *Candida* isolates and only 2 strains were isolated from blood cultures. Contrary to this, clade 2 had twice as much invasive strains as commensals. In case of clade 3, the situation was vice versa – it contained only 3 invasive, but 6 commensal strains. These findings seemed to be promising; however, none of these results was statistically significant. The additionally performed eBURST analysis, comparing all strains containing BC isolates with all strains in the database (see Figure 15 and Figure 16), showed that the distribution of invasive strains to the eBURST clusters was equal to the general distribution. Clade 1, generally the largest cluster [32], was also the largest "invasive" cluster. DST 69, the commonest strain, was also the most frequent strain in blood culture isolates. This finding does not oppose the fact that clade 1 strains are especially well adapted to commensalism, but highlights the finding that patients most likely acquire candidal infections from their own colonizing flora – as previously stated (see 1.3.2) [48], colonization is an independent risk factor for disseminated candidiasis in the susceptible host.

4.3.3 Different strokes for different folks?

In our study we disposed of two cases where we could investigate an oral as well as an invasive isolate from the same patient. In both cases these isolates shared one MLST profile, suggesting an endogenous transmission of the disseminated infection. As already mentioned, MLST detects differences and not similarity of strains; nevertheless, it is very likely that the isolates are at least closely related. If there were differences, undetected by MLST, these differences might be due to microvariations, a mechanism which increases the fitness of the fungus as it maximizes its genetic diversity [32]. Our findings strengthen those of previous investigations – invasive candidal infections develop more likely from the patients' own commensal strains [32]. While healthy individuals often show mixed *Candida* populations of very closely related strains, patients with superficial infections are colonized more likely by a single strain [107]. This suggests that epithelial invasion is promoted through overgrowth of one certain *C. albicans* strain [107]. It seems to be more likely that a certain strain, except microvariations, remains the same in one patient throughout the time [108-110].

4.3.4 Limitations and future perspectives

Phylogenetic analysis showed some limitations in previous studies. All 7 fragment sequences have to be concatenated prior to analysis. It has been shown that the statistical analysis with UPGMA sometimes arranged isolates with very similar polymorphisms to one clade, but which are no close relatives in fact, as these polymorphisms were on different gene fragments [82]. This was true for some of our strains as well, as we discovered by means of an eBURST analysis. An eBURST analysis including these new strains showed congruence with the UPGMA and neighbor-joining analysis in 30 strains. However, 11 strains differed, especially singletons and smaller clusters.

The absence of gene fragments from chromosomes 3, 5, and 7 has already been mentioned as a limitation of MLST (see 1.4.3). The ideal MLST method for *C. albicans* could be a scheme of 24 primer pairs, encoding for gene fragments from each end and the central part of each chromosome, respectively [32]. Investigations with MLST, in particular with meaningful amounts of isolates, require a lot of time and financial as well as staffing efforts (7 PCR reactions – 7 purification steps – 14 sequencing reactions – 14 purification steps for each strain). Free hardware capacities – especially for the sequencing, which needs a lot of time – are required as well as skilled personnel, also for the analysis of the raw data with specialized software. A 24-gene-fragment MLST scheme does hence not seem to be in step with actual practice.

As the crucial financial and time factors are the sequencing and the post-sequencing purification step, a high-throughput sequencing method appears to be promising [111]. By the time it is appropriately adapted to diploid organisms, and accurate software has been developed, this method could constitute a cost-effective alternative to the customary MLST method [111].

Outsourcing of the sequencing and purification steps to commercial providers may be an alternative, especially for small laboratories. The sequence data are of good quality and the results are obtained quickly. However, this method requires more steps prior to shipment, such as gel electrophoresis and correct packaging, apart from the considerable payment. We recently started to implement this method to test higher amounts of isolates; however, we experienced some problems which should be resolved prior to further investigations. We used barcoded 96-well-plates (microsynth, Balgach, Switzerland) and essayed a high-throughput method with 96 isolates and 3 different primer pairs, respectively. Despite of accurate PCR-preparations beneath an UV-cleaned work bench, and storage on ice during preparation, one third up to half of the reactions did not contain sufficient amounts of DNA. We tried to raise the reaction volumes at the expense of water, as well as the annealing temperatures and the cycles, but these attempts did not yet solve the problem. The investigation of more isolates in less time may remain an aim of further investigations. Nonetheless it is unclear whether the broadening of MLST to more *C. albicans* isolates is encouraging. It has been shown that the increase of the internet database – to date it includes more than 2000 strains – did not reveal new insights but strengthened the statistical significance of already established findings [100]. The elucidation of clinical issues, for example, similarity testing in clinical outbreaks, or strain typing in isolates which differ in resistance; may remain an application of MLST in future investigations [100]. However, this is also limited due to the already mentioned fact that strain typing was developed to highlight differences, not similarity. In the specific case of *C. albicans*, future efforts should therefore preferably attempt in the development of whole-genome-sequencing arrays [32]. As a matter of course, these techniques should be objective, proportionately easy to perform, and accessible via internet.

Invasive fungal infections, with *C. albicans* in a leading position, are still an important global health problem. Despite all the efforts which have been made within a wide range of studies, we can be sure that future investigations in the molecular typing and genomics of *C. albicans* remain exciting.

5 REFERENCES

[1] Mora C, Tittensor DP, Adl S, Simpson AG, Worm B. How many species are there on Earth and in the ocean? PLoS Biol 2011; Aug 9 [Epub ahead of print].

[2] Mayer FL, Wilson D, Hube B. Candida albicans pathogenicity mechanisms. Virulence 2013; 4: 119-128.

[3] de Hoog GS, Guarro J, Gené J, Figueras MJ. Atlas of Clinical Fungi. Electronic version 3.1 ed. Utrecht, The Netherlands: Centraalbureau voor Schimmelcultures; 2011.

[4] Brown GD, Denning DW, Levitz SM. Tackling human fungal infections. Science 2012; 336: 647.

[5] Denis O, Vincent M, Havaux X, De Prins S, Treutens G, Huygen K. Induction of the specific allergic immune response is independent of proteases from the fungus Alternaria alternata. Eur J Immunol 2013; 43: 907-917.

[6] Cavalier-Smith T. A revised six-kingdom system of life. Biol Rev Camb Philos Soc 1998; 73: 203-266.

[7] Calderone RA editor. *Candida* and Candidiasis. 1st ed. Washington, DC; USA: ASM Press; 2002.

[8] Lavoie K, Touchette M, St-Gelais D, Labrie S. Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. Dairy Sci Technol 2012; 92: 455-468.

[9] Eddouzi J, Hofstetter V, Groenewald M, Manai M, Sanglard D. Characterization of a new clinical yeast species, Candida tunisiensis sp. nov., isolated from a strain collection of Tunisian hospitals. J Clin Microbiol 2012 Oct 17 [Epub ahead of print].

[10] Hui FL, Niu QH, Ke T, Liu Z. Candida ficus sp. nov., a new yeast species found in the gut of Apriona germari larvae. Int J Syst Evol Microbiol 2012 Jun 22 [Epub ahead of print].

[11] Hui FL, Niu QH, Ke T, Li YX, Lee CF. Candida sanyaensis sp. nov., an ascomycetous yeast species isolated from soil. Antonie Van Leeuwenhoek 2012 Aug 7 [Epub ahead of print].

[12] James SA, Carvajal BEJ, Portero Barahona P, Cross K, Bond CJ, Roberts IN. Candida ecuadorensis sp. nov., a novel ascomycetous yeast species found in two separate regions of Ecuador. Int J Syst Evol Microbiol 2012 Oct 26 [Epub ahead of print].

