Glossary

Allele: a variant of a locus
Auxotrophy: inability of an organism to grow in the absence of a given organic compound
Chemokines: type of cytokines that attracts specific celltypes to the site where they are secreted
Clade: evolutionary term for a group of organisms including their common ancestor and all its descendants
C-type lectin: a type of lectin that requires Ca^{2+} for proper function
Cytokine: small proteins that are secreted by various type of cells and play role in communication and interaction between cells
Dectin-1: a C-type lectin playing important role in fungal recognition
Desaturase: an enzyme responsible for converting saturated chemical bonds to unsaturated ones
Diploid: a term used for organisms possessing two copies of each chromosome
Endothel: thin layer of endothelial cells covering the inner wall of blood - and lymphatic vessels
Epithel: thin layer of epithelial cells covering the inner and outer surfaces of ther body
Ergosterol: a type of sterol found in the fungal cell membrane to increase rigidity
Glucan: a D-glucose polymer in the fungal cell wall
Heterozygosity: a state of being heterozygous
Heterozygote/ Heterozygous: the organism possesses two different alleles in a given locus.
Homology: similarity of biological properties between species derived from the same ancestor
Homozygote/Homozygous: the organism possesses the same alleles in the given locus
Hybrid: the resulting descendant of mating between two different species
In silico: any approach (search, calculation, prediction, *et cetera*) that utilises a computer
In vitro: any experiment performed outside of a living organsim
In vivo: any experiment performed on a living organism
Lectin: a protein that recognizes and binds carbohydrate molecules
Lipase: an enzyme that degrades fatty acids via hydrolysis
Locus: a given region of a genome
Lysosome: internal organelles filled with hydrolytic enzymes
Macrophage: antigen presenting phagocytes in the tissues
Mannan (O-, N-linked): a glucose polymer consisting of a linear backbone (α-1,6-bonds) with possible (α-1,2- or α-1,3-bonds) branches. It can be attached to –OH groups of Serine or Threonine of proteins that is called O-linked mannan, or to $-NH_2$ group of Asparagine that is known as N-linked mannan.
Mating (yeast): a well regulated process by which two individual cells (belonging to the opposite mating type) fuse together
Mb: megabase, unit of the size of DNA. 1 megabase is 1.000.000 bases.
Meiosis: a type of cell division, where the initial chromosome number (characteristic to the parent cell) is halved (in the daughter cell)
MIC: minimum inhibitory concentration is the lowest concentration of a specific drug that inhibits the growth of the microbe of interest.
Mitochondrion: an organelle in the eukaryotic cell responsible for providing energy
Mitosis: a type of cell division, where the initial chromosome number (characteristic to the parent cell) does not change (in the daughter cell)
Neutropenia: a condition where the concentration of neutrophil granulocytes is less than 1,500 * μl^{-1}
Neutrophil granulocyte: short-lived white blood cells being present in the largest number in the blood of most mammals

Null-mutant: a strain of an organism lacking both alleles of a given locus
Onycomycoses: infection of the nails caused by a fungus
Orthology (genome): a type of homology where the homolog region is defined across species
Paralogy (genome): a type of homology where the homolog region is defined in one species. It is typically represented by gene duplications.
PBMC: peripheral blood mononuclear cell, any blood cell possessing a round shaped nucleus
PCR: polymerase chain reaction, a method used for multiply a specific region of the genome or DNA sequence
Phagocyte: a type of cells in the body that is capable of engulfing various organic or inorganic particles (fungus, bacteria, cell debris *et cetera*)
Phagosome: the orgenelle formed as a result of phagocytosis
Phospholipases: enzymes responsible for hydrolyzing ester and ether bonds of phospholipids
Phylogenetic reconstruction: an *in silico* process by which the most probable relationship between species can be defined
Plasmid: a circular extrachromosomal element
Primer: a single oligonucleotide that act as starting point for DNA synthesis during PCR
Promoter: a DNA region found before a gene. This is where transcription factors can bind and therefore determine whether a gene is turned on or off.
Prototroph: an organism that is capable of growing in the absence of organic nutrients
QRT-PCR: quantitative real-time PCR, a PCR based method to determine the amount of a desired nucleotide sequence
Receptor: a protein that can recieve and transfer chemical - or physical signals from the environment to promote a regulated cell resposne
Recombinase: an enzyme catalysing the recombination procedure
Recombination: a regulated process where different regions of the chromosomes are combined
SNP: single nucleotide polymorphism or point mutation. A type of alteration in the nucleotide sequence where only one nucleotide is changed compared to a reference sequence.
Tetraploid: a term used for organisms possessing four copies of each chromosome
T_h cell: special type of immune cell being responsible for regulating the immune response by secreting specific cytokines
Transcription: a process by which the information encoded by a gene is converted into RNA
Transcription factor: proteins that regulate the transcription
Wild-type: any isolate without a genetic modification

Candida parapsilosis sensu lato - General introduction

Candida parapsilosis sensu lato species are diploid yeasts belonging to the so-called CUG clade of the Class *Saccharomycetes* (Division of *Ascomycota*). The term "*C. parapsilosis sensu lato*" refers to three very closely related but distinct species: *C. parapsilosis sensu stricto, C. orthopsilosis,* and *C. metapsilosis*, formerly known as *C. parapsilosis* Group I, II and III, respectively. Their genome sizes range between ~ 12.6 - 13.3 Mb and encode ~ 5,700 - 6,300 genes. Although they conserve remnants of mating related genes in their genomes, no complete sexual cycle has yet been observed in this group. Instead, recent results suggest that genetic diversity is increased through hybrid formation. These species are generally considered as human commensals, often associated with healthy human skin and mucosa. However, in the setting of immune compromise, these fungi cause illnesses ranging from mild superficial or mucosal infections to life-threatening systemic diseases. Strikingly, *C. parapsilosis sensu stricto* has a strong predilection for disease in low and very low birth weight neonates and is responsible for numerous outbreaks in neonatal intensive care units. Additional predisposing risk factors for disease from these pathogens include the use of

intravenous catheters, antibiotics, neutropenia, HIV infection, cancer therapy and prolonged hospitalization. After identification, azole derivatives are generally administered, alternatively amphotericin B may be used or, if susceptible, echinocandins. Although significant advances in the understanding of the pathobiology of these yeasts have been made, there remain many questions regarding the specific tools that they harness to enable human infection. The virulence factors that have been identified to date, include adhesins and secreted hydrolytic enzymes (lipases and proteinases). In turn, host receptors involved with recognition and interaction with these yeasts include Toll-like receptor 4 (TLR4), Dectin-1, and Galectin-3. Additional insights have suggested that *C. parapsilosis sensu stricto* can modulate the host immune response by inhibiting the Th1/Th17 host response and inducing antigen production to hide from the host immune system. There are many unexplained questions regarding the biology of *Candida parapsilosis sensu lato* species, but their increased medical importance over the past 2 decades has resulted in numerous active investigations intended to elucidate a deeper understanding the pathogenesis of these species in order to develop improved preventative and therapeutic strategies.

Candida parapsilosis sensu lato complex - History

The evolution of the nomenclature of the *Candida parapsilosis sensu lato* species is somewhat convoluted and emerged out of a lack of scientific tools as well as the absence of standardization of scientific naming. Moreover, communication between doctors, biologists and other scientists was very limited and ponderous in the late 1800s and early 1900s. At that time macroscopic/microscopic morphology and the capability of an isolate to grow or not to grow on/in different mediums were the basis for species identification (Castellani and Chalmers (1919), Stovall and Bubolz (1932), Ciferri and Redaelli (1929)). The limitation of these methods and the disarray of species naming are well represented by the publication of Ciferri and coworkers who found 121 synonym names for *Candida albicans* in the literature in 1938 (Cifferi (1938)). In fact, the majority of *Candida* species were mostly described as *Monilia* in the literature 19th and the first half of the 20th century until the 1940s when a group of scientists proposed a fundamental change in the nomenclature based on the hosts these microbes infect. Since then, *Monilia* species were considered as plant pathogens and the name *Candida* was dedicated to yeasts infecting animals (Mackinnon and Artagaveytia-Allende (1945), Skinner (1947)). There was also an attempt to standardize the classification process. Besides morphology, various cultivation methods were applied and fermentation properties were examined that helped avoid misidentification of the *Candida* species (Martin and Jones (1940)).

The first appearance of the name *Candida parapsilosis* in the literature was in 1932 by Langeron and Talice. However, this organism had actually been reported 6 years before by Pollacci and Nann in their scientific paper on a microbe called *Monilia onychophila*, that later turned out to be *C. parapsilosis*. Unfortunately, many of these early reports are not available directly, but are known through references in subsequent publications (Talice (1932), Martin and Jones (1940), Talice (1932)). The earliest accessible publication is from 1928 written by a doctor named Ashford who described a patient suffering from diarrhea whose stool grew an interesting yeast. This microbe differed in its fermentation capabilities from the well known yeast at that time called *Monilia psilosis*, which had previously been isolated from stool and other patient samples. Ashford named this microbe *Monilia parapsilosis* and this is today known as *Candida parapsilosis* while *Monilia psilosis* is called *Candida albicans* (Ashford (1928), Robin (1923), Rogers (1922)).

