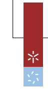

Joana Isabel Carvalho Pereira **Development of biomarkers for the identification of pathogenic *Candida* species**

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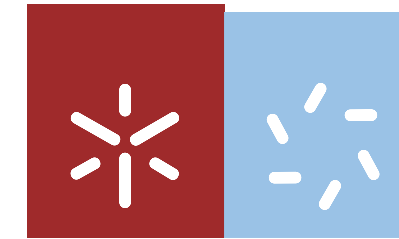

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Escola de Ciências

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Development of biomarkers for the identification of pathogenic *Candida* species

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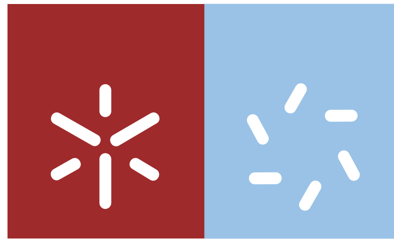

Universidade do Minho
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Development of biomarkers for the identification of pathogenic *Candida* species

Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho realizado sob a orientação da
Doutora Ana Paula Fernandes Monteiro Sampaio Carvalho
e da
Doutora Célia Sacramento Santos Pais

Outubro de 2012

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É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA TESE, APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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Development of biomarkers for the identification of pathogenic *Candida* species

Abstract

In the last decades the incidence of fungal infections has increased exponentially, due to the development of more aggressive therapeutic techniques, which increase the number of immunocompromised and in risk individuals. The *Candida* species are the most common etiological agents isolated from opportunistic fungal infections in these patients and *Candida albicans* appears to be the most common species isolated. However, infections caused by non-*albicans* species, such as *C. parapsilosis, C. glabrata, C. tropicalis* and *C. krusei*, are increasing alarmingly. It is thought that the susceptibility to antifungal drugs varies according to species, thus, the rapid and correct identification of infecting species are crucial. Microsatellite sequences have been largely used as molecular targets to differentiate and characterize strains. However, no studies have been performed using microsatellite DNA for *Candida* species identification. Therefore, the main objectives of this work were the evaluation of the potential of microsatellite markers for species differentiation and for identification of specific *C. albicans* lineages.

After an intensive search for described microsatellite markers for the main *Candida* species, several markers were selected for *C. albicans*, *C, glabrata*, *C. parapsilosis, C. tropicalis* and *C. krusei*. These markers were combined into a multiplex strategy and this new developed system tested in 81 strains from 10 different species. All tested loci amplified correctly in single and multiplex conditions, except for the *C. tropicalis* selected locus that was unable to amplify in multiplex. After removal of the *C. tropicalis* primers, this system showed 100% specificity and 100% sensibility.

To test if the microsatellites were able to identify specific *C. albicans* lineages two microsatellite markers were used, CAI and CAVIII. These markers are located in two repeat-containing ORFs, CAI is located in the terminal-3' of RLM1 gene and CAVIII in the terminal-3' of SAP8 gene. CAI microsatellite has been previously described and CAVIII was described for the first time in this study. CAVIII demonstrated to be highly specific for *C. albicans* strains and presented a discriminatory power of 0.72. The two microsatellite markers were tested in 144 unrelated *C. albicans* strains isolated from different body locations, allowing the statistical differentiation of strains from oral cavity, vulvovaginal infections and from extra-mucosal (respiratory tract and

urine) infections. The number of extra-mucosal strains was increased to 224 and statistical analysis based in CAI genotypes, demonstrated significant differences between genotypes of strains isolated from superficial (oral and vagina) and invasive infections (respiratory tract, urine and blood).

The results obtained allowed to conclude that the microsatellite loci analysis can be used to differentiate the most common *Candida* species, being an alternative in clinical diagnosis. Moreover, it was also possible observe that analysis of repeat containing ORFs, such as RLM1 and SAP8 is able to differentiate lineages of *C. albicans.*

Desenvolvimento de biomarcadores para a identificação de espécies patogénicas de *Candida*

Resumo

Nas ultimas décadas a incidência das infecções provocadas por fungos têm aumentado exponencialmente. A principal razão proposta para esta mudança incide no desenvolvimento de métodos terapêuticos mais agressivos, responsáveis pelo aumento do número de indivíduos imunocomprometidos. As espécies de *Candida* são os agentes etiológicos mais frequentemente isolados de amostras de pacientes com infeções fúngicas oportunistas, sendo *Candida albicans* a espécie mais comum. Contudo, as infecções provocadas por outras espécies, nomeadamente *C. parapsilosis, C. glabrata, C. tropicalis* e *C. krusei*, têm aumentado em grande escala. Sabe-se que a susceptibilidade ás terapêuticas antifúngicas varia de acordo com a espécie causadora da infecção, assim, a correta identificação destes organismos é essencial. As sequências de DNA microssatélite têm sido frequentemente utilizadas como alvos para a diferenciação de estirpes. Contudo, não têm sido realizados estudos que utilizem o DNA microssatélite na identificação das espécies patogénicas de *Candida*. Desta forma, o principal objectivo deste trabalho consistiu na avaliação do potencial dos marcadores de microssatélites na diferenciação de espécies, assim como na diferenciação de linhagens de *C. albicans*.