[13] Poomtien J, Jindamorakot S, Limtong S, Pinphanichakarn P, Thaniyavarn J. Two new anamorphic yeasts species, Cyberlindnera samutprakarnensis sp. nov. and Candida thasaenensis sp. nov., isolated from industrial wastes in Thailand. Antonie Van Leeuwenhoek 2012 Sep 26 [Epub ahead of print].

[14] Yuan B, Hu N, Sun J, Wang SA, Li FL. Purification and characterization of a novel extracellular inulinase from a new yeast species Candida kutaonensis sp. nov. KRF1(T). Appl Microbiol Biotechnol 2012 May 24 [Epub ahead of print].

[15] Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog 2010 Jan 8 [Epub ahead of print].

[16] Saghrouni F, Ben Abdeljelil J, Boukadida J, Ben Said M. Molecular methods for strain typing of Candida albicans: a review. J Appl Microbiol 2013; 114: 1559-1574.

[17] Falagas ME, Apostolou KE, Pappas VD. Attributable mortality of candidemia: a systematic review of matched cohort and case-control studies. Eur J Clin Microbiol Infect Dis 2006; 25: 419-425.

[18] Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, et al. A higher-level phylogenetic classification of the Fungi. Mycol Res 2007; 111: 509-547.

[19] Gow NAR, de Veerdonk FLv, Brown AJP, Netea MG. Candida albicans morphogenesis and host defence: discriminating invasion from colonization. Nature Reviews Microbiology; 10: 112-122.

[20] Fleck CB, Schobel F, Brock M. Nutrient acquisition by pathogenic fungi: Nutrient availability, pathway regulation, and differences in substrate utilization. Int J Med Microbiol 2011 May 6 [Epub ahead of print].

[21] Kirkpatrick CH. Chronic mucocutaneous candidiasis. Pediatr Infect Dis J 2001; 20: 197-206.

[22] Saunte DM, Klingspor L, Jalal S, Arnau J, Arendrup MC. Four cases of Candida albicans infections with isolates developing pink colonies on CHROMagar Candida plates. Mycoses 2005; 48: 378-381.

[23] Arkowitz RA, Bassilana M. Polarized growth in fungi: Symmetry breaking and hyphal formation. Semin Cell Dev Biol 2011 Sep 1 [Epub ahead of print].

[24] Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR. Nonfilamentous C. albicans mutants are avirulent. Cell 1997; 90: 939-949.

[25] Kumamoto CA. Molecular mechanisms of mechanosensing and their roles in fungal contact sensing. Nat Rev Microbiol 2008 Sep; 6: 667-673.

[26] Brand A, Shanks S, Duncan VM, Yang M, Mackenzie K, Gow NA. Hyphal orientation of Candida albicans is regulated by a calcium-dependent mechanism. Curr Biol 2007 Feb 20; 17: 347-352.

[27] Slutsky B, Staebell M, Anderson J, Risen L, Pfaller M, Soll DR. "White-opaque transition": a second high-frequency switching system in Candida albicans. J Bacteriol 1987; 169: 189-197.

[28] Berman J. Candida albicans. Curr Biol 2012; 22: R620-2.

[29] Morschhauser J. Regulation of white-opaque switching in Candida albicans. Med Microbiol Immunol 2010 Apr 14 [Epub ahead of print].

[30] Arendrup M, Horn T, Frimodt-Moller N. In vivo pathogenicity of eight medically relevant Candida species in an animal model. Infection 2002; 30: 286-291.

[31] Fanning S, Mitchell AP. Fungal biofilms. PLoS Pathog 2012; 8: e1002585.

[32] Odds FC. Molecular phylogenetics and epidemiology of Candida albicans. Future Microbiol 2010; 5: 67-79.

[33] Bougnoux ME, Pujol C, Diogo D, Bouchier C, Soll DR, d'Enfert C. Mating is rare within as well as between clades of the human pathogen Candida albicans. Fungal Genet Biol 2008; 45: 221-231.

[34] Diogo D, Bouchier C, d'Enfert C, Bougnoux ME. Loss of heterozygosity in commensal isolates of the asexual diploid yeast Candida albicans. Fungal Genet Biol 2009; 46: 159-168.

[35] Bennett RJ, Johnson AD. Completion of a parasexual cycle in Candida albicans by induced chromosome loss in tetraploid strains. EMBO J 2003; 22: 2505-2515.

[36] Berman J, Hadany L. Does stress induce (para)sex? Implications for Candida albicans evolution. Trends Genet 2012; 28: 197-203.

[37] Marchetti O, Bille J, Fluckiger U, Eggimann P, Ruef C, Garbino J, et al. Epidemiology of candidemia in Swiss tertiary care hospitals: secular trends, 1991-2000. Clin Infect Dis 2004; 38: 311-320.

[38] Krause R, Schwab E, Bachhiesl D, Daxbock F, Wenisch C, Krejs GJ, et al. Role of Candida in antibiotic-associated diarrhea. J Infect Dis 2001; 184: 1065-1069.

[39] Lehrnbecher T, Frank C, Engels K, Kriener S, Groll AH, Schwabe D. Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. J Infect 2010; 61: 259-265.

[40] Arendrup MC. Epidemiology of invasive candidiasis. Curr Opin Crit Care 2010; 16: 445-452.

[41] Chalmers C, Gaur S, Chew J, Wright T, Kumar A, Mathur S, et al. Epidemiology and management of candidaemia--a retrospective, multicentre study in five hospitals in the UK. Mycoses 2011; 54: e795-800.

[42] Bae GY, Lee HW, Chang SE, Moon KC, Lee MW, Choi JH, et al. Clinicopathologic review of 19 patients with systemic candidiasis with skin lesions. Int J Dermatol 2005; 44: 550-555.

[43] Oude Lashof AM, Rothova A, Sobel JD, Ruhnke M, Pappas PG, Viscoli C, et al. Ocular manifestations of candidemia. Clin Infect Dis 2011; 53: 262-268.

[44] Ullmann AJ, Akova M, Herbrecht R, Viscoli C, Arendrup MC, Arikan-Akdagli S, et al. ESCMID* guideline for the diagnosis and management of Candida diseases 2012: adults with haematological malignancies and after haematopoietic stem cell transplantation (HCT). Clin Microbiol Infect 2012; 18 Suppl 7: 53-67.

[45] Rammaert B, Desjardins A, Lortholary O. New insights into hepatosplenic candidosis, a manifestation of chronic disseminated candidosis. Mycoses 2012; 55: e74-84.

[46] Hall AM, Poole LA, Renton B, Wozniak A, Fisher M, Neal T, et al. Prediction of invasive candidal infection in critically ill patients with severe acute pancreatitis. Crit Care 2013; 17: R49.

[47] Muskett H, Shahin J, Eyres G, Harvey S, Rowan K, Harrison D. Risk factors for invasive fungal disease in critically ill adult patients: a systematic review. Crit Care 2011; 15: R287.

[48] Magill SS, Swoboda SM, Johnson EA, Merz WG, Pelz RK, Lipsett PA, et al. The association between anatomic site of Candida colonization, invasive candidiasis, and mortality in critically ill surgical patients. Diagn Microbiol Infect Dis 2006; 55: 293-301.

[49] van de Veerdonk FL, Kullberg BJ, Netea MG. Pathogenesis of invasive candidiasis. Curr Opin Crit Care 2010; 16: 453-459.

[50] Dongari-Bagtzoglou A, Kashleva H. Candida albicans triggers interleukin-8 secretion by oral epithelial cells. Microb Pathog 2003; 34: 169-177.

[51] Brown GD. Innate Antifungal Immunity: The Key Role of Phagocytes. Annu Rev Immunol 2010 Apr 5 [Epub ahead of print].