C. parapsilosis gained importance in the 1970s when the number of medical case reports related to this yeast started to rise. Further reports underscored that the predilection for disease varied in *C. parapsilosis* compared to *C. albicans* as, for example, *C. parapsilosis* is often

associated with neonatal candidiasis (Anonymus (1977), Faix (1983), Baley (1986)). It was during this time period that significant advances in molecular biology techniques occurred that facilitated the use of genetic markers and related methods for species characterization and identification. By applying techniques such as restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD), the genetic background of *C. parapsilosis* was revealed to have diversity as demonstrated by sequence variances in the internal transcribed spacers (ITS) as well as in mitochondrial DNAs and rRNA domains. These data led to *C. parapsilosis* being separated into three different groups, called Group I, II and III (Scherer and Stevens (1987), Lehmann (1992), Lin (1995), Nosek (2002b), Kurtzman and Robnett (1998), Kurtzman and Robnett (1997), Rycovska (2004)). In 2005, Tavanti and coworkers used a multilocus sequence typing (MLST) technique to more deeply molecularly characterize these three groups and their work suggested that the *C. parapsilosis sensu lato* group should be divided into three individual species: Group I became *C. parapsilosis sensu stricto*, Group II was renamed to *C. orthopsilosis* and Group III was called *C. metapsilosis* (Tavanti (2005)). The three species are impossible to distinguish based on macroscopic or microscopic characteristics. In general they are all incapable of fermenting maltose and present as round or elongated cells, which may form pseudohyphae (Ashford (1928)). Colonies are usually shiny, white or yellowish in color, but this feature depends on the media used for cultivation. The morphology of *C. parapsilosis sensu lato* colonies is very diverse (smooth, rough, snowball, crepe, crater and concentric) and can be influenced by compounds or chemicals used in the media (Enger (2001), Laffey and Butler (2005)). The species grow well at 30 °C and 37 °C, but none grow at 42 °C. The holotypes have similar minimal inhibitory concentration (MIC) values against antifungal agents, and they also do not differ in either their biofilm forming capabilities or sugar assimilation profiles. Since morphological characterization cannot distinguish between these species, molecular methods are applied for species identification. Standard identification approaches are based on the localization of single nucleotide polymorphisms (SNPs) in putative alanyl-tRNA synthetase and secondary alcohol dehydrogenase genes compared to the historically most ancient species, *C. parapsilosis sensu stricto*. Primer pairs have also been developed that do not amplify any fragment from *C. metapsilosis* and *C. orthopsilosis* genome (Tavanti (2005)).

Candida parapsilosis sensu lato - Epidemiology

The *Candida* strains that scientists studied in the 1940-50s were mostly isolated from oral, vaginal and stool samples and the outcome of these illnesses were rarely fatal (Seelig (1966)). Although *C. albicans* was the most abundant among them, some other, so-called non-*albicans* species were also represented. The emergence of *Candida* species as a significant human pathogen began in the decade of "Penicillin era", when healthcare workers began to encounter super-infections. This term was used when a patient was admitted to a hospital with a bacterial infection and was treated with an antibiotic that eliminated the pathogen, but a secondary infection occurred (a.k.a. a super-infection, an infection caused a microbe other than the original one). One of the early well-known pathogens responsible for super-infections was *Staphylococcus aureus* but later fungi also started to emerge (Colgan (1956), Scales (1956), Woods (1951)). The possible connection between the use of broad-spectrum antibiotics and super-infection was first discussed in 1949 by Harris, who proposed that antibiotic drugs eradicated the microbes causing the presenting disease, but also eliminated or at least attenuated normal gut colonizing bacteria facilitating the development of super-infections by certain organisms, such as *Candida* species (Harris (1949)). Other reports subsequently provided additional support for this concept of antibiotics disrupting homeostasis of normal flora (Bartels and Buchbinder (1945), Pappenfort and Schnall (1951), Janke (1952), Huppert (1953), Sharp (1954)). Moreover, experimental evidence was provided

by Foley and Winter in 1949, who demonstrated that the administration of penicillin *in vivo* to chicken embryos enhanced the lethality of *C. albicans* clinical strains (Foley and Winter (1949)).

In addition to modification by antimicrobials, the general health of the host's immune system was identified as a critical factor in resistance to *Candida.* Alterations in host immunity that facilitated *Candida* infection ranged from the increased access to the bloodstream via intravenous catheters to devastation of host effector cells through primary or secondary immune destruction. Along this line, by the late 1970s and early 1980s, two independent effects leading to immunodeficiency were recognized as being closely connected to candidiasis: 1) organ transplantation associated with artificial attenuation of the immune system to avoid organ rejection and 2) patients with acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) (Sanger (1962), Klein (1984)). The increasing rates of *Candida* species among immunocompromised patients and their association with nosocomial infections resulted in a fundamental change in research priorities. Early publications in the *Candida* field had focused primarily on species identification, characterization and case reports, but the rapid increase in clinical cases expanded investigations into *Candida* virulence, pathogenesis and molecular characteristics. Infection models were established, the essential compounds required for growth were identified and the chemical properties of the cell wall were determined (Salvin (1952), Eisman (1953), Gebhardt and Hill (1956), Roth (1957), Johnson (1954), Sikl (1964)). Since *C. albicans* has long been the dominant *Candida* species causing systemic infections, most of these publications report results primarily on *C. albicans*; however, scientific papers from the 1950s began to explore other *Candida* species, including *C. tropicalis*, *C. krusei*, *C. glabrata* and *C. parapsilosis* (De Eshougues (1951), Monod (1956), Rotter and Staib (1958), Rook and Brand (1950), Wickerham (1957), Manchester and Georg (1959)).

C. parapsilosis sensu lato species are associated with humans (Weems (1992)). Interestingly, although *C. parapsilosis sensu stricto* has also been isolated from non-human sources like plants, seawater and soil, there have been no environmental *C. orhopsilosis* or *C. metapsilosis* strains isolated to date, but this does not necessarily mean that these species do not have a niche unrelated to humans (Fell and Meyer (1967)). There are differences in tissue colonization by these species with *C. parapsilosis sensu stricto* and *C. orthopsilosis* colonizing normal skin, *C. parapsilosis sensu stricto* and *C. metapsilosis* oral mucosa and *C. parapsilosis sensu stricto* vaginal mucosa (Underhill and Iliev (2014), Findley (2013), Ghannoum (2010), Nyirjesy (2005)). However, they are opportunistic pathogens as local and systemic disease can arise from colonized tissues. In addition to the above described risks of immunodeficiency and use of antibiotics as predisposing factors for disease secondary to these species, prolonged hospitalization, intravenous catheters, indwelling medical devices, surgical interventions, malignancy, neutropenia and burn injuries are all associated with invasive infections (Law (1984), Pan (2012) and reviewed in Trofa (2008)). Additionally, parenteral nutrition, intravenous lipid emulsion and intubation are also important risk factors for infections due to *C. parapsilosis sensu stricto*, particularly in neonatal disease (Saiman (2000)). *C. parapsilosis sensu stricto* is the predominant species identified in clinical disease followed distantly by *C. othopsilosis* and *C. metapsilosis* (Gomez-Lopez (2008), Lockhart (2008), Tay (2009), Mirhendi (2010), Orsi (2010), de Toro (2011)). Nevertheless, *C. orthopsilosis* and *C. metapsilosis* species have been isolated from ascites fluid, cerebrospinal fluid, joint fluid, abscesses, lung, blood, skin, nail, urine, central venous catheter and from superficial candidiasis (Lockhart (2008), Chen (2010), de Toro (2011), Asadzadeh (2016), Feng (2012), Silva (2009a), Schroder (2016)).

C. parapsilosis sensu lato species cause candidemia (fungemia caused by *Candida* species), which is a dangerous condition when the fungus gets into the blood and therefore

can disseminate throughout the body. Interestingly there is a remarkable difference between *C. albicans* and *C. parapsilosis sensu stricto* in this process. *C. albicans* is a normal colonizer of the gut and the female genitals and is transmitted from mother to child during and shortly after birth (called vertical transmission) (Nucci and Anaissie (2001), Tiraboschi (2010), Farr (2015)). Thus, people who develop *C. albicans* candidemia are usually infected by the strain they are colonized with. In contrast, *C. parapsilosis sensu stricto* does not need to undergo colonization after adhesion to cause such a disease. These infections are often associated with hospital environments (nosocomial infections), where medical devices and healthcare workers contribute to the horizontal transmission of this yeast (discussed in Trofa (2008)). Nosocomial acquisition of *C. parapsilosis* has been deemed responsible for 9 – 39 % of adult infections (reviewed in detail in Trofa (2008)). There are relatively few reports of candidemia due to *C. metapsilosis* and *C. orthopsilosis* (Gomez-Lopez (2008), Chen (2010), Blanco-Blanco (2014), Constante (2014), Ziccardi (2015)); however, this could be due to inaccurate speciation within the *C. parapsilosis sensu lato* group.