Após uma intensa pesquisa por marcadores de microssatélites previamente descritos, cinco marcadores foram selecionados para a identificação de *C. albicans*, *C, glabrata*, *C. parapsilosis, C. tropicalis* e *C. krusei*. Estes marcadores foram combinados numa reação de PCR-multiplex e o sistema desenvolvido foi testado em 81 estirpes de 10 espécies diferentes. Todos os marcadores apresentaram uma amplificação específica em reação singleplex e multiplex, porém, o marcador selecionado para a identificação de *C. tropicalis* não foi capaz de amplificar em reação de multiplex. Após remover o marcador para a *C. tropicalis* do sistema de identificação foi obtida uma especificidade e sensibilidade de 100%.

Com o objectivo de verificar a utilidade da análise do DNA microssatélite na diferenciação de linhagens de *C. albicans* foram utilizados dois marcadores, CAI e CAVIII, localizados no terminal 3' das regiões codificantes dos genes RLM1 e SAP8, respectivamente. O primeiro, CAI, já tinha sido previamente descrito apresentando elevada estabilidade e especificidade,

enquanto que o CAVIII foi descrito pela primeira vez neste trabalho. O microssatélite CAVIII demonstrou ter elevada especificidade para as estirpes de *C. albicans* e apresentou um poder discriminatório de 0.72. Ambos os microssatélites foram testados utilizando 144 estirpes de *C. albicans* isoladas a partir de diferentes locais, e a análise combinada dos genótipos obtidos com os dois microssatélites permitiram diferenciar as estirpes provenientes da cavidade oral, de infecções vulvovaginais e de infeções invasivas. Porém, o número de estirpes provenientes de infecções invasivas foi aumentado numa fase posterior do estudo, e a análise estatística foi realizada novamente utilizando apenas os genótipos obtidos com o marcador CAI. Esta análise demonstrou diferenças significativas entre as estirpes provenientes das infecções superficiais e estirpes provenientes das infecções invasivas, demonstrando a sua utilidade na diferenciação das linhagens de *C. albicans*.

Os resultados obtidos permitiram concluir que a análise de DNA microssatélite pode ser útil para diferenciar as espécies de *Candida* mais comuns, sendo uma excelente alternativa para o diagnóstico clínico. Para além disso, é também possível observar que a análise combinada com os marcadores CAI e CAVIII permite a diferenciação de linhagens de *C. albicans.*

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List of Abbreviations

5-FC	5-fluorocytosine
Ab	Antibody
Ag	Antigen
Als	Agglutinin-like sequence
ATCC	American type culture collection
BC	Blood culture
BSA	Bovine Serum Albumin
CBS	Centraalbureau voor Schimmelcultures
CD4	Cluster of differentiation 4
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DP	Discriminatory power
DTT	Dithiothreitol
ECMM	European Confederation of Medical Mycology
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Epa	Epithelial adhesins
FAM	6-carboxyfluorescein
HCl	Hydrochloric acid
HEX	Hexachlorofluorescein
HIV	Human Immunodeficiency Virus
ITS	Internal transcribed spacer
KCl	Potassium chloride
LA	Latex agglutination
Lip	Lipases
$MgCl_2$	Magnesium chloride
NEMIS	National Epidemiology of Mycosis Survey
OPC	Oropharyngeal candidiasis
ORF	Open reading frame

PCR	Polymerase chain reaction
Pga30	Glycophosphatidylinositol-anchored protein 30
Pl	Phospholipase
PYCC	Portuguese yeast culture collection
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPLA	Reverse passive latex agglutination
RT	Respiratory tract
RVCC	Recurrent vulvovaginal candidiasis
Sap	Secreted aspartyl proteinase
SSR	Simple sequence repeats
STR	Short tandem repeats
Ta	Annealing temperature
TAMRA	6-carboxytetramethylrhodamine
TET	Ortetrachloro-6-carboxyfluorescein
UPGMA	Unweighted pair group method with arithmetic mean
URT	Upper respiratory tract
USA	United States of America
VE	Vaginal exudate
VVC	Vulvovaginal candidiasis

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CHAPTER I

General Introduction

1. Candida and candidiasis

In the last two decades fungal infections have caused many difficulties in clinical practice. The main concerns about this problem are their prevalence, in which an alarming increase in number of cases, as well as the variety and complexity of the etiological agents involved (Guarro *et al.* 1999). Several reasons have been proposed to explain the increased incidence, including the increase lifespan in the populations of the developed world, the age related loss of immune-competence, as well as the use of more aggressive therapeutic methods, such as chemotherapeutic agents, bone marrow or solid-organ transplants, immunomodulatory agents, broad-spectrum antibiotics and more aggressive surgeries (Peres-Bota *et al.* 2004; Benjamin *et al.* 2010).