[52] Gantner BN, Simmons RM, Underhill DM. Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. EMBO J 2005; 24: 1277-1286.

[53] Ghosh S, Navarathna DH, Roberts DD, Cooper JT, Atkin AL, Petro TM, et al. Arginine-induced germ tube formation in Candida albicans is essential for escape from murine macrophage line RAW 264.7. Infect Immun 2009 Apr; 77: 1596-1605.

[54] Wuthrich M, Deepe GS,Jr, Klein B. Adaptive immunity to fungi. Annu Rev Immunol 2012; 30: 115-148.

[55] LeibundGut-Landmann S, Wuthrich M, Hohl TM. Immunity to fungi. Curr Opin Immunol 2012; 24: 449-458.

[56] Cuenca-Estrella M, Verweij PE, Arendrup MC, Arikan-Akdagli S, Bille J, Donnelly JP, et al. ESCMID* Guideline for the Diagnosis and Management of Candida Diseases 2012: Diagnostic Procedures. Clinical Microbiology and Infection 2012 09-25; 18: 9-18.

[57] Mikulska M, Calandra T, Sanguinetti M, Poulain D, Viscoli C, Third European Conference on Infections in Leukemia Group. The use of mannan antigen and anti-mannan antibodies in the diagnosis of invasive candidiasis: recommendations from the Third European Conference on Infections in Leukemia. Crit Care 2010; 14: R222.

[58] Kullberg BJ, Verweij PE, Akova M, Arendrup MC, Bille J, Calandra T, et al. European expert opinion on the management of invasive candidiasis in adults. Clin Microbiol Infect 2011; 17 Suppl 5: 1-12.

[59] Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, et al. ESCMID* guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients. Clin Microbiol Infect 2012; 18 Suppl 7: 19-37.

[60] Ruhnke M, Rickerts V, Cornely OA, Buchheidt D, Glockner A, Heinz W, et al. Diagnosis and therapy of Candida infections: joint recommendations of the German Speaking Mycological Society and the Paul-Ehrlich-Society for Chemotherapy. Mycoses 2011; 54: 279-310.

[61] Pappas PG, Kauffman CA, Andes D, Benjamin J,Daniel K., Calandra TF, Edwards J,John E., et al. Clinical Practice Guidelines for the Management of Candidiasis: 2009 Update by the Infectious Diseases Society of America. Clinical Infectious Diseases 2009; 48: 503-535.

[62] Kett DH, Shorr AF, Reboli AC, Reisman AL, Biswas P, Schlamm HT. Anidulafungin compared with fluconazole in severely ill patients with candidemia and other forms of invasive candidiasis: support for the 2009 IDSA treatment guidelines for candidiasis. Crit Care 2011; 15: R253.

[63] Tripathi N, Watt K, Benjamin DK,Jr. Treatment and prophylaxis of invasive candidiasis. Semin Perinatol 2012; 36: 416-423.

[64] Hope WW, Castagnola E, Groll AH, Roilides E, Akova M, Arendrup MC, et al. ESCMID* Guideline for the Diagnosis and Management of Candida Diseases 2012: Prevention and Management of Invasive Infections in Neonates and Children Caused by Candida spp. Clinical Microbiology and Infection 2012; 18: 38-52.

[65] Suzuki T, Uno T, Chen G, Ohashi Y. Ocular distribution of intravenously administered micafungin in rabbits. J Infect Chemother 2008; 14: 204-207.

[66] Mochizuki K, Sawada A, Suemori S, Kawakami H, Niwa Y, Kondo Y, et al. Intraocular Penetration of Intravenous Micafungin in Inflamed Human Eyes. Antimicrob Agents Chemother 2013 May 20 [Epub ahead of print].

[67] Mochizuki K, Suemori S, Udo K, Komori S, Ohkusu K, Yamada N, et al. Intraocular penetration of micafungin in patient with Candida albicans endophthalmitis. J Ocul Pharmacol Ther 2011; 27: 531-533.

[68] Kaufman DA. "Getting to Zero": Preventing invasive Candida infections and eliminating infection-related mortality and morbidity in extremely preterm infants. Early Hum Dev 2012; 88: 45-49.

[69] Pfaller MA, Messer SA, Woosley LN, Jones RN, Castanheira M. Echinocandin and triazole antifungal susceptibility profiles of opportunistic yeast and mould clinical isolates (2010-2011): Application of new CLSI clinical breakpoints and epidemiological cutoff values to characterize geographic and temporal trends of antifungal resistance. J Clin Microbiol 2013 May 29 [Epub ahead of print].

[70] Desnos-Ollivier M, Bretagne S, Raoux D, Hoinard D, Dromer F, Dannaoui E, et al. Mutations in the *FKS1* gene in Candida albicans, C. tropicalis, and C. krusei correlate with elevated caspofungin MICs uncovered in AM3 medium using the method of the European Committee on Antibiotic Susceptibility Testing. Antimicrob Agents Chemother 2008; 52: 3092-3098.

[71] Balashov SV, Park S, Perlin DS. Assessing resistance to the echinocandin antifungal drug caspofungin in Candida albicans by profiling mutations in *FKS1*. Antimicrob Agents Chemother 2006; 50: 2058-2063.

[72] Park S, Kelly R, Kahn JN, Robles J, Hsu MJ, Register E, et al. Specific substitutions in the echinocandin target *FKS1*p account for reduced susceptibility of rare laboratory and clinical Candida sp. isolates. Antimicrob Agents Chemother 2005; 49: 3264-3273.

[73] Hunter PR. Reproducibility and indices of discriminatory power of microbial typing methods. J Clin Microbiol 1990; 28: 1903-1905.

[74] Spampinato C, Leonardi D. Molecular fingerprints to identify Candida species. Biomed Res Int 2013 Jun 6 [Epub ahead of print].

[75] L'ollivier C, Labruere C, Jebrane A, Bougnoux ME, d'Enfert C, Bonnin A, et al. Using a Multi-Locus Microsatellite Typing method improve phylogenetic distribution of Candida albicans isolates but failed to demonstrate association of some genotype with the commensal or clinical origin of the isolates. Infect Genet Evol 2012 Aug 17 [Epub ahead of print].

[76] Gago S, Lorenzo B, Gomez-Lopez A, Cuesta I, Cuenca-Estrella M, Buitrago MJ. Analysis of strain relatedness using high resolution melting in a case of recurrent candiduria. BMC Microbiol 2013 Jan 23 [Epub ahead of print].

[77] Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A 1998; 95: 3140-3145.

[78] Bougnoux ME, Aanensen DM, Morand S, Theraud M, Spratt BG, d'Enfert C. Multilocus sequence typing of Candida albicans: strategies, data exchange and applications. Infect Genet Evol 2004; 4: 243-252.

[79] Bougnoux ME, Tavanti A, Bouchier C, Gow NA, Magnier A, Davidson AD, et al. Collaborative consensus for optimized multilocus sequence typing of Candida albicans. J Clin Microbiol 2003 Nov;41:5265-5266.

[80] Bougnoux ME, Morand S, d'Enfert C. Usefulness of multilocus sequence typing for characterization of clinical isolates of Candida albicans. J Clin Microbiol 2002; 40: 1290-1297.

[81] Aanensen DM, Spratt BG. The multilocus sequence typing network: mlst.net. Nucleic Acids Res 2005; 33: W728-33.

[82] Tavanti A, Davidson AD, Fordyce MJ, Gow NA, Maiden MC, Odds FC. Population structure and properties of Candida albicans, as determined by multilocus sequence typing. J Clin Microbiol 2005; 43: 5601-5613.