The clinical relevance and manifestations of *C. parapsilosis sensu stricto* range from superficial to disseminated infections. Fungal meningitis, arthritis, ocular and urinary tract infection rarely occur due to *C. parapsilosis sensu stricto*. Fungal peritonitis is also a rare, but serious condition with high mortality rate, mostly caused by *Candida* species, among which *C. parapsilosis sensu stricto* has been found to be responsible for 26 – 34% of cases (Chen (2004), Wang (2000), Warady (2000), Manzano-Gayosso (2003)). *Candida* species are the second or the third most common cause of fungal nail infection (onycomycoses) (Mugge (2006), Jayatilake (2009)). Although globally *C. albicans* is the dominant species causing onycomycoses, *C. parapsilosis sensu stricto* is the most prevalent species of the genus in some regions (Mugge (2006), Mujica (2004), Segal (2000), Fich (2014)). Vulvovaginal candidiasis is a condition that 50% of the women over 25 years of age will experience at least once. It is mostly caused by *C. albicans* (85-95%) while *C. parapsilosis sensu stricto* varies between 1.2 - 8.9% (Geiger (1995), Sobel (2007), Nyirjesy (2005) and reviewed in Trofa (2008)). *C. parapsilosis sensu stricto* is also the second most common cause of fungal endocarditis, with a mortality rate of approximately 50% (Martin (1979), Ellis (2001), Garzoni (2007)). The most striking observation regarding *C. parapsilosis sensu stricto* epidemiology, however, is its remarkable tendency to cause disease in low birth weight (less than 2500 grams) premature infants (Cheng and Yu (1970), Rubinstein (1975), Brunner (1975), Gugnani (1978), Yarchoan (1979), English (1972), Falagas (2010)). As noted above, parenteral nutrition, intravenous lipid emulsion and intubation are major risk factors for neonatal *C. parapsilosis sensu stricto* disease (Saiman (2000)). In this specific group of patients, the prevalence of *C. parapsilosis sensu stricto* varies between 15.5 – 65.1% (reviewed in Trofa (2008)). In these neonates, the infection can affect almost all the body sites including the lungs, urine, retina and central nervous system (Carter (2008)). In one study of a 15 year period (from 1981 to 1995) the prevalence of candidemia increased by 10-fold in a neonatal intensive care unit in the USA, with *C. albicans* as the dominant species during the first 10 years, but *C. parapsilosis sensu stricto* was responsible for up to 60% of the cases in the last 5 years (Kossoff (1998)). Although this is an extreme example, the dominance of *C. parapsilosis sensu stricto* is obvious as it was demonstrated by a large scale study published in 2013 focusing specifically on neonatal candidemia. The authors summarized the results of 37 publications and found the average proportion of *C. parapsilosis sensu stricto* of all neonatal *Candida* infection was 33.4%. The infection did not show a homogeneous geographic distribution, it varied between 19.1 and 35.7% among continents (Europe 19.1%, Asia 24.7%, South America 29.1%, North America 33.8% and Australia 35.8%). The average mortality rate among neonates was approximately 10% (reviewed in Trofa (2008), Pammi (2013)). However, the data presented a reciprocal correlation between birth weight and

mortality rate, i.e. lower the birth weight the higher the mortality rate. The mortality rates among infants weighing less than 1500 grams varies between 20-45%, and those who recover frequently suffer from long-term neurodevelopmental issues (Benjamin (2006), Fridkin (2006), Benjamin (2010), Miranda (2012), Friedman (2000)). *C. metapsilosis* and *C. orthopsilosis* are infrequently reported as causes of neonatal candidemia (Oliveira (2014)). Despite the clear association of *C. parapsilosis sensu stricto* with neonatal invasive disease, the ability of this fungus to preferentially affect this specific patient population remains unresolved.

Candida parapsilosis sensu lato - Diagnostics and treatment

Until the early 1950s superficial and systemic *Candida* infections, independent of the species, were difficult to cure due to the lack of effective antimycotics (Duhig and Mead (1951)). The first antifungal drug available was nystatin that was followed by amphotericin B soon after (Hazen and Brown (1950), Oura (1955), Sloane (1955)). These drugs are polyenes that function by destabilizing cell membrane ergosterol in fungi, causing an imbalance in ion homeostasis (reviewed in Ellis (2002), Hammond (1974)). These polyenes are challenged by their lack of absorption orally and their intravenous use is complicated by side effects, such as renal injury (Bell (1962), Giddings (1962), Haber and Joseph (1962)). Nevertheless, amphotericin B was to the first antifungal that was able to cure systemic candidiasis, and newer formulations with less toxicity have been developed, particularly by admixing with lipids or intercalating the drug into liposomes (summarized in Dismukes (2000)). *C. parapsilosis sensu lato* isolates are susceptible to amphotericin B with very few exceptions, although *C. parapsilosis sensu stricto* is somewhat less susceptible than the other two members of the complex (Lockhart (2008), Silva (2009a), Ziccardi (2015), Lotfali (2016)). Historically polyenes were followed by flucytosine (5-Fluorocytosine or 5-FC) in the late 60's to treat candidiasis (Tassel and Madoff (1968)). This chemical is a fluorinated analogue of the base cytosine and can be uptaken by fungal cells. 5-FC itself does not possess antifungal activity however in the fungal cell it can be converted into derivatives inhibiting fungal RNA and DNA synthesis (Polak and Scholer (1975), Waldorf and Polak (1983)). 5-FC has excellent oral bioavailability and can be effective in the setting of disseminated candidiasis (Tassel and Madoff (1968)). However, rapid resistance can occur with monotherapy, which limits the use of 5-FC (Hoeprich (1974), Ostrosky-Zeichner (2003)). In the 1980s, azoles (that interfere with the ergosterol biosynthesis of fungi) became available and their ease of administration by the oral route has made them highly utilized (Sobel (1989)). The azoles include ketoconazole, fluconazole, posaconazole, itraconazole, isavuconazole, and voriconazole. Of these, fluconazole is the most commonly used drug to treat *C. parapsilosis sensu lato* infections, since most of the isolates tested so far are susceptible (Pappas (2004)). Although a publication from 2008 found that *C. parapsilosis sensu stricto* minimal inhibitory concentrations (MICs) were slightly lower than the ones of *C. orthopsilosis* and *C. metapsilosis*, a 2015 study established an opposite correlation (Ziccardi (2015), Lockhart (2008)). These differences might be due to the limited sample numbers examined (especially in the case of *C. metapsilosis* and *C. orhopsilosis*) or it can be a geographical characteristic, but it is also possible that susceptibility is changing over time. At present, the echinocandins (anidulafungin, caspofungin, micafungin) are also frequently used prior to speciation of *Candida* identified in invasive disease (see Pappas (2016b)). Echinocandins interfere with the β-1,3-glucan synthase 1, an enzyme responsible for cell wall biosynthesis of specific fungi, like *Candida*. *C. parapsilosis sensu stricto* displays a higher MIC value compared to *C. metapsilosis* and *C. orthopsilosis* as well as *C. albicans* (Ostrosky-Zeichner (2003), Pfaller (2006), Lockhart (2008)). A possible explanation for this phenomena could be the diverse sequences of *FKS1* (the gene that encodes the synthase enzyme) orthologs in the three species

(Garcia-Effron (2011)). Moreover some authors reported strains of *C. parapsilosis sensu stricto* resistant to caspofungin (Ataides (2015), Ziccardi (2015)). Due to the different mechanisms of these drugs, combination therapy utilizing antifungals with different mechanisms of action can be considered (Rex (2000), Vasquez (2002) and summarized in detail in Pappas (2004) and Pappas (2016a)).

Because of the different susceptible profiles of pathogens, accurate and rapid identification of the species is crucial for efficient antimicrobial therapy. To identify *Candida* species from clinical samples one can utilize molecular and non-molecular methods. The latter involves differential medias, carbohydrate assimilation/fermentation tests and microscopic examinations. Although these methods are relatively cost-efficient they are time consuming. There were also detection kits commercially available making these processes faster and more accurate but still the identification may take longer than one day. Molecular methods involving peptide nucleic acid – fluorescent *in situ* hybridization (PNA - FISH) designed to 26S RNA, polymerase chain reaction (PCR) applied alone or in combination with DNA digestion, quantitative real-time – PCR (qRT-PCR) with targeting dedicated genomic region of a specific pathogen were introduced, but these methods were much time intensive and costly (Rigby (2002), Flahaut (1998), Morace (1997), Maaroufi (2003)). Due to the lack of special equipment and standardized protocols in many hospitals still non-molecular methods are in use (Hospenthal (2006) and reviewed in Silva (2012)). More recently, techniques such as Luminex xTAG Fungal Analyte system, matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI - TOF MS) and loop-mediated isothermal amplification (LAMP) methods are being utilized in some institutions due to their high specificity and sensitivity (Babady (2011), Buchan and Ledeboer (2013), Haas (2016), Trabasso (2015)).