The most common etiological agents involved in fungal infections are *Candida* spp., *Aspergillus* spp. and *Cryptococcus* spp., although, other agents, such as *Malasezzia* spp., *Fusarium* spp. or *Trichosporon* spp. may also be involved (Fridkin and Jarvis 1996). Several pathogenic species have emerged in the last years but the ubiquitous *Candida* species remain the most common cause of serious fungal infections (Fridkin and Jarvis 1996; Tortorano *et al.* 2004). More than 200 species of *Candida* spp. have been isolated but only nearly 20 species have been identified as being associated with human infections such as, *C. albicans, C. parapsilosis, C. glabrata, C. krusei, C. dubliniensis, C. tropicalis, C. guilliermondii, C. metapsilosis, C. bracarensis, C. kefyr* among others (Guarro *et al.* 1999). Many of these pathogenic species are present in the commensal flora of genitourinary system, gastrointestinal tract, skin or oral cavity of healthy individuals (30-60% of humans), and only in disorders of the normal flora balance or when the immune defences are compromised, they may cause opportunistic infections, denominated candidiasis (Sanchez-Martinez and Perez-Martin 2001).

1.1. Clinical manifestations of candidiasis

Usually candidiasis is an endogenous infection, caused by prior colonization of mouth, gastrointestinal tract, vagina or skin. In these cases an unusual growth of normal flora occurs and the immune system is unable to react to this condition. However, the source of candidiasis may also be exogenous, and many species of *Candida* spp. have been isolated from hospital

environmental such as the floor, countertops, doorknobs, food and other inanimate surfaces (Perlroth *et al.* 2007).

Candida spp. is capable of causing a range of infections, from less-severe superficial lesions in mucosa and skin (including nails and hair) to life-threatening disseminated mycosis, characterized by the spread of fungi through the tissues and blood circulation (Fridkin and Jarvis 1996).

Superficial candidiasis affects mucosal epithelial tissues and is frequent in individuals with prior colonization when host physical barriers or immune system integrity are compromised. In the majority of these cases, the patient is symptom free and unaware of a problem, however, it can also cause a burning sensation, discomfort or pain (Jayatilake 2011). The most common clinical manifestations of superficial candidiasis are oropharyngeal candidiasis (OPC) and vulvovaginal candidiasis (VVC) although, infections in the urinary tract and skin are also observed (Jayatilake *et al.* 2009; Sobel *et al.* 2011).

Candida species are frequently associated with normal oral carriage in humans, occurring in the mouth of up to 80% of healthy individuals, but changes in the oral cavity environment can enhance the *Candida* infection. Oropharyngeal candidiasis (OPC) is an acute condition often affecting new-born babies due to the immature immune system and individuals infected with HIV (Samaranayake and Holmstrup 1989; Blignaut 2007). VVC is the most common vaginal infection and more than 75 % of women will have had at least one episode during their lives. It is known that about 40-50% of these women experience a recurrence, and up to 5% suffer more than four episodes during 1 year (recurrent vulvovaginal candidiasis – RVVC) (Buitron Garcia-Figueroa *et al.* 2009; Kalkanci *et al.* 2012). The presence of *Candida* species in urine is a common clinical finding, particularly in hospitalized patients, and several studies indicate that at least 10%–15% of hospital acquired urinary tract infections are caused by *Candida* species (Sobel *et al.* 2011).

Invasive candidiasis is verified only in severe cases of patient debilitation or immune compromisation and can involve the infection and spread of *Candida* cells via the bloodstream (candidaemia) to multiple organs, such as the brain, kidneys, heart, lungs and liver (Jayatilake 2011). This condition is more significant because of its associated high mortality rate (46-75%) and high morbidity in patients who survive the infection.

The route of bloodstream infection can occur through the “natural” way where yeast cells penetrate epithelial cells, the iatrogenic way through the use of medical devices (central venous catheters, peritoneal dialysis and cardiovascular devices) in which the formation of biofilms on its surface is important, or through the damage of defence barriers (polytrauma, surgery, drug treatment) (Figure 1.1) (Mavor *et al.* 2005).