[83] Arendrup MC, Cuenca-Estrella M, Lass-Florl C, Hope W. EUCAST Technical Note on the EUCAST Definitive Document EDef 7.2: Method for the determination of broth dilution minimum Inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clinical Microbiology and Infection 2012; 18: E246-247.

[84] Tavanti A, Gow NA, Senesi S, Maiden MC, Odds FC. Optimization and validation of multilocus sequence typing for Candida albicans. J Clin Microbiol 2003; 41: 3765-3776.

[85] Odds FC, Jacobsen MD. Multilocus sequence typing of pathogenic Candida species. Eukaryot Cell 2008; 7: 1075-1084.

[86] Douglas CM, D'Ippolito JA, Shei GJ, Meinz M, Onishi J, Marrinan JA, et al. Identification of the *FKS1* gene of Candida albicans as the essential target of 1,3-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother 1997; 41: 2471-2479.

[87] Johnson AD. An extended IUPAC nomenclature code for polymorphic nucleic acids. Bioinformatics 2010 Mar 03 [Epub ahead of print].

[88] Odds FC, Bougnoux ME, Shaw DJ, Bain JM, Davidson AD, Diogo D, et al. Molecular phylogenetics of Candida albicans. Eukaryot Cell 2007; 6: 1041-1052.

[89] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 2011 05-04 [Epub ahead of print].

[90] Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A 2004; 101: 11030-11035.

[91] Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol 2004; 186: 1518-1530.

[92] Pfaller MA, Espinel-Ingroff A, Canton E, Castanheira M, Cuenca-Estrella M, Diekema DJ, et al. Wild-Type MIC Distributions and Epidemiological Cutoff Values for Amphotericin B, Flucytosine, and Itraconazole and Candida spp. as Determined by CLSI Broth Microdilution. J Clin Microbiol 2012 Mar 29 [Epub ahead of print].

[93] Odds FC, Jacobsen MD. Multilocus Sequence Typing of Pathogenic Candida Species. Eukaryotic Cell 2008; 7: 1075 -1084.

[94] Hall BG. Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol 2013; 30: 1229-1235.

[95] Feierl G, Buzina W, Massoud-Landgraf L. Resistenzbericht 2010.

[96] Feierl G, Buzina W, Massoud-Landgraf L. Resistenzbericht 2012.

[97] Pfaller MA, Jones RN, Doern GV, Sader HS, Hollis RJ, Messer SA. International surveillance of bloodstream infections due to Candida species: frequency of occurrence and antifungal susceptibilities of isolates collected in 1997 in the United States, Canada, and South America for the SENTRY Program. The SENTRY Participant Group. J Clin Microbiol 1998; 36: 1886-1889.

[98] Trick WE, Fridkin SK, Edwards JR, Hajjeh RA, Gaynes RP, National Nosocomial Infections Surveillance System Hospitals. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989-1999. Clin Infect Dis 2002; 35: 627-630.

[99] Odds FC. In Candida albicans, resistance to flucytosine and terbinafine is linked to MAT locus homozygosity and multilocus sequence typing clade 1. FEMS Yeast Res 2009; 9: 1091-1101.

[100] Odds FC, Jacobsen MD. Multilocus sequence typing of pathogenic Candida species. Eukaryot Cell 2008; 7: 1075-1084.

[101] Beyda ND, Lewis RE, Garey KW. Echinocandin resistance in Candida species: mechanisms of reduced susceptibility and therapeutic approaches. Ann Pharmacother 2012; 46: 1086-1096.

[102] Castanheira M, Woosley LN, Diekema DJ, Messer SA, Jones RN, Pfaller MA. Low prevalence of *FKS1* hot spot 1 mutations in a worldwide collection of Candida strains. Antimicrob Agents Chemother 2010; 54: 2655-2659.

[103] Laverdiere M, Lalonde RG, Baril JG, Sheppard DC, Park S, Perlin DS. Progressive loss of echinocandin activity following prolonged use for treatment of Candida albicans oesophagitis. J Antimicrob Chemother 2006; 57: 705-708.

[104] Slater JL, Howard SJ, Sharp A, Goodwin J, Gregson LM, Alastruey-Izquierdo A, et al. Disseminated Candidiasis caused by Candida albicans with amino acid substitutions in *FKS1* at position Ser645 cannot be successfully treated with micafungin. Antimicrob Agents Chemother 2011; 55: 3075-3083.

[105] Katiyar S, Pfaller M, Edlind T. Candida albicans and Candida glabrata clinical isolates exhibiting reduced echinocandin susceptibility. Antimicrob Agents Chemother 2006; 50: 2892-2894.

[106] Wiederhold NP, Najvar LK, Bocanegra RA, Kirkpatrick WR, Patterson TF. Caspofungin dose escalation for invasive candidiasis due to resistant Candida albicans. Antimicrob Agents Chemother 2011; 55: 3254-3260.

[107] MacCallum DM, Castillo L, Nather K, Munro CA, Brown AJ, Gow NA, et al. Property differences among the four major Candida albicans strain clades. Eukaryot Cell 2009; 8: 373-387.

[108] Da Matta DA, Melo AS, Guimaraes T, Frade JP, Lott TJ, Colombo AL. Multilocus sequence typing of sequential Candida albicans isolates from patients with persistent or recurrent fungemia. Med Mycol 2010 Jan 28 [Epub ahead of print].

[109] Cliff PR, Sandoe JAT, Heritage J, Barton RC. Use of multilocus sequence typing for the investigation of colonisation by Candida albicans in intensive care unit patients. J Hosp Infect 2008; 69: 24-32.

[110] Odds FC, Davidson AD, Jacobsen MD, Tavanti A, Whyte JA, Kibbler CC, et al. Candida albicans strain maintenance, replacement, and microvariation demonstrated by multilocus sequence typing. J Clin Microbiol 2006; 44: 3647-3658.

[111] Boers SA, van der Reijden WA, Jansen R. High-throughput multilocus sequence typing: bringing molecular typing to the next level. PLoS One 2012; 7: e39630.

6 SUPPLEMENTARY MATERIAL

No	Source	Susceptibility testing								
		5FC	AMB	FCA	ITR	VRC	CS	POS	AND	MYC
4	oral	S	S	S	S	S	S	S	S	S
5	BAL	S	S	S	S	S	S	S	S	S
6	oral	S	S	S	S	S	S	S	S	S
7	?	S	S	S	S	S	S	S	S	S
8	oral	S	S	S	S	S	S	S	S	S
9	BAL	S	S	S	S	S	S	S	S	S
10	BAL	S	S	S	S	S	S	S	S	S
11	oral	S	S	S	S	S	S	S	S	S
12	oral	S	S	S	S	S	S	S	S	S
13	BAL	S	S	S	S	S	S	S	S	S
14	oral	S	S	S	S	S	S	S	S	S
15	tracheostomy	S	S	S	S	S	S	S	S	S
16	oral	S	S	S	S	S	S	S	S	S
22	oral	S	S	S	S	S	S	S	S	S
23	BAL	S	S	S	S	S	S	S	S	S
25	oral	S	S	S	S	S	S	S	S	S
27	oral	S	S	S	S	S	S	S	S	S
28	oral	S	S	S	S	S	S	S	S	S
29	oral	S	S	S	S	S	S	S	S	S
30	?	S	S	S	S	S	S	S	S	S
32	oral	S	S	S	S	S	S	S	S	S
33	oral	S	S	S	S	S	S	S	S	S
34	oral	S	S	S	S	S	S	S	S	S
35	oral	S	S	S	S	S	S	S	S	S
37	oral	S	S	S	S	S	S	S	S	S
38	tracheal	S	S	S	S	S	S	S	S	S
39	oral	S	S	S	S	S	S	S	S	S
40	oral	S	S	S	S	S	S	S	S	S
41	oral	S	S	S	S	S	S	S	S	S
42	tracheal	S	S	S	S	S	S	S	S	S
43	oral	**R***	S	S	S	S	S	S	S	S
44	oral	S	S	S	S	S	S	S	S	S
47	oral	S	S	S	S	S	S	S	S	S
48	oral	S	S	S	S	S	S	S	S	S
49	oral	S	S	S	S	S	S	S	S	S
50	oral	S	S	S	S	S	S	S	S	S
51	oral	S	S	S	S	S	S	S	S	S
56	oral	S	S	S	S	S	S	S	S	S
57	oral	S	S	S	S	S	S	S	S	S
58	oral	S	S	S	S	S	S	S	S	S
59	oral	S	S	S	S	S	S	S	S	S
60	?	S	S	S	S	S	S	S	S	S
61	?	S	S	S	S	S	S	S	S	S
62	?	S	S	S	S	S	S	S	S	S