Candida parapsilosis sensu lato - Phylogeny

As noted, *C. parapsilosis sensu lato* species complex belongs to the Class *Saccharomycetes* (Division of *Ascomycota*/Kingdom *Fungi*) and they are members of the CUG clade, a group of yeasts that differs from most other organisms in how it translates the CUG codon during protein synthesis. The universal codon usage CUG nucleotide triplet is generally translated to the hydrophobic amino acid leucine, but these fungi incorporate the hydrophilic amino acid serine instead (Santos and Tuite (1995)). The CUG clade also includes *Lodderomyces elongisporus* and *Debaryomyces hansenii* and certain *Pichia* species as well as other *Candida* species, like *C. albicans*, *C. dubliniensis*, *C. tropicalis* (Pryszcz (2015)). Computational analyses have revealed that *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis* are a closely related species complex in the CUG clade irrespective of the genomic regions chosen or the methods applied for calculations. By changing these parameters, however, the topology of the *C. parapsilosis sensu lato* species might be different in one publication compared to another. For example using only internal transcribed spacer 1 (ITS1) (a short non-coding genomic region located between 18S and 5.8S rDNA coding sequences) in combination with neighbor joining for phylogenetic reconstruction, *C. orthopsilosis* and *C. metapsilosis* clustered together separately from *C. parapsilosis sensu stricto* (Tavanti (2005)). This topology was also found when the ITS1 - 5.8S rDNA - ITS2 region was the subject of analysis with the same approach (Robl (2014)) or when the partial sequences of more than 1000 genes were analyzed using a maximum likelihood method (Riccombeni (2012)). However, when this latter algorithm was applied for genomic sequences of mitochondrial proteins, the basal position of *C. metapsilosis* was established, while *C. orthopsilosis* and *C. parapsilosis sensu stricto* seem to be more closely related (Wu (2009)).

Similar results were obtained by a recent analysis applying the sophisticated PhylomeDB pipeline for all the ortholog genes of the three species (Pryszcz (2015)).

Candida parapsilosis sensu lato - Genomics

Current methods allow for the whole genome sequencing of an organism within a few weeks. Understanding a species genome not only facilitates genetic manipulation, but the data can also be used for comparative analysis with other genomes to reveal genomic differences between species and even strains, and the data helps retrace evolutionary connections and trends. The genomes of *C. parapsilosis sensu lato* species complex conserves remarkable genetic evidences of how a species evolved on the way to becoming a human pathogen. Remarkably, all three members of the *C. parapsilosis sensu lato* group went through different evolutionary paths to become the pathogenic species that we know today. The first whole genome sequence available of the species complex was of *C. parapsilosis sensu stricto*. The genome of the clinical strain CDC 317 (isolated from a hand of a health care worker in the USA) was published in 2009 (Kuhn (2004), Butler (2009)). This was followed by *C. orthopsilosis* and *C. metapsilosis* in 2012 and 2015, respectively (Riccombeni (2012), Pryszcz (2015)). These three species are considered to be diploid organisms and they have not been observed to undergo either mating or meiosis. Mating requires two mating competent cells belonging to opposite mating types, as is well described process in *Saccharomyces cerevisiae*. These types are determined by two loci in *S. cerevisiae* called mating type (MAT). The MAT locus can be either an „a" or an „α" idiomporph in this species (Haber (2012)). Homologous sequences of MATa and MATα idiomorphs can be found in other yeasts like *C. albicans* and they are also present (sometimes only partially) in the *C. parapsilosis* species complex where they are called mating-type-like (MTLa and MTLα) locus (Sai (2011)).

Candida parapsilosis sensu stricto - the single

Although more than 200 isolates of *C. parapsilosis* have been genetically analyzed, no MTLα idiomorphs have been found; therefore, there is genetically no chance for mating. Not having been found of course does not mean it does not exist, maybe it is very rare, or it has a specific geographic preference. However, the complete lack of a mating competent strain encoding MTLα idiomorph in its genome is just one reason explaining why mating might not occur in this species. Besides, a1 gene encoded within the MTLa idiomorph turned out to be a pseudogene (possibly due to a recent event), therefore it is not functional (Sai (2011), Logue (2005)). Many authors have reported on the poor genetic variability among different *C. parapsilosis sensu stricto* clinical isolates, which points to the fact that this species might be highly clonal, meaning that the originally environmental species adapted to the human host only one time with the isolates causing human disease representing descendants of the same ancient strain (Lasker (2006), Butler (2009), Tavanti (2010)). The first comprehensive whole genomic analysis of *C. parapsilosis sensu stricto* revealed molecular evidences suggesting that this transition might have actually happened more than once. The comparative genomic approach involved the reference strain CDC 317 and three newly sequenced strains: two environmental isolates (CBS 1954 from Italy and CBS 6318 from the USA), and one clinical strain GA1 (isolated from bloodstream infection in Hamburg, Germany) (Pryszcz (2013), Butler (2009), van Rij and Verona (1949), Valach (2012), Gacser (2005)). Pryszcz and coworkers established expansions of the arsenite transporter 3 (*ARR3*) gene (that encodes a protein required in baker's yeast to survive under high arsenic conditions) in clinical isolates (Ghosh (1999)). One can assume that an organism living on the human skin does not have to face the selection pressure of arsenite, as it is highly toxic to the host. Therefore, the increased number of this gene presumably provides evolutionary advantage in the environment but not in/on the host. According to the molecular analysis these genetic expansions in the two

clinical isolates occurred independently, meaning that the two strains might have been created in nature. If this is true, the "environment to human" transition of *C. parapsilosis sensu stricto* might not have been a unique event and might have happened at least twice (Pryszcz (2013)). Further analysis established few (5,147) SNPs and 40 copy number variations (including deletions and duplications) between the four strains. Predicted gene numbers and genome sizes varied (between 5,665 and 6,293 genes and 13.030 and 13.138 Mb) mostly due to copy number variations. Heterogeneous distribution of SNPs, the footprints of flanking regions of deletions, suggest that recombination events likely happened in *C. parapsilosis sensu stricto* (Pryszcz (2013)). This is in line with the finding of Fundyga and colleagues, who had proposed that the minor sequence variations in the sequenced alleles could have been introduced by recombination events (Fundyga (2004)). All these results taken together describe a largely homogenous genome and (in evolutionary steps) a very young species (estimated age of the *C. parapsilosis sensu stricto* species is about 10^6 years) (Pryszcz (2013), Fundyga (2004)).

***Candida orthopsilosis* - keep on hybridizing**

C. orthopsilosis species seems to consist of (at least) two different subspecies called Type 1 and Type 2. The latter is very likely formed due to a hybridization event. The signs of divergence and recombination within the species were described in 2005 and 2007 by Tavanti and coworkers by using RAPD and amplified fragment length polymorphism (AFLP) analysis (Tavanti (2005), Tavanti (2007)). Also in 2005, Iida and colleagues analyzed ITS sequences of *C. parapsilosis sensu lato* species, and identified two previously unknown groups of the complex. One was called Group IV, which showed the highest similarity to Group II (*C. orthopsilosis*) and one more group (named Group V) that seemed to be closely related to Group II and Group IV (Iida (2005)). The genome of *C. orthopsilosis* (strain 90-125, a Type 2 isolate) was published in 2012. The 12.6 Mb genome contains 5,700 protein-coding genes distributed on eight chromosomes (Riccombeni (2012)). Based on the strains and sequences available, the genome of *C. orthopsilosis* is the only one in the complex so far that conserves evidence of mating in terms of MTL loci. This was established by Sai and colleagues, who investigated 16 *C. orthopsilosis* isolates and found both MATa/MATa (9 out of 16), MATα/MATα (5 out of 16) homozygous and MATa/MATα heterzygous (2 out of 16) isolates in both the subspecies, but no mating was observed under various laboratory conditions. One possible explanation was the incorrect maturation of the transcripts of MTLa1 and MTLa2 regulatory proteins (Sai (2011)). Further evidence of mating was found a few years later with a comparative analysis of two *C. orthopsilosis* isolates (Riccombeni (2012), Pryszcz (2014)). Surprisingly the newly sequenced MCO456 (ATCC96141) Type 1 (isolated in Texas, USA) strain showed a high degree of heterozygosity, as heterozygous sites affected 17% of the genome compared to only 0.1% in the reference 90-125 Type 2 strain (Tavanti (2005), Pryszcz (2014)). *In silico* data revealed a mixed genome as rDNA cluster came from a close relative of Type 2 strain, the ITS region and MTL locus possibly derived from a Type 1 ancestor, suggesting that it is most likely that MCO456 Type 1 strain is a consequence of mating between two parental lineages (Pryszcz (2014)). The minor (0.3%) divergence found between mitochondrial genomes of the subspecies also supports the mating hypothesis, as mitochondrion inheritance was found to be uniparental in *Candida* species (Ni (2011)). Besides MCO456, another Type 1 isolate, AY2 (isolated in Singapore) was also investigated (Chan (2011)). Interestingly there was over 99.9% identity found between the two Type 1 hybrids. This phenomenon that strains isolated from very distant geographic regions (Texas, USA and Singapore) share almost the same genome, point to the fact that the hybrid might have formed once, obtained evolutionary advantage in virulence and might have spread worldwide. However, this hypothesis had two weak points: 1) the sample number was limited,