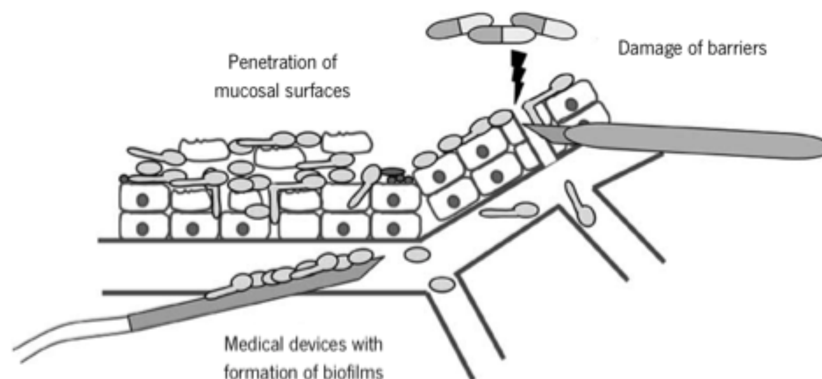

Figure 1.1. Routes of entry into the bloodstream by *Candida.* Adapted from Mavor *et al.* 2005.

Numerous studies have identified common risk factors for patients developing candidiasis, and most of these causes are extremely common in hospitalized patients, increasing the possibility of development of nosocomial infections. These reasons include immunosuppression (chemotherapy, malnutrition, malignancy and neutropenia), prior colonization, disruption of normal skin barriers (intravascular catheters, extensive burns, invasive surgery, parenteral nutrition) and broad-spectrum antibiotics, since they disrupt the competition of bacterial flora (Peres-Bota *et al.* 2004; Benjamin *et al.* 2010). However, not all the predisposing factors equally favour superficial and invasive candidiasis, since immune protection of human host is site-specific. T- cell immune responses are important in protection against superficial candidiasis but resistance against systemic disease is more often associated with a functional phagocytic response (Calderone and Fonzi 2001). For example, HIV infected individuals suffer frequently from oral infections and onychomycosis due to a reduction in CD4+ cells counts, but rarely developed systemic infections (Mavor *et al.* 2005). Moreover, this feature is also supported by the fact that neutropenic patients are particularly susceptible to systemic infections (Koh *et al.* 2008).

1.2. Epidemiology of candidiasis

Fungal infections are an increasingly encountered threat among critically ill patients and are a significant cause of morbidity and mortality. Moreover, *Candida* species are the most common etiological agents of fungal infections, causing superficial or invasive candidiasis (Table I.I) (Pfaller and Diekema 2007).

Table I.I. Agents of opportunistic mycosis. Adapted from Pfaller *et al.* 2007.

Organism(s)[b]	No. of cases/ million/yr	Case/fatality ratio (%)
Yeasts		
Candida species	72.8	33.9
Cryptococcus species	65.5	12.7
Other yeasts		
Hyaline molds		
Aspergillus species	12.4	23.3
Zygomycetes	1.7	30.0
Other hyalohyphomycetes	1.2	14.3
Dematiaceous molds	1.0	0
Pneumocystis jiroveci		

Over the past decades several epidemiologic studies have been performed in European countries and in USA to evaluate the incidence of superficial and invasive candidiasis. These studies suggested that *Candida* infections are the third most common urinary tract infections with an incidence of ≈20%, and that the majority of episodes of *Candida* urinary tract infections occur in hospitalized patients with indwelling bladder catheters (Sobel *et al.* 2011). Moreover, *Candida* species are also responsible for 13.2% of all intra-abdominal infections, 70% of all onychomycosis (Jayatilake *et al.* 2009) and may colonize about 70% of women vagina (Sobel 2007). The incidence of invasive *Candida* infections have been studied by several multi-institutional surveys, such as European Confederation of Medical Mycology (ECMM) (Tortorano *et al.* 2004) survey, National Epidemiology of Mycosis Survey (NEMIS) (Rangel-Frausto *et al.* 1999), among others. These studies concluded that the frequency of candidaemia among hospitalized patients has doubled during these two decades and candidaemia is now the third most common nosocomial blood-stream infection.

Data from ECMM indicate that *C. albicans* remains the most common species isolated from the blood of patients with invasive fungal infection (Tortorano *et al.* 2004). However, infections caused by non-*albicans* species are increasing (Lass-Florl 2009). This trend may be explained by the introduction of fluconazole in 1990 (Rodloff *et al.* 2011), since it was demonstrated that patients with candidaemia caused by non-*albicans* species received prophylactic antifungal

agents before the onset of their infections more frequently than patients with candidaemia by *C. albicans* (Hachem *et al.* 2008).