63	oral	S	S	S	S	S	S	S	S	S
64	oral	S	S	S	S	S	S	S	S	S
66	BAL	S	S	S	S	S	S	S	S	S
67	oral	S	S	S	S	S	S	S	S	S
68	oral	S	S	S	S	S	S	S	S	S
70	oral	S	S	S	S	S	S	S	S	S
71	BAL	S	S	S	S	S	S	S	S	S
72	oral	S	S	S	S	S	S	S	S	S
73	BAL	S	S	S	S	S	S	S	S	S
74	BAL	S	S	S	S	S	S	S	S	S
75	perianal	S	S	S	S	S	S	S	S	S
76	oral	S	S	S	S	S	S	S	S	S
77	perianal	S	S	S	S	S	S	S	S	S
78	oral	S	S	S	S	S	S	S	S	S
79	BAL	S	S	S	S	S	S	S	S	S
80	oral	S	S	S	S	S	S	S	S	S
81	pharynx	S	S	S	S	S	S	S	S	S
82	BAL	S	S	S	S	S	S	S	S	S
83	BAL	S	S	S	S	S	S	S	S	S
84	oral	S	S	S	S	S	S	S	S	S
85	BAL	S	S	S	S	S	S	S	S	S
86	oral	S	S	S	S	S	S	S	S	S
87	perianal	S	S	S	S	S	S	S	S	S
88	oral	S	S	S	S	S	S	S	S	S
89	BAL	S	S	S	S	S	S	S	S	S
90	BAL	S	S	S	S	S	S	S	S	S
91	oral	S	S	S	S	S	S	S	S	S
92	oral	S	S	S	S	S	S	S	S	S
93	oral	S	S	S	S	S	S	S	S	S
94	perianal	S	S	S	S	S	S	S	S	S
95	perianal	S	S	S	S	S	S	S	S	S
96	oral	S	S	S	S	S	S	S	S	S
97	BAL	S	S	S	S	S	S	S	S	S
98	BAL	S	S	S	S	S	S	S	S	S
100	oral	S	S	S	S	S	S	S	S	S
103	oral	S	S	S	S	S	S	S	S	S
104	oral	S	S	S	S	S	**R***	S	**R**	**R***
106	BC	S	S	S	S	S	S	S	S	S
107	tracheal	S	S	S	S	S	S	S	S	S
108	BC	S	S	S	S	S	S	S	S	S
109	oral	S	S	S	S	S	S	S	S	S
110	punction	S	S	S	S	S	S	S	S	S
111	cyst	S	S	S	S	S	S	S	S	S
112	CVC	S	S	S	S	S	S	S	S	S
113	BC	S	S	S	S	S	S	S	S	S
114	BC	S	S	S	S	S	S	S	S	S
115	BC	S	S	S	S	S	S	S	S	S
116	BC	S	S	S	S	S	S	S	S	S
117	BC	S	S	S	S	S	S	S	S	S
118	BC	S	S	S	S	S	S	S	S	S
120	BC	S	S	S	S	S	S	S	S	S
121	BC	S	S	S	S	S	S	S	S	S
122	BC	S	S	S	S	S	S	S	S	S

124	BC	S	S	S	S	S	S	S	S	S
125	perianal	S	S	S	S	S	S	S	S	S
126	pharynx	S	S	S	S	S	S	S	S	S
127	BC	S	S	S	S	S	S	S	S	S
128	BC	S	S	S	S	S	S	S	S	S
129	BC	S	S	S	S	S	S	S	S	S
130	oral	S	S	S	S	S	S	S	S	S
131	perianal	S	S	S	S	S	S	S	S	S
132	BAL	S	S	S	S	S	S	S	S	S
133	BAL	S	S	S	S	S	S	S	S	S
134	oral	S	S	S	S	S	S	S	S	S
135	perianal	S	S	S	S	S	S	S	S	S
136	CVC	S	S	S	S	S	S	S	S	S
137	oral	S	S	S	S	S	S	S	S	S
138	oral	S	S	S	S	S	S	S	S	S
139	perianal	S	S	S	S	S	S	S	S	S
140	BAL	S	S	S	S	S	S	S	S	S
141	BAL	S	S	S	S	S	S	S	S	S
142	oral	S	S	S	S	S	S	S	S	S
143	oral	S	S	S	S	S	S	S	S	S
144	BAL	S	S	S	S	S	S	S	S	S
145	BAL	S	S	S	S	S	S	S	S	S
146	oral	S	S	S	S	S	S	S	S	S
148	perianal	S	S	S	S	S	S	S	S	S
150	BAL	S	S	S	S	S	S	S	S	S
152	BAL	S	S	S	S	S	S	S	S	S
154	oral	S	S	S	S	S	S	S	S	S
156	perianal	S	S	S	S	S	S	S	S	S
158	BAL	S	S	S	S	S	S	S	S	S
159	oral	S	S	S	S	S	S	S	S	S
161	oral	S	S	S	S	S	S	S	S	S
162	oral	S	S	S	S	S	S	S	S	S
163	perianal	S	S	S	S	S	S	S	S	S
165	perianal	S	S	S	S	S	S	S	S	S
167	BAL	S	S	S	S	S	S	S	S	S
169	oral	S	S	S	S	S	S	S	S	S
171	oral	S	S	S	S	S	S	S	S	S
173	BC	S	S	S	S	S	S	S	S	S
176	oral	S	S	S	S	S	S	S	S	S
177	Port	S	S	S	S	S	S	S	S	S
178	BC	S	S	S	S	S	S	S	S	S
180	?	S	S	S	S	S	S	S	S	S
181	oral	S	S	S	S	S	S	S	S	S