and 2) no homozygous Type 1 strain was involved in the analysis (Pryszcz (2014)). This gap was filled in 2016, when a large scale comprehensive analysis of *C. orthopsilosis* isolates was performed involving 27 newly sequenced strains deriving from all over the world and associated with human infection. Among them only one isolate was found to be homozygous (like strain 90-125), whereas all the others were heterozygous. Phylogenetic reconstruction based on the SNP distribution in heterozygous regions clustered the isolates into four distinct groups. This indicates that hybridization in the *C. orthopsilosis* species is not a unique event, but occurred at least four times. A similar approach revealed that all the investigated isolates derived from hybridization events between the same parental lineages. To unify the nomenclature of the parental strains it was suggested to use "Parent A" and "Parent B" instead of "Type 1" and "Type 2". "Parent A" homozygous strain is equal to Type 2 isolate represented by 90-125. "Parent B" is a hypothetical strain that has not yet been identified. "Parent A" and "Parent B" are 5% different in genome sequence, and they possibly went through hybridization and formed "Type 1" hybrids. Upon hybridization the tetraploid state can be resolved by random chromosome loss resulting in a (nearly) diploid genome, which is a known process in *C. albicans* (Bennett and Johnson (2003)). It is likely that similar events could have happened to *C. orthopsilosis* hybrids, but the involvement of sexual processes can not be completely excluded (Schroder (2016)).

***Candida metapsilosis* - the virulent hybrid**

C. metapsilosis is the third member of the species complex and is least frequently associated with clinical disease (Gomez-Lopez (2008), Silva (2009a), Canton (2011), Feng (2012)). Perhaps this was the reason why its genome was a missing link right until 2015, when the comparative analysis of the genome of eleven clinical isolate was published (Pryszcz (2015)). Nearly 6000 protein-coding genes were identified in the 13.3 Mb genome. The results, similarly to *C. orthopsilosis*, revealed hybridization between two parental strains, being approximately 4.5% divergent in sequence. Molecular evidence suggests that all 11 strains involved in the analysis are derivatives of the same hybrid. However, as with *C. orthopsilosis*, it is not clear whether sexual processes were involved in the hybridization event. Although no MTLa genes had previously been found in 18 *C. metapsilosis* isolates by PCR, whole genome sequencing revealed strains carrying at least portions of both mating type loci; therefore, mating can be considered as a possible mechanism for hybrid formation (Sai (2011), Pryszcz (2015)). The fraction of the heterozygous regions varied between 54.5%-61.3% and 63.4%-68.5% across strains depending on the parameters used for *in silico* analysis, meaning that either the hybridization event is very recent or this heterozygous state is stable and loss of heterozygosity occurs slowly. Moreover, 84 deletions and 87 duplications were identified among the samples in question. This is in agreement with the results of Hensgens and colleagues who found *C. metapsilosis* species to be highly heterogeneous using AFLP genotyping (Hensgens (2009)). Since all eleven *C. metapsilosis* isolates derived from clinical sources and they most likely represent descendents of the same hybrid, one might consider that by this process a new species emerged that was capable of adapting to human host and eventually acting as a pathogen under given circumstances (Pryszcz (2015)). This hypothesis would have been supported further if the parental isolates were available, but unfortunately no such strains have yet been found. It is also not known if the parental strains are pathogenic or even still exist in the first place.

Candida parapsilosis sensu lato - Genetic manipulation

The first genetically altered mutant of the complex was created in 2002. It was a galactokinase auxotroph strain of *C. parapsilosis* complemented with the functional allele of the gene cloned into a circular plasmid (Nosek (2002a)). Since then, many homozygous

deletion mutants were generated in *C. parapsilosis sensu stricto* according to the methods developed and improved during the last 15 years (Gacser (2005), Ding and Butler (2007), Gacser (2007), Holland (2014)). Efficient genetic manipulation requires three parameters optimized: 1) a selectable marker, 2) a target locus, 3) a method for transformation.

Selectable markers are also referred as reporter genes, as they "report" if they (and most likely the construct they are the part of) are in the cell or not. The ones used in *C. parapsilosis sensu lato* can be divided into two groups: dominant selectable markers and auxotrophy markers. The huge advantage of dominant selectable markers is that they can be used in prototroph strains (i.e. in practically any isolates). The first attempt of using such a system in *C. parapsilosis sensu stricto* was in 2005. Gacser and colleagues adopted a technique used in *C. albicans* that consisted of a drug called mycophenolic acid that can be neutralized by the enzyme inosine monophosphate dehydrogenase 3. The gene is encoded in *C. albicans*, and must be artificially overexpressed to take effect against the chemical (Kohler (1997), Gacser (2005)). The major problem with dominant markers is that since *C. parapsilosis sensu lato* species are diploid, to delete both alleles of a given gene, two rounds of transformation have to be performed. But once the heterozygote is resistant to a given drug, how can one use the same drug again to create the homozygote? To resolve the problem, a system was developed that used a so-called recyclable selection marker. In *C. albicans,* the auxotrophy marker *URA3* is subsequently replaced with a dominant marker (nourseothricin acetyl transferase). The construct carrying the marker gene is flanked by two recognition sites of a site specific recombinase called flippase that upon activation removes all the sequences between the recognition sites, including the flippase, its promoter and most importantly the marker gene; therefore, the same cassette can be used again to remove the second allele (Kilby (1993), Morschhauser (1999), Staib (1999), Shen (2005)). This technique was adopted by two different groups and used to perform the first targeted gene deletions in *C. parapsilosis sensu stricto* (Ding and Butler (2007)¸ Gacser (2007)). Besides dominant selectable markers, auxotrophy markers are also possible to use. The drawback of this approach is the need of an auxotroph strain. Obviously if one decides to delete the same region in different prototroph isolates, this system is not usable. Pioneering work was done by Nosek and coworkers, who created the first auxotrophy selection system in *C. parapsilosis sensu stricto* utilizing a galactose auxotroph recipient strain and the galactokinase gene of *C. parapsilosis sensu stricto* isolated and cloned into a plasmid (Nosek (2002a)). However to create knock out mutants (without the use of recyclable markers), the recipient strain must be a double auxotroph, which requires more work to create. But once the double auxotroph strain is available, the mutant generation is much more efficient then the recyclable technique in terms of time and effort. This method was elaborated by Noble and Johnson for *C. albicans* and used later by Holland and coworkers in *C. parapsilosis sensu stricto* to create large scale gene deletion libraries in these species (Noble and Johnson (2005), Holland (2014)).

Circular plasmids encoding an autonomously replicative sequence (ARS) can exist in *C. parapsilosis sensu stricto* (without integration into the genome), although they are not mitotically stable, meaning that without selection, they are eliminated rapidly (Nosek (2002a)). Interestingly some circular constructs are stable without the ARS, while others are not. It is possible that the stable ones possess a region that can act as an "ARS-like" element (Nosek (2002a), Gacser (2005)). In contrast to circular replicative elements, linear constructs (linearized plasmids or PCR products, etc.) tend to integrate by recombination into the genome and therefore be mitotically stable, which can be a targeted process or occur randomly. Randomly integrating constructs (or at least some of them) might have a special preference to dedicated regions of the genome, called hotspots (Gacser (2005)). Targeted gene deletion can be achieved by adding homologous sequences of the flanking regions of the targeted locus in question to the ends of the linear constructs used for transformation. This can

be performed by classical molecular techniques (amplification of homolog flankings by PCR, digesting and ligating them around the marker construct) or by PCR-mediated fusion as reported recently (Gacser (2007), Holland (2014)). In general approximately 150 base pairs were found to be efficient, but later 400-500 or even 700 base pair long homologous sequences were used (Ding and Butler (2007), Gacser (2007), Horvath (2012), Holland (2014), Grozer (2015)). Even though it is called targeted, ectopic integration (when the construct does not integrate into the target locus, but some other region) can occur.