The most common non-*albicans Candida* species are *C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei,* however, the incident rates of these organisms vary according to patient population (Table I.II) (Tortorano *et al.* 2004).

Table I.II. Species distribution of *Candida* bloodstream isolates. Adapted from Tortorano *et al*. 2004.

Species	Range[a]
C. albicans	42.7–67.0
C. glabrata	8.3–16.1
C. parapsilosis	6.9–30.0
C. tropicalis	2.1–10.0
C. krusei	0–3.1
C. guilliermondii	0–2.1
C. lusitaniae	0–2.0
C. kefyr	0–1.6
C. pelliculosa	0–1.2
C. famata	0–1.4
C. dubliniensis	0–1.8
C. lipolytica	0–0.7
C. norvegensis	0–0.5
C. inconspicua	0–0.5
C. utilis	0–0.2
C. sake	0–0.5

C. parapsilosis has been associated with parental nutrition, neonatal population, intravenous catheters or contaminated prosthetic devices. The contamination may be caused by health care workers since *C. parapsilosis* is the most common species isolated from the hands of nurses (Pappas *et al.* 2003; Trofa *et al.* 2008). *C. glabrata* has a natural resistance to commonly used antifungals due to the constitutively expression of drug efflux pumps (Parkinson *et al.* 1995) and is commonly isolated from surgical patients, patients with urinary catheters, neutropenia and bone marrow transplant patients (Fidel *et al.* 1999). *C. tropicalis* is frequently responsible for invasive infections in patients with hematologic malignancies and neutropenia. Finally, *C. krusei* represents a significant challenge to clinicians due to the inherent resistance to azole drugs due to an altered target enzyme, and affects more frequently leukemic patients and bone marrow transplant recipients (Orozco *et al.* 1998).

Reported mortality rates from candidaemia range from 30 to 75% in European surveys, depending on species and geographic location studied (Table I.III) (Pappas *et al.* 2003; Tortorano *et al.* 2006; Lass-Florl 2009).

Table I.III. Mortality rates of *Candida* bloodstream infections. Adapted from Tortorano *et al.* 2006.

Candida species	ECMM survey	
	No. of episodes	Mortality rate, % (range)[a]
C. albicans	1090	38.5 (29–45)
C. glabrata	269	45.0 (25–64)
C. parapsilosis	263	25.9 (0–34)
C. tropicalis	140	41.4 (20–44)
C. krusei	38	55.3 (20–67)
Total	1942[b]	37.9 (27–40)

ECMM, European Confederation of Medical Mycology.
[a] Range reflects data from all participating countries.
[b] Includes 142 other less common *Candida* species not reported in detail.

Small differences in the incidence of candidiasis in Europe and USA were found, probably due to differences in patient demographics or differences in medical practices (Pfaller and Diekema 2007). Candidaemia not only is associated with increased mortality and morbidity rates but also prolongs hospitalization and increases medical cares costs. Systemic *Candida* infections have been associated with an attributable intensive care unit cost of US $21,590 (Tortorano *et al.* 2004).

1.3. Virulence factors of *Candida* species

In order to establish an infection, an opportunistic pathogen have to colonise a host, penetrate the surface, survive and divide in the host environment, and avoid the immune response. Although some *Candida* species are commensal organisms of the normal flora, the ability to adapt to different environments, including changes in oxygen and carbohydrate levels, pH, osmolality, availability of nutrients and temperature, improves the development of *Candida* infections. The mechanisms required for the occurrence of these processes are designated as virulence factors (Mavor *et al.* 2005).

Candida species have developed an effective battery of putative virulence factors and specific strategies to assist in their ability to colonize host tissues, cause disease and overcome host defences (Yang 2003). The *Candida* virulence factors most studied are adhesion capacity, production of hydrolytic enzymes, hyphae formation and phenotypic switching. However, the virulence factors expressed may vary depending on the type, the site and the stage of infection, and the nature of the host response (Naglik *et al.* 2003).

1.3.1. Adhesion

The colonization and infection of *Candida* species are dependent on the ability to adhere to host cells, tissues and medical devices in different stages of infection. However, the extent of adhesion is dependent on microbial, host and abiotic surface proprieties, such as cell-surface hydrophobicity and cell wall composition (Silva *et al.* 2011).

An important element that is correlated with the adhesion ability of *Candida* species is the presence of specific cell-wall proteins, denominated adhesins. These proteins are defined as biomolecules that promote the adherence of *Candida* species to host cells or host cell ligands (Calderone and Fonzi 2001; Trofa *et al.* 2008; Silva *et al.* 2011). Mutants deficient in the genes encoding these adhesins exhibit decreased adherence to host substrates *in vitro* as well as a corresponding reduction in virulence in several experimental models of candidiasis (Sheppard *et al.* 2004).