Table 13: Susceptibility testing results

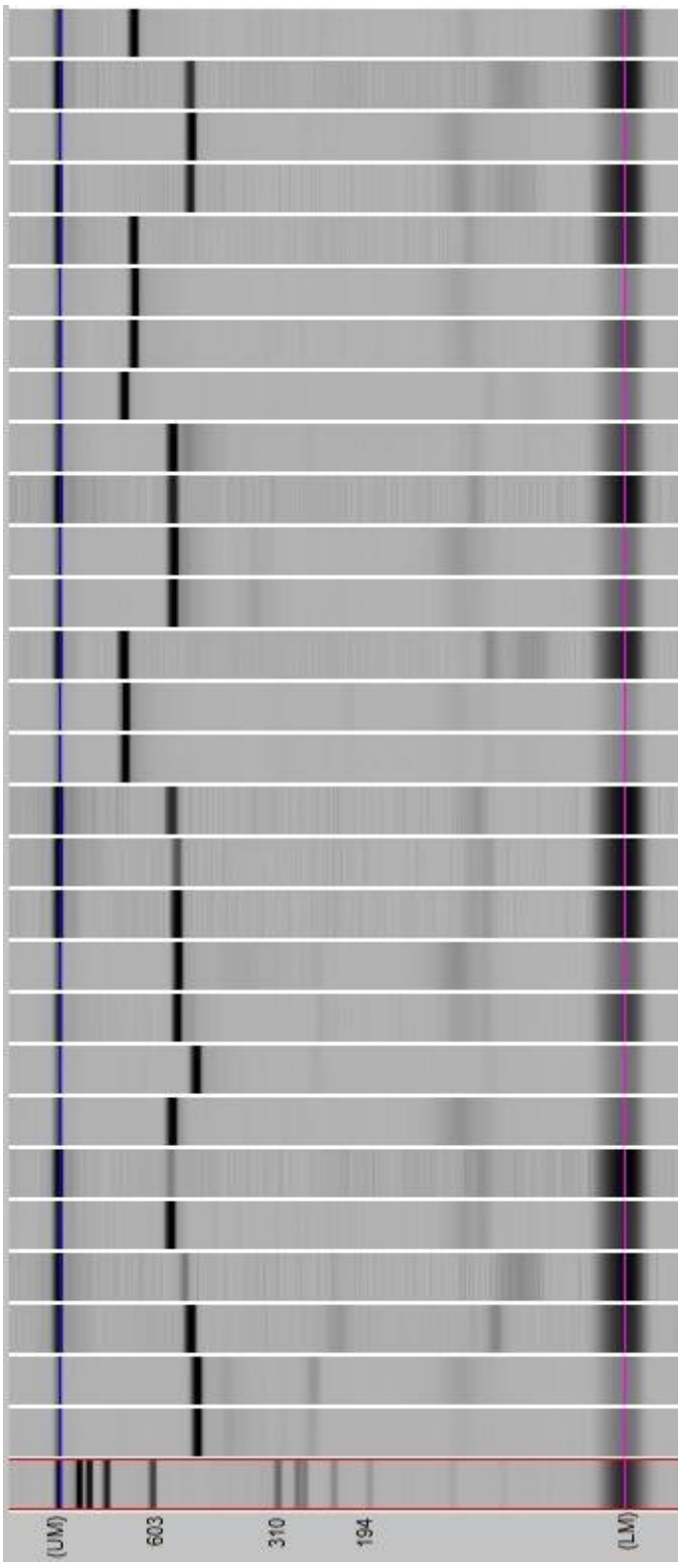


Figure 18: example of agarose gel controls

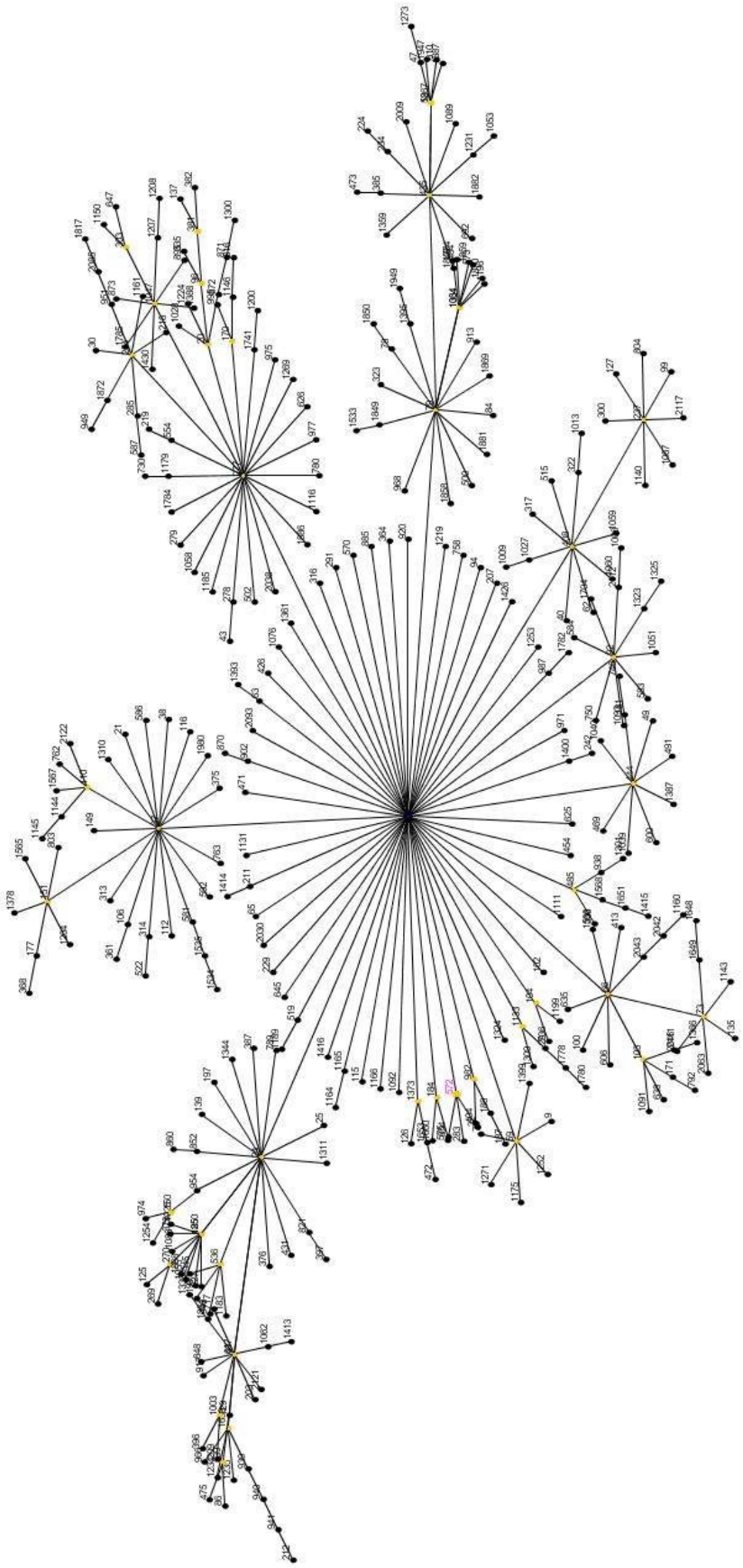


Figure 19: Clade 1 as determined by eBURST with all DSTs as of October 2013

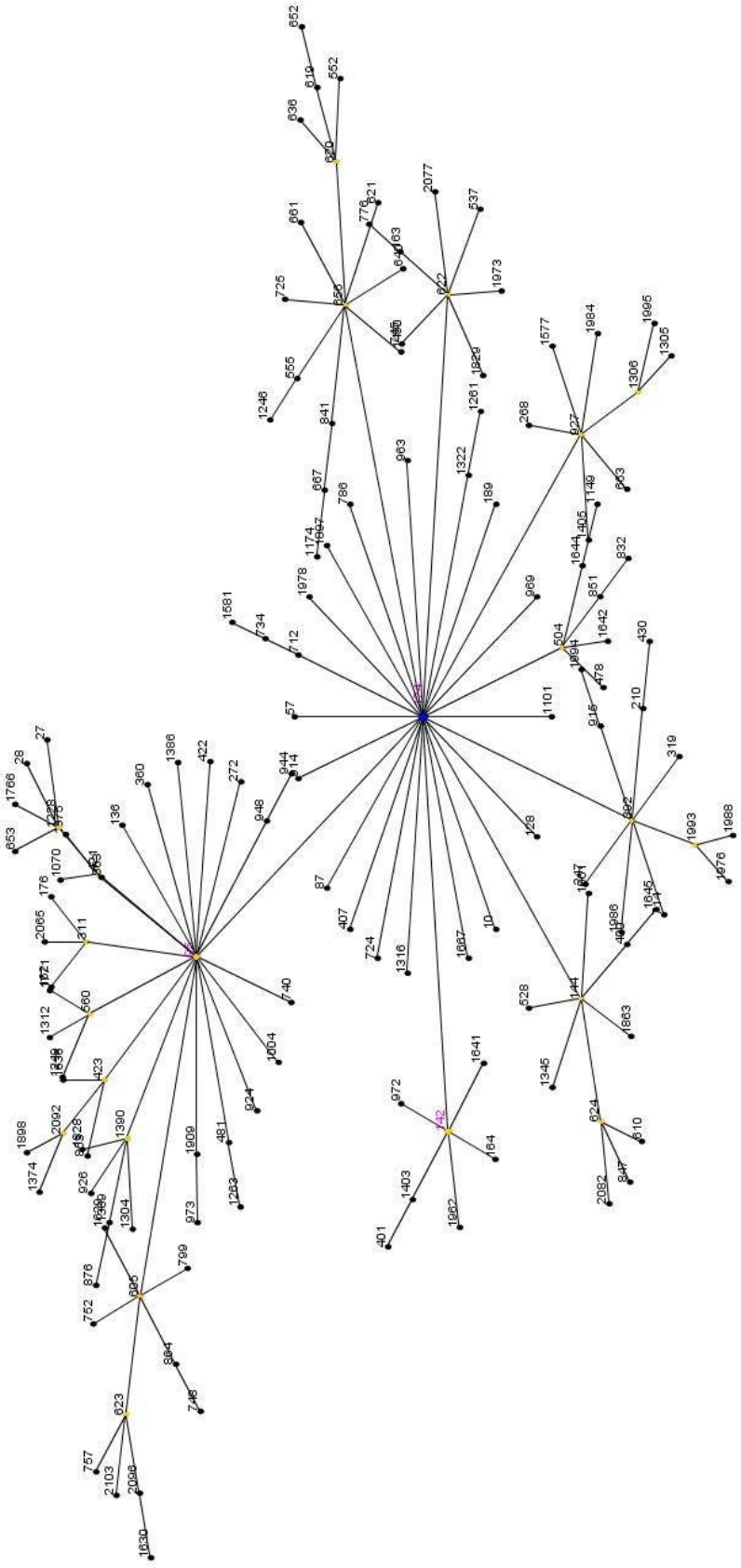


Figure 20: Clade 2 as determined by eBURST with all DSTs as of October 2013

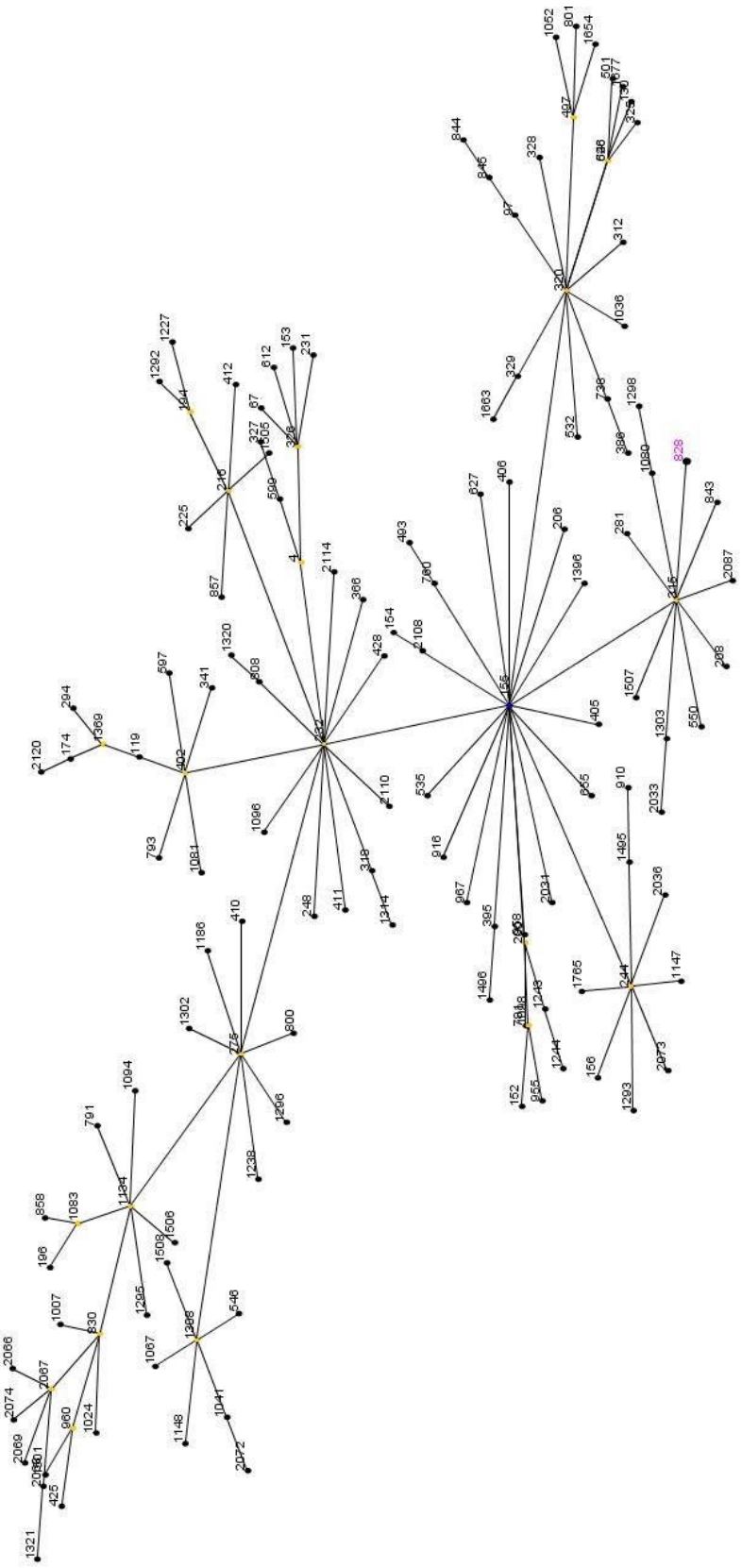


Figure 21: Clade 3 as determined by eBURST with all DSTs as of October 2013

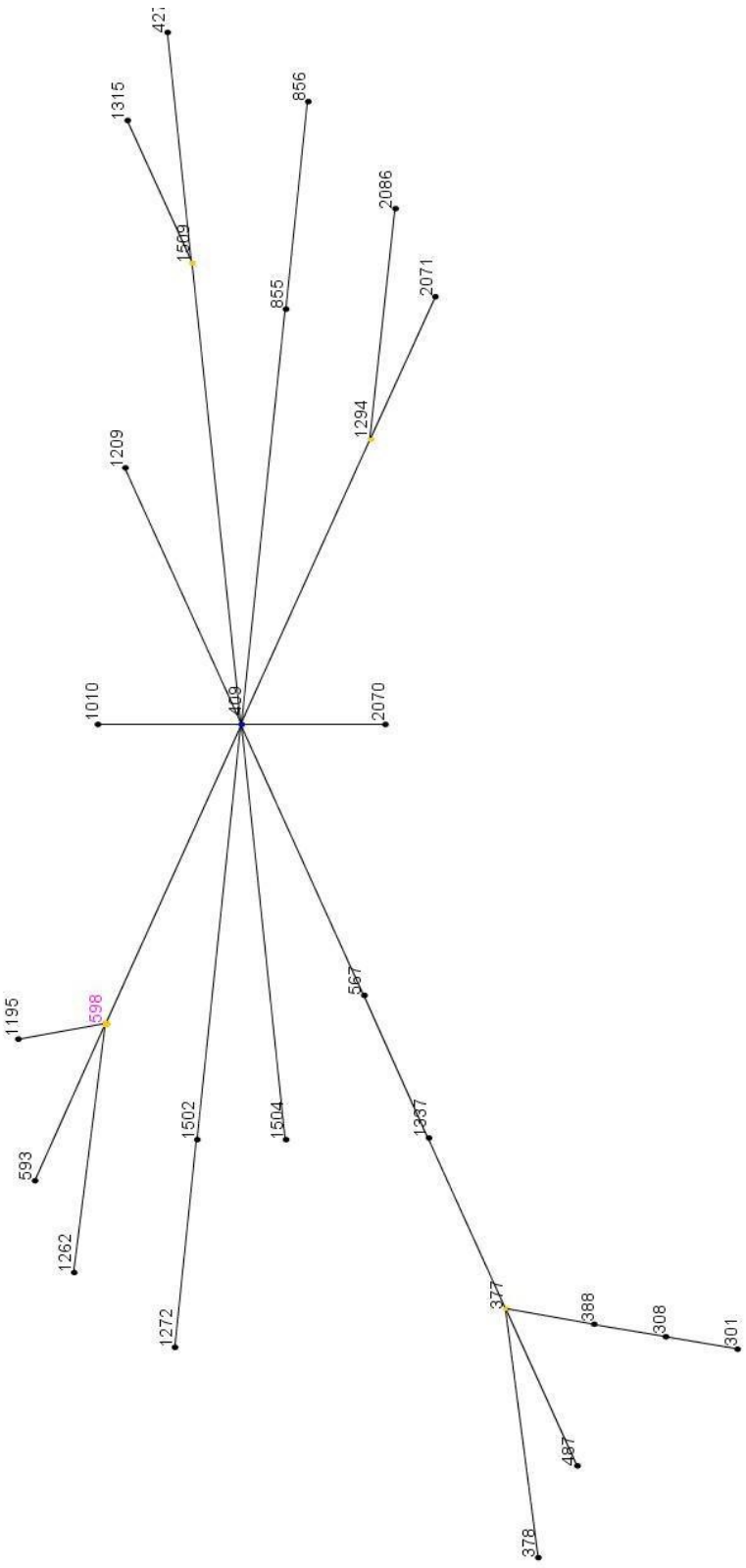