Once the construct is ready for transformation a reliable method is needed to allocate the DNA into the cell. To do so three methods were adopted and optimized for transforming *C. parapsilosis sensu lato*: 1) biolistics, 2) chemical transformation, and 3) electroporation. Biolistics (often referred as "gene gun") is a physical method that utilizes heavy metal (wolfram or gold) particles coated with the DNA construct that are accelerated by a rapid helium gas burst. The metal particles (and the DNA) reach a speed so great that they can hit through not only the membrane but also the cell wall of the yeast cells (Klein (1992)). Although there is a publication of using biolistics to transform *C. parapsilosis sensu stricto* with replicative elements, it was not efficient and the procedure required special equipment (Zemanova (2004)). Instead, chemical transformation and electroporation have become the standard everyday protocols to follow. Chemical transformation by the Lithium-acetate/single-strand DNA/polyethylene glycol method was the first approach successfully used to genetically modify *C. parapsilosis sensu stricto*. The advantage of the method is that it does not require a dedicated machine or equipment. This method yielded a transformation efficiency of 10^3 transformants μg^{-1} DNA using a non-integrative construct (Nosek (2002a)). Although it was approximately ten times more efficient than biolistics it was still less than what had been achieved in other *Candida* species using electroporation (Zemanova (2004), Voronovsky (2002)). The principles of electroporation were discussed by Shigekawa and Dower where the electroporator device applies a short direct current pulse that temporary opens pores in the plasma membrane through of which DNA molecules can pass (Shigekawa and Dower (1988)). As the cell wall represents an additional barrier, it must be weakened by (Tris-(hydroxymethyl)-aminomethane Ethylene-diamine-tetraacetate) Tris-EDTA, Lithium-acetate and (Dithiothreitol) DTT treatment. Combining these methods resulted in an increase of two orders of magnitude μg^{-1} DNA in the transformation efficiency compared to chemical transformation, leading to the general use of this approach (Zemanova (2004), Nguyen (2011), Horvath (2012), Grozer (2015)). However, it was subsequently determined that the efficiency of the electroporation and chemical method is highly strain dependent. For example, *C. parapsilosis sensu stricto* SR23 and GA1 isolates are more effectively transformed by using electroporation, whereas CLIB 214 and its derivatives are more amenable to chemical transformation (Zemanova (2004), Horvath (2012), Holland (2014), Perez-Garcia (2016)).

Additionally, there is a study describing the first deletion mutant in *C. orthopsilosis*, which was generated using a recyclable nourseothricin selection marker that was introduced to the cells by electroporation to create *his1* and *leu2* homozygous deletion strains to screen possible mating events (Sai (2011)). In contrast, no *C. metapsilosis* transformant has been reported to date.

Candida parapsilosis sensu lato - Virulence

Virulence is neither a property of a pathogen nor the host, rather it is a term that characterizes the outcome of the interaction between them. Virulence depends on the susceptibility of the host and properties related to the pathogen. These latter ones are called virulence factors, which, when altered, cause attenuated damage in a given system upon interaction (Casadevall and Pirofski (1999)). Cellular organisms developed protective

mechanisms during evolution against obligate or opportunistic pathogens to maintain their own integrity. In higher eukaryotes the immune system is responsible for performing such tasks. It has humoral - (water soluble molecules, for example antibodies, complement proteins, defensins, *et cetera*) and cellular components (phagocytes, B-cells, T-cells, *et cetera*). It also can be divided to an ancient innate (monocytes, neutrophils, etc.) component and an evolutionary more recent adaptive branch (represented by B-cells and T-cells) (Batista and Harwood (2009), Sarma and Ward (2011), Ganz (2003), Dale (2008), Crotty (2015)).

Candida cells are often associated with the skin and medical devices. In terms of pathogenesis, yeast cells have to overcome immune system defenses, most usually epithelial cells and phagocytes, to cause disease; therefore studies examining *C. parapsilosis sensu lato* virulence primarily focus on properties related to such interactions. Although overall tendency in virulence ranges from *C. parapsilosis sensu stricto* to *C. orthopsilosis* and *C. metapsilosis* in a decreasing order, the virulence attributes within species is a strain dependent characteristic (Sabino (2011), Nemeth (2013)). Historically, the first attempt to gain insight into *C. parapsilosis sensu stricto* - host interaction *in vivo* was in 1962 by Andriole and Hasenclever, who investigated the survival of alloxan treated mice inoculated with different *Candida* species. They established that *C. parapsilosis sensu stricto* caused 40% mortality after one day of intravenous infection irrespectively of the alloxan treatment, and suggested the high dosage (10^8 yeast cells) as the most possible factor (Andriole and Hasenclever (1962)). This was proven a few years later by Goldstein and colleagues, who intravenously infected mice with lower inocula and monitored the animals for 21 days. They found that *C. parapsilosis sensu stricto* was cleared from all the organs examined (lung-heart, liver-spleen, kidneys, brain). However, clearance was significantly delayed when mice were treated with steroid (cortisone) (Goldstein (1965)).

Morphology

C. parapsilosis sensu lato species typically grows as yeast-like cells, but *C. parapsilosis sensu stricto* and *C. orthopsilosis* strains are also able to form pseudohyphae (Nemeth (2013)). The prefix "pseudo" is used because these filament-like structures are elongated, similar to true hyphae of *C. albicans*, but the two formations are fundamentally different. Pseudohyphae consist of mostly ellipsoid shaped cells that are attached to each other at the site of septation, while true hyphae are tube like structures with parallel sides without obvious constrictions between the cells and the first septum is located in the tube-like structure distant from the mother cell (reviewed in Berman and Sudbery (2002)). Filamentation can be induced by growing *C. parapsilosis sensu lato* species in the presence of 5% (V/V) CO_2 or in fetal bovine serum (FBS) containing media (Sabino (2011), Nemeth (2013)). Different cell morphologies can affect colony morphology and biofilm forming abilities as well (discussed subsequently) (Laffey and Butler (2005)). Although the pseudohypha forming capabilities of the *C. parapsilosis sensu lato* species is poorly studied, it appears that in contrast to *C. parapsilosis sensu stricto* and *C. orthopsilosis*, *C. metapsilosis* is unable to form pseudohyphae. However, among *C. parapsilosis sensu stricto* and *C. orthopsilosis* isolates both pseudohypha producers and non-producers have been identified (Sabino (2011), Nemeth (2013), Gago (2014)). Interestingly significantly larger proportion of *C. parapsilosis sensu stricto* strains cultivated from blood cultures formed filaments than environmental isolates (Sabino (2011)). Pseudohypha formation might be important in host - pathogen interactions since pseudohypha-positive *C. orthopsilosis* isolates are less efficiently killed by J774.2 murine macrophages compared to pseudohypha negative cells. Additionally, this observation is also the case when the producer and non-producer strains of the species group are compared (Nemeth (2013)).

Adherence and biofilm formation

C. parapsilosis sensu stricto has a strong predilection to adhere to medical devices, including implants, and the yeast has been frequently isolated from hands of healthcare workers, particularly in outbreak investigations (Ramage (2006), Bonassoli (2005)). *C. parapsilosis sensu stricto* tends to form lower amounts and less compact biofilms than *C. albicans*. *C. albicans* biofilm on silicon elastomer is shallow and contains yeast-like, pseudohyphal – and hyphal elements embedded in a rich extracellular matrix (Andes (2004), Ramage (2004)). In contrast, *C. parapsilosis sensu stricto* biofilms are thin and consists of yeast-like cells with a basal yeast layer but without significant extracellular matrix (Kuhn (2002)). Interestingly biofilm forming capability of *C. parapsilosis sensu stricto* cells depends on their morphology. Different morphotypes correlate with distinct adhesion properties and varied colony morphologies (smooth, rough, snowball, crepe, crater and concentric) between which fungal cells can undergo a morphological "switch", a process known as phenotypic switching. Morphology is not conserved among strains, but it can be changed and/or facilitated by adding specific chemicals (Lott (1993), Enger (2001), Laffey and Butler (2005)). In addition to morphology, adherence and biofilm formation among *Candida* species strongly depends on the medium, pH and oxygen tension (Hawser and Douglas (1994), Ramage (2006), Silva (2009b)). Although biofilms are formed by the different *C. parapsilosis sensu lato* species, there is significant variability in their biofilm forming capabilities. *C. orthopsilosis* and *C. parapsilosis sensu stricto* biofilms on silicon elastomer material are similar in terms of dry weight in mature biofilms (48h), but in contrast to *C. orthopsilosis*, *C parapsilosis sensu stricto* tends to have a solid lag phase, while *C. orthopsilosis* biofilms form rapidly. In contrast, *C. metapsilosis* forms less biofilm (Lattif (2010)). Similarly, *C. parapsilosis sensu stricto* and *C. orthopsilosis* are more adherent and form more biofilm *in vitro* (with human buccal epithelial) and *in vivo* (in experimental mouse vaginal candidiasis model) compared to *C. metapsilosis* (Bertini (2013)). However, the overall adherent properties of *C. parapsilosis sensu stricto* are weak or moderate compared that to those of *C. albicans* in vascular endothelium and human epithelial models (Klotz (1983), Bendel (1995)). Notably, the factors involved in *C. parapsilosis sensu stricto* adherence are poorly understood. Agglutinin-like sequences (Als) are glycosylphosphatidylinositol (GPI) anchored cell surface proteins responsible for adhesion, and they compose a large and well characterized family in *C. albicans*, and Als3 is one of the best characterized adhesin (Hoyer (2001), Phan (2007), Hoyer (2008), Hoyer and Cota (2016)). A recent study reported the involvement of the *C. albicans ALS3* ortholog gene CPAR2_404800 in *C. parapsilosis sensu stricto* adhesion. The homozygous mutant lacking CPAR2_404800 was attenuated in its ability to adhere to buccal epithelial cells *in vitro* and to bladder epithelium in a murine urinary tract infection model compared to wild-type (Bertini (2016)). Additionally, the regulation of biofilm formation in *C. parapsilosis sensu stricto* is still unclear. However, the ortholog of *C. albicans BCR1* gene (a regulator of biofilm formation) in *C. parapsilosis sensu stricto* (*CpBCR1*) encodes a transcription factor that is required for adhesion and biofilm formation on silicone surface. In *C. albicans* Bcr1 is a positive regulator of *HWP1* that encodes a GPI-anchored protein responsible for adhesion to buccal epithelial cells (Staab (1999), Nobile and Mitchell (2005), Nobile (2006b), Mayer (2013)). Although *C. parapsilosis sensu stricto* lacks the ortholog of *HWP1*, it encodes one of its close relatives, *CpRBT1*, and this gene has been found to be under the regulation of CpBcr1 (Braun (2000), Ding and Butler (2007)). Interestingly, while in *C. albicans* Bcr1 promotes the expression of *ALS1* and *ALS3* in addition to *HWP1*, CpBcr1 appears to promote *CpRBT1*, but not the *CpALS* genes (Nobile (2006a), Ding and Butler (2007)). This suggests that the induction of *ALS3* genes in the two pathogens occurs through different regulatory pathways.