Several genes encoding cell wall adhesins of *Candida* species have been identified. The most common adhesins studied are from the agglutinin-like sequence (Als) protein family, encoded by eight *ALS* genes (*ALS1-7* and *ALS 9*) (Yang 2003). Three domains characterize these proteins and differences in N-terminal domain among distinct Als proteins are responsible for differences in their function. For example, Als1p has been shown to mediate binding to human vascular endothelial cells and epithelial cells in early stages of infection, whereas Als5p confers adherence to collagen, fibronectin, bovine serum albumin and laminin (Sheppard *et al.* 2004). The *ALS* genes are differentially expressed depending on the growth conditions or on the species analysed. Several strains of *C. albicans* express all eight ALS genes, however, in *C. parapsilosis* and *C. tropicalis* only five and three ALS genes were found, respectively (Silva *et al.* 2011).

Others adhesins have been identified, including the Epa (epithelial adhesin) family in *C. glabrata,* the glycophosphatidylinositol-anchored protein 30 (Pga30) in *C. parapsilosis* or Hwp1 in *C. albicans* (Nobile *et al.* 2006; Silva *et al.* 2011).

1.3.2. Morphogenesis

Some *Candida* species are polymorphic yeasts that are able to undergo morphogenic switching from the unicellular budding yeast forms (blastopores) to the filamentous forms (hyphae or pseudohyphae). This transition is regulated by a complex network of signal transduction

pathways, which includes transcription factors such as Efg1, Cph1 and Tup1. The transcription factors are activated by morphogenetic stimuli such as the presence of serum or the interaction with innate immune cells (Heilmann *et al.* 2011). The yeast-to-hyphae transition is the most prominent morphological change in the *Candida* (especially *C. albicans*) life cycle and two important functions of hyphae formation have been suggested, including the ability to penetrate into tissue surfaces and the capacity to escape from host cells following internalization (Gow *et al.* 2002). In order to penetrate the epithelial tissue and to provide resistance to phagocytosis, the hyphae produce mechanical forces. The expression of adhesins, such as Hwp1p or Als3, for anchoring the *Candida* cells to host tissue is probably a prerequisite for hyphae invasion (Kumamoto and Vinces 2005). Another trend in hyphae penetration consists in the secretion of enzymes able to degrade proteins, lipids and other cellular components, facilitating the infiltration into solid substrates and tissues (Gow *et al.* 2002).

Although the hyphae formation is considered an important virulence factor in *Candida* virulence, most lesions are populated by both morphological forms, suggesting that both have a role in the development and progression of disease (Calderone and Fonzi 2001). It has been suggested that yeast cells are better suited for dissemination while hyphae are important for tissue and organ invasion and for adaptation to different host niche conditions (Mavor *et al.* 2005; Lim *et al.* 2012).

This ability is observed in species such as *C. albicans, C. parapsilosis* or *C. tropicalis* and is considered to be crucial for virulence (Lim *et al.* 2012). *C. glabrata* is generally described as incapable to form hyphae and pseudohyphae. However, the ability of pseudohyphae formation was suggested in numerous studies, where this feature was observed in some strains (Odds *et al.* 1997; Csank and Haynes 2000; Lachke *et al.* 2002). Regarding *C. krusei*, no consistent filamentous studies have been performed.

1.3.3. Hydrolytic enzymes

The secretion of hydrolytic enzymes during the development of candidiasis may be required as a virulence attribute. This virulence factor may be involved in adhesion by degrading host cell surface molecules, invasion by digesting host cell membranes, resistance to host immunity by attacking the immune system, and nutrient acquisition. The three most significant extracellular

hydrolytic enzymes secreted by *Candida* species include secreted aspartyl proteinases (Sap), phospholipases and lipases (Mavor *et al.* 2005; Jayatilake 2011).

The secretion of secreted aspartyl proteinases (Sap) by *Candida* species is recognized as an important virulence factor since they facilitate invasion and colonization of host tissue. A family of 10 *SAP* genes encodes the Sap proteins and the virulence mechanism of Sap involves the disruption of host mucosal membranes and degradation of important immunological and structural defence proteins, such as immunoglobulin G heavy chains, C3 protein, collagen, fibronectin, albumin, haemoglobin, keratin among others (Yang 2003; Trofa *et al.* 2008). The expression of *SAP* genes during infection has been studied by their disruption in several models and differential expression profiles under various conditions it has been observed (Naglik *et al.* 2003; Naglik *et al.* 2008; Correia *et al.* 2010). Schaller and co-workers (Schaller *et al.* 2001) demonstrated that *SAP* genes family is differentially expressed in the yeast, hyphal and phenotypically switched states. SAP1-3 is predominantly expressed on cell walls and cytoplasm of blastopores, SAP4-6 is localized at the tips of hyphae and SAP1 and SAP3 are expressed by phenotypically switched cells. Moreover, Sap8 is predominantly detected in yeast cells grown at 25°C and Sap9 is preferentially expressed in later growth phases (Yang 2003). Hereupon, the versatility of SAP genes expression may prove to be vital to the success of *Candida* as an opportunistic pathogen, by allowing the fungus to survive and cause infections on a variety of tissues (Naglik *et al.* 2003).