Figure 22: Clade 13 as determined by eBURST with all DSTs as of October 2013

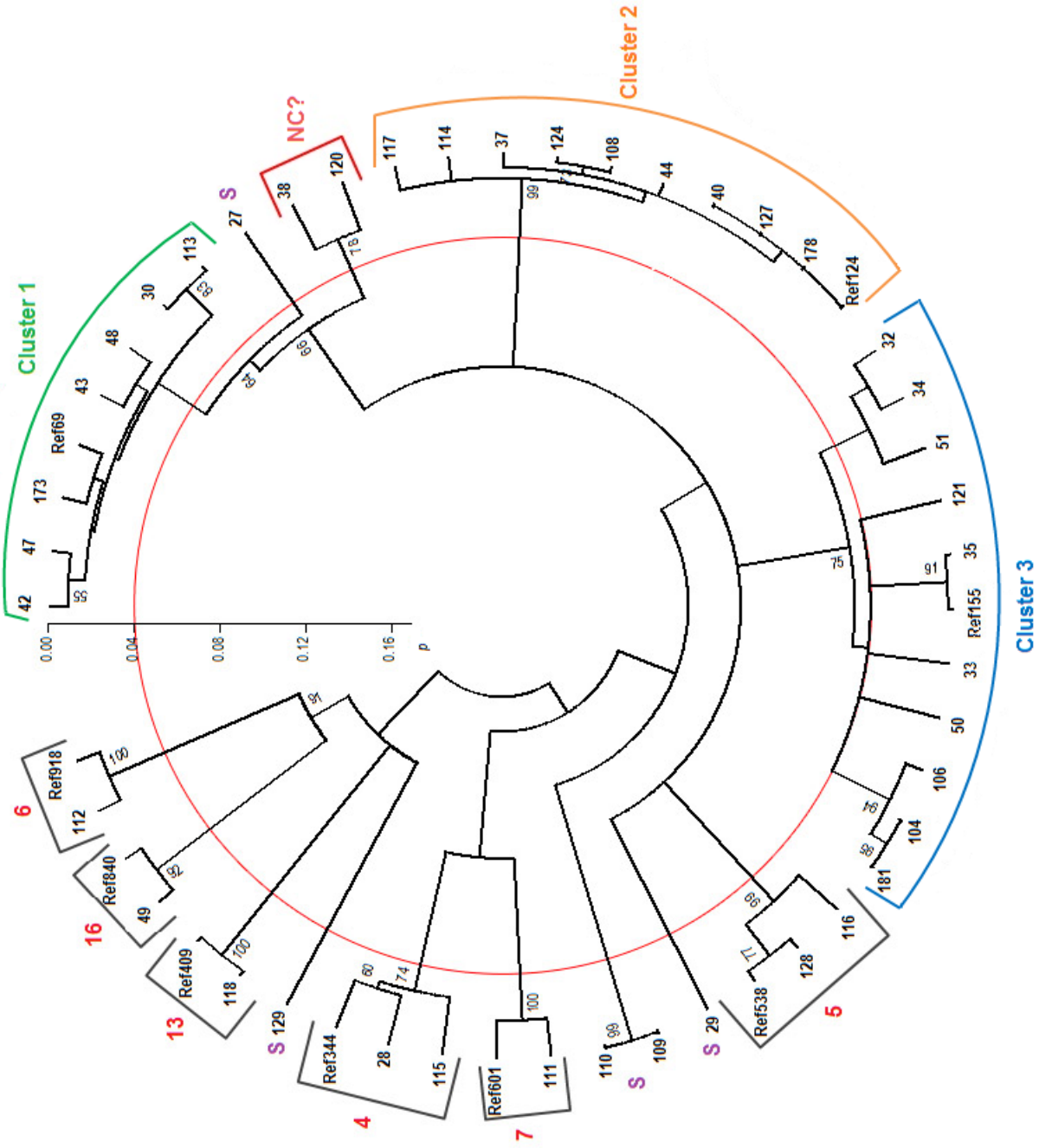


Figure 23: magnification of Figure 13: Neighbor-joining tree

6.1 Links

http://blast.ncbi.nlm.nih.gov/Blast.cgi

Nucleotide and protein blast queries by National Center for Biotechnology Information

http://calbicans.mlst.net

MLST database for *C. albicans*

http://www.candidagenome.org/

Assembly of *Candida* genome, sequence and primer data

http://www.ebi.ac.uk/Tools/msa/clustalo/

http://www.ebi.ac.uk/Tools/msa/muscle/

Multiple sequence alignment

www.eucast.org

The European Committee on Antimicrobial Susceptibility Testing

http://eburst.mlst.net

http://www.megasoftware.net

http://pubmlst.org/software/analysis/start2/

Programs for phylogenetic analysis

http://www.ebi.ac.uk/Tools/st/emboss_transeq

Nucleotide – to amino acid translation and vice versa

http://technelysium.com.au/

Chromas lite, free DNA sequencing software

http://inkscape.org/

Open source graphic software