Secreted enzymes

Secreted enzymes play vital roles in nutrition acquisition in nature and during interaction with the host. Indeed, the host environment is limited in accessible carbon and nitrogen sources. To digest such macromolecules, *Candida* species secrete proteinases, lipases and phospholipases. The production of phospholipase in *C. parapsilosis sensu stricto* is strain dependent and their involvement in virulence remains in question (Mohan das and Ballal (2008), Pakshir (2013), Ramos Lde (2015), Shirkhani (2016)). However, proteinases and lipases are well characterized and linked to virulence. Genes of these proteins expanded and compose gene families in certain pathogenic species that have resulted in the divergence of the enzymes in terms of substrate specificity, inducibility and expression pattern (Monod (1994), Hube (2000), Naglik (2004), Schaller (2005)).

Genomic analysis of the *C. parapsilosis sensu lato* species complex revealed a remarkable expansion of secreted aspartyl proteinase (in *parapsilosis*) (*SAPP*) genes. While in *C. orthopsilosis* 11 such ORF were identified, the genome of *C. metapsilosis* and *C. parapsilosis sensu stricto* encode 14 potential *SAPP*s (Pryszcz (2015)). The ability of *C. parapsilosis sensu stricto* to secrete proteinases and their association with virulence was already established in the 1980s (Macdonald (1984), Ruchel (1984), Ruchel (1986)). By the 1990s, secreted proteinases were shown to degrade secretory Immunoglobulin A, and isolates from patients with *C. parapsilosis sensu stricto* vulvovaginits were found to secrete significantly higher amount of proteinases than isolates from asymptomatic carriers (Douglas (1988), Agatensi (1991)). Although proteinase secretion is a general property of *C. parapsilosis sensu stricto*, examinations of *C. metapsilosis* and *C. orthopsilosis* have revealed both producers and non-producers (Sabino (2011), Nemeth (2013), Gago (2014)). In *C. parapsilosis sensu stricto SAPP1* and *SAPP2* have been investigated in detail. The proteins encoded by these genes differ in their substrate specificity, enzymatic activity and expression patterns (Merkerova (2006), Hruskova-Heidingsfeldova (2009)). *SAPP1* is duplicated, the paralogs are distinguished as *SAPP1a* and *SAPP1b*, and they are similar in nucleotide sequence (Horvath (2012)). The mature protein localizes to the cell wall, and it is expressed only when exogenous protein is present as the sole nitrogen source in the media (Vinterova (2011), Hruskova-Heidingsfeldova (2009)). The disruption of the alleles of *SAPP1a* and *SAPP1b* (*Δ/Δsapp1*) in *C. parapsilosis sensu stricto* increased the resulting mutant's susceptibility to human serum and reduced its resistant to primary human phagocytes (peripheral blood mononuclear cells (PBMCs) and PBMC-derived macrophages (PBMC-DM)) in terms of phagocytosis and killing compared to wild-type yeasts (Horvath (2012)). The expression of *SAPP2* appears to be constitutive, as it has been detected in every media examined, but the expression is significantly increased in the *Δ/Δsapp1* mutant compared to wild-type (Hruskova-Heidingsfeldova (2009), Horvath (2012)).

Based on computational predictions, lipases represent another major secreted family of pathogenic *Candida* species. In *C. albicans* there are 10 lipase genes identified to date, and some of them have been associated with virulence (Hube (2000), Stehr (2004), Schofield (2005)). *C. parapsilosis sensu stricto* have been biochemically studied since the 1990s, and more recent studies have examined their potential utilization for biotechnological purposes (Briand (1995), Vaysse (1997), Neugnot (2002), Rodrigues (2016), Kannoju (2017)). In the *C. parapsilosis sensu lato* group, the predicted numbers of lipase genes are 4 (*C. parapsilosis sensu stricto* and *C. orthopsilosis*) and 5 (in *C. metapsilosis*) (Pryszcz (2015)). The higher number of lipase genes in *C. metapsilosis* is in contrast to laboratory observations, as no lipase producer *C. metapsilosis* strain has yet been identified, suggesting that the predicted genes are not functional (Nemeth (2013)). However, it is possible that the induction media were not specifically optimized for *C. metapsilosis*. *C. parapsilosis sensu stricto* and *C. orthopsilosis* have both lipase positive and negative isolates. Notably, *C. parapsilosis sensu*

stricto and *C. orthopsilosis* lipase producer strains are able to cause significantly more damage to J774.2 macrophages *in vitro* than non-producer ones (Nemeth (2013)). Targeted removal of *Candida parapsilosis sensu stricto* lipase genes (*CpLIP*) *CpLIP1* and *CpLIP2* demonstrated their role in nutrient acquisition and virulence. Although both genes were removed, reintroduction of only one allele of wild-type *CpLIP2* totally complemented both the examined phenotypes, suggesting that *CpLIP1* (and other predicted lipase genes) might not be actively transcribed or proteolytically active under specific circumstances (Neugnot (2002)). In the *Δ/Δcplip1-cplip2* mutant the overall lipase production was abolished (suggesting that other predicted lipases might not be expressed) and an apparent growth deficiency in mediums containing fatty acids as a sole carbon source was observed. Moreover *Cplip2* is also involved in biofilm formation on polyethylene, silicon and polystyrene surfaces and, more importantly, in virulence both *in vitro* and *in vivo*. This null-mutant was less resistant to phagocytosis and killing by murine macrophages, mature and immature human PBMC derived dendritic cells and PBMC-DM *in vitro,* and in a peritoneal mouse and a rat neonate model (Gacser (2007), Nagy (2011), Toth (2014a), Trofa (2011)). Moreover, the phagocytes responded with higher chemokine and inflammatory cytokine expression on RNA and protein levels in *in vitro* interactions (Nagy (2011), Toth (2014a)). The possible reason of the more efficient uptake was investigated by Toth and colleagues, who found an increase in macrophage (J774.2) migration towards lipase null-mutant fungal cells compared that to the wild-type yeasts (Toth (2015)). In another *in vitro* system utilizing PBMC-DMs, the *Δ/Δcplip1-cplip2* strain avoided phagosome - lysosome fusion (and consequently macrophage killing) less effectively than wild-type cells, and induced a significantly higher expression and secretion of TNFα, IL-1β, and IL-6 inflammatory cytokines (Toth (2014a)). These findings indicate that secreted lipases might be the tools *C. parapsilosis sensu stricto* utilizes to attenuate host inflammatory responses.