The secretion of Saps is recognized as an important virulence factor, however, the expression of all ten *SAP* genes is only observed in *C. albicans* strains, whereas only four (SAP1-4) and three (SAP1-3) genes have been identified in *C. tropicalis* and *C. parapsilosis,* respectively (Trofa *et al.* 2008; Silva *et al.* 2011). Regard *C. glabrata* and *C. krusei*, some proteinase activity was detected, however, the number of these proteinases have not been well defined (Yang 2003). Although the expression of SAP genes has been recognized as an important virulence factor, Correia and co-workers (Correia *et al.* 2010) demonstrated that other factors must be the major contributors to invasion and cell damage in this model.

Phospholipases (PLs) are enzymes that hydrolyse phospholipids to fatty acids and glycerol. Depending on the different ester bonds cleaved, these enzymes have been classified into PLs A, B, C and D. However, only proteins encoded by the phospholipase B family (PLB1-5) seem to be extracellular, especially *PLB1* that is essential for virulence in animal models of

candidiasis (Calderone and Fonzi 2001; Silva *et al.* 2011). The presence of PLs during infection could contribute to host cell membrane damage and adherence of *Candida* species. Jayatilake and co-workers (Jayatilake *et al.* 2005) and Gahnnoum and co-workers (Ghannoum 2000) demonstrated that PLs are expressed at the tips of *Candida* hyphae and initial buds of *C. albicans* during invasion. These studies confirm that PLs of *Candida* are involved in the pathogenesis of candidiasis by facilitating the tissue penetration. Recent studies have indicated that *C. tropicalis* and C. *parapsilosis* are able to produce extracellular PLs, however, at much lower levels than *C. albicans*. For *C. glabrata* and *C. krusei* very few studies were performed and no clear PL activity was observed.

Lipases are involved in the hydrolysis and synthesis of triacylglycerols. These enzymes are encoded by ten *LIP* genes (*LIP1-10*) differentially expressed at different stages and sites of infection. In *C. albicans* and *C. tropicalis* ten *LIP* genes (*LIP1-10*) were detected. However, for *C. parapsilosis*, only two lipase genes, *LIP1* and *LIP2*, have been reported (Trofa *et al.* 2008). Moreover, no studies have been performed to investigate the expression of *LIP* genes in *C. glabrata* and *C. krusei* (Silva *et al.* 2011). Gácser and co-workers (Gacser *et al.* 2007) demonstrated the significance of lipases, showing that the use of lipase inhibitors significantly reduce tissue damage during infection in reconstituted human tissue models.

1.3.4. Phenotypic switching

The colonies of *Candida* species can reversibly switch between different morphologies, and this process is known as phenotypic switching. The ability to undergo phenotypic switching is thought to aid survival in different microenvironments, and evasion from the host immune response. Moreover, phenotypic switching also affects adhesion, hyphal formation, sensitivity to neutrophils and increase the resistance to antifungals (Mavor *et al.* 2005). However, the basic mechanism of phenotypic switching and the involvement of this switching in the virulence are not clear (Calderone and Fonzi 2001).

The white-opaque switching in strain WO-1 of *C. albicans* is the most studied phenotypic switching. In this case, the smooth and white colonies with round-ovoid cells can switch to flat and grey colonies with elongated or bean-shaped cells (Morschhauser 2010). The ultrastructural observations of white and opaque phenotypes have revealed differences in the cell shape, cell surface structures and germination at 37°C, suggesting that phenotypic switching could affect the behaviour of the organism. For instance, opaque phase cells have

higher ability to colonize the skin whereas white cells are more virulent in a systemic animal model (Calderone and Fonzi 2001).

Although the phenotypic switching of *C. albicans* is the most studied, *C. glabrata, C. parapsilosis* and *C. tropicalis* also present this capability. Laffey and co-workers (Laffey and Butler 2005) identified four core phenotypes in *C. parapsilosis*, including the crepe, concentric, smooth and crater phenotypes and demonstrated their relation with biofilms formation. Moreover, Lachke and co-workers (Lachke *et al.* 2002) identified four phenotypes in *C. glabrata* (White, Dark Brown, very Dark Brown and Light Brown) and França and co-workers (Franca *et al.* 2011) demonstrated the presence of four possible phenotypes in *C. tropicalis* (Smooth, Rough, Ring, Semi-Smooth). The phenotypic switching of *C. krusei* has not been studied.