Host interaction

Recognition of pathogens upon interaction occurs between so-called pathogen associated molecular patterns (PAMP) and the receptors of the host cells called pattern recognition receptors (PRR). PAMPs are evolutionary conserved molecules that are indispensable components of a given pathogen (i.e. double stranded RNA of certain viruses, lipopolysaccharide of bacteria, β-glucan components of the fungal cell wall, etc.). PRRs are germ-line encoded receptors that can bind one or a few types of PAMPs, which triggers a cell response leading to phagocytosis and/or secretion of cytokines and other soluble factors. Such soluble factors are, for example, small molecular weight defensins, which are secreted primarily by neutrophils (α-defensins) and epithelial cells (β-defensins). Defensins are positively charged, cysteine rich proteins, often acting as pore-forming agents (reviewed in Ganz (2003) and Lehrer and Lu (2012)). *C. parapsilosis sensu stricto* induces significant release of α-defensins from primary human neutrophils and interactions with a human intestinal cell line, Caco-2, leads to an increased expression and secretion of human β-defensin 2 (HBD-2), although the difference compared to the non-infected Caco-2 controls was not significant (Gacser (2014)).

In addition to the secretion of biologically active molecules, phagocytosis of pathogens and intracellular killing are effective mechanisms for eradicating invading microbes. After engulfment, a pathogen can be eliminated through various mechanisms including reactive chemical compounds, enzymatic digestion, ion chelators (Babior (2002), Aratani (1999), Vazquez-Torres (1996), Samaranayake (2001), Nikawa (1993)). The first step of host-pathogen interaction is the recognition of the microbe by the host effector cell. Successful pathogens have evolved numerous, specific paths to avoid the interaction between their PAMPs and the PRRs of the host. Although the involvement of *C. albicans* cellular

components on host interactions has been intensively studied, our knowledge on such components of *C. parapsilosis sensu lato* remains limited (Ruiz-Herrera (2006), Chaffin (2008), McKenzie (2010)). *C. parapsilosis sensu stricto* cells are ingested and efficiently killed by J774.2 murine macrophages *in vitro*, and, during the interaction, the phagocytes respond with the transcription of inflammatory cytokines (IL-1β and TNFα) (Nemeth (2014)). In terms of the components the cell walls of the *C. parapsilosis sensu lato* species, they appear to molecularly be similar to the ones present in *C. albicans*, but their proportions and, consequently, the overall structures are diverse. Interestingly the cell wall porosity levels of the species complex is approximately twice as much as that of *C. albicans*, which is due to the shorter mannan chains attached to cell wall proteins in the *C. parpasilosis sensu lato* species (Shibata (1995), Estrada-Mata (2015)). In line with this, the *C. parpasilosis sensu lato* species possess more glucan underneath a mannan layer, possibly as a result of a known compensatory mechanism to maintain cell wall strength (Estrada-Mata (2015), Walker (2013)). The PRRs involved in the recognition and subsequent cytokine secretion playing role in *C. parapsilosis sensu lato* - phagocytes interaction are the C-type lectins dectin-1 and mannose receptor (MR), which recognize β-1,3 glucan and N-linked mannans respectively, and toll-like receptor 4 (TLR4), binds O-linked mannans (Brown and Gordon (2001), Medzhitov (1997), Tada (2002), Garner (1994), Yamamoto (1997), Netea (2006)). All of these receptors are involved in TNFα secretion, while IL-1β production is MR and dectin-1 dependent (Estrada-Mata (2015), Toth (2013)). The lack of N-linked mannans in the outer layer leads to increased secretion of IL-1β, TNFα, IL-10 (anti inflammatory cytokine) and IL-6 from human PBMCs, and this compound is not necessary to efficient phagocytosis. However, the involvement of dectin-1 in IL-1β secretion might be a strain specific property since some reports describe TLR4 and Dectin-1 dependent IL-1β production (Toth (2013), Estrada-Mata (2015), Netea (2010), Perez-Garcia (2016), Toth (2013)). Indeed the involvement of receptors in cytokine secretion induced by different isolates of the same species is a known phenomenon in *C. albicans* - host interactions (Marakalala (2013), Netea (2010)). Notably, *C. orthopsilosis* differed from the other two members of the group in terms of decreased TLR4 dependent IL-6 and MR dependent IL-10 secretion, which is consistent with the observation of the highly exposed glucan and chitin content of *C. orthopsilosis* compared to *C. parapsilosis sensu stricto* and *C. metapsilosis* (Estrada-Mata (2015)).

C. parapsilosis sensu stricto and *C. albicans* induce the differentiation of distinct T-helper subpopulations. In contrast to PBMCs secreting increased amounts of Th1 and Th17 cytokines (IFNγ, IL-1β and IL-17, IL-22 respectively) upon challenge with *C. albicans*, interactions with *C. parapsilosis sensu stricto* induces the release of IL10, a typical Th2 cytokine. Although the investigation of the T-helper response to *C. parapsilosis sensu stricto* is a very new field, recent work demonstrates that *C. parapsilosis sensu stricto* is able to switch the Th1/Th17 - Th2 bias towards the latter with a yet unknown mechanism to avoid the antifungal activity induced by Th1/Th17 cytokines (Toth (2013)). Moreover, Galectin-3, another lectin type receptor that can be both anchored onto the cell surface and secreted, is involved with *C. parapsilosis sensu stricto* recognition in a systemic mouse infection model, as the fungal burden and the damage in the kidney of Galectin-3 knock-out mutants were higher compared to the ones of the wild-type mice. Interestingly, this study also found that the level of Galectin-3 in the sera of human neonates was significantly lower than in the samples of adults, which could be a possible explanation for why newborns are more susceptible to *C. parapsilosis sensu stricto* infection than adults (Linden (2013)).

Successful pathogens utilize mechanisms by which they can avoid elimination by host mechanisms. It has already been established that *C. parapsilosis sensu stricto* is capable of replicating, forming pseudohyphae, aborting host cell mitosis and inducing exocytosis, therefore escaping from phagocytes and endothelial cells (Toth (2014b), Glass (2015)).

Additionally, endothelial cells might act as fungal reservoirs during infection. *C. parapsiolsis sensu stricto* can be uptaken by these cells, and once internalized they can be hidden away from patrolling neutrophil granulocytes (Glass (2015)). As shown above, *C. parapsilosis sensu stricto* secreted lipases are able to attenuate PBMC derived dendritic cell and PBMC-DM cytokine secretion, phagosome - lysosome fusion and affect phagocyte migration (Nagy (2011), Toth (2014a), Toth (2014b)). Moreover, lipases might further modulate the immune response, such as through altering the prostaglandin profile of the host (Toth (2014a)). Phospholipases can cleave membrane bound lipids resulting in the release of arachinodic acid, which can be processed further by the host to synthesize prostaglandins that can have either pro- or anti-inflammatory effects (Funk (2001), Harris (2002)). *C. parapsilosis sensu stricto* can produce prostaglandin E2 (PGE2), which is known to mediate T-cell response by promoting a Th2-type differentiation that, as mentioned earlier, is a poorly effective response to *Candida* (Grozer (2015), Harris (2002)). In fact, *C. parapsilosis sensu stricto* induces human PBMC-DMs to produce more IL-10 and less IL-1β, IFN$_{\gamma}$, IL-17 and IL-22 compared to PBMC-DMs challenged with *C. albicans* (Toth (2013)). In contrast to *C. albicans*, removal of the gene *OLE2* (putative Δ9 fatty acid desaturase) from the genome of *C. parapsilosis sensu stricto* did not decrease PGE2 production. However, the *de novo* fatty acid biosynthesis changed, as demonstrated by an increase in palmitoleic acid and oleic acid, and the mutant was less resistant to human PBMC-DMs. Moreover it induced higher IL-10 (Th2 cytokine) secretion than the wild-type (Grozer (2015)). Changes in the fatty acid synthesis can also lead to an impaired capacity to form biofilms on polysterene and silicone surfaces as well as a decreased resistance against macrophage killing (Nguyen (2009)). The involvement of fungal fatty acid synthesis in virulence is also elucidated by the fact that *OLE1* (a fatty acid desaturase) is responsible for maintaining cell membrane integrity and invasive growth of *C. parapsilosis sensu stricto*, as an *OLE1* mutant was less resistant to human serum and phagocytosis *in vitro* and showed attenuated virulence in intravenous mouse infection model (Nguyen (2011)). The interactions of *C. metapsilosis* and *C. orthopsilosis* with host cells are poorly studied. However, *C. metapsilosis* is significantly less resistant to host effector cells compared to *C. parapsilosis sensu stricto* and *C. orthopsilosis* (Orsi (2010), Sabino (2011), Nemeth (2013)), which is in line with the limited clinical cases of disease due to *C. metapsilosis*. Interestingly, BV-2 mouse microglial cells challenged with *C. metapsilosis* effectively formed phagosomes containing the yeast cells, whereas *C. parapsilosis sensu stricto* partially inhibited this process (Orsi (2010)). Studies in a Greater wax moth (*Galleria mellonella*) larvae survival model further demonstrated that *C. metapsilosis* is significantly less virulent compared to the other members of the *sensu lato* species and *C. metapsilosis* was also more effectively phagocytosed by *Galleria mellonella* hemocytes compared to the other two members of the complex (Nemeth (2013), Gago (2014)).

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