1.3.5. Biofilm formation

The attachment of *Candida* cells to host or medical devices followed by cell division and proliferation is called biofilm. Biofilms are complex and well organized microbial communities with fungal cells embedded within a mainly polysaccharide extracellular matrix (Lim *et al.* 2012). Biofilm formation is considered as an important virulence factor in the development of infection. The presence of biofilms confers significant tolerance to antifungal therapy and host immune responses, and causes the failure of indwelling medical devices (Trofa *et al.* 2008). Numerous *Candida* species produce biofilms, including *C. albicans, C. tropicalis, C. glabrata* and *C. parapsilosis,* and their presence during infection has been linked to higher mortality rates. However, the biofilm formation is dependent on several factors, such as the species, strains and environmental conditions (pH, medium composition and oxygen) (Silva *et al.* 2011).

Estivill and co-workers (Estivill *et al.* 2011) studied the biofilm formation by *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei* on three clinical materials. This study demonstrated that *C. parapsilosis* showed great biofilm formation capacity and its ability to cause nosocomial infections can be related with this feature. Moreover, this study also demonstrated that the capacity of *C. krusei* to form biofilms is limited.

1.4. Treatment of candidiasis

The increasing incidence of fungal infections, including *Candida* infections, as well as the increasing variety of pathogenic species have contributed significantly to the mortality in immunosuppressed patients. In order to reverse this condition several antimycotics agents have been developed, however, numerous species remain difficult to treat due to delayed diagnosis, drug toxicity, antifungal drug resistance, drug bioavailability and lack of oral or intravenous preparations. Recent epidemiological trends have confirmed the increasing importance of infections caused by resistant fungal species (Lass-Florl 2009). Thereby, it is crucial to understand the antifungal drug resistance and develop effective therapeutics.

The antifungal agents are classified into different groups according to the antifungal mechanism of action, namely polyenes, azoles, echinocandins and others antifungal agents (Figure 1.2) (Mathew and Nath 2009).

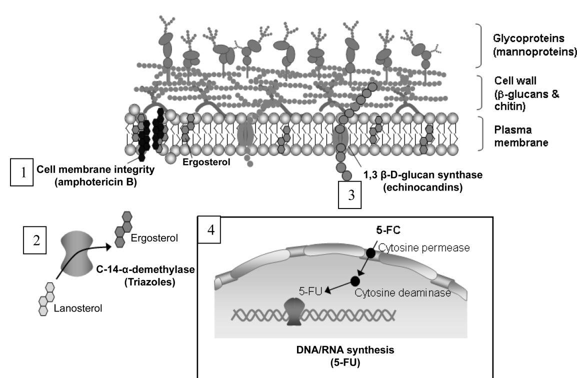

Figure 1.2. Mechanisms of action of (1) polyenes, (2) azoles, (3) echinocandins and (4) 5-FU.

1.4.1. Polyenes

Polyenes are the major class of antifungal agents and are isolated from *Streptomyces* species. The mechanism of action of polyenes is based on their interaction with ergosterol components of the fungal membrane. The complex polyene-esterol formed provides an aqueous pore and affect cell permeability, which causes cell leakage and cell death (Mathew and Nath 2009). The polyenes with therapeutic application are amphotericin B, nystatin, pimaricin and candicidin, however only the first two are commonly used. Amphotericin B has long been considered the gold standard for the treatment of fungal infections. This agent is active against most fungal pathogens, namely *Trichosporan beigelii, Aspergillus terreus, Pseudallesheria boydii, Malassezia furfur* and *Fusarium* species (Masia Canuto and Gutierrez Rodero 2002).

Regarding candidiasis, this agent is active against most *Candida* species and can be used in the treatment of invasive or superficial candidiasis. However, the cytotoxicity associated with amphotericin B demanded the development of new formulations, which use liposomes or lipid complexes as delivery systems (Chen and Sorrell 2007).

The acquisition of polyenes resistance by *C. albicans* and other *Candida* species is unusual however, numerous reports have demonstrated resistance to amphotericin B by *C. albicans* in patients previously treated with polyenes (Mokaddas *et al.* 2007). The molecular mechanisms involved in polyene resistance are the decrease in the total ergosterol content of the cell, replacement of some or all of the polyene-binding sterols, and reorientation or masking of existing ergosterol (Masia Canuto and Gutierrez Rodero 2002).

1.4.2. Azoles

Azoles are the second most studied antifungal agents and their mechanism of action is based in the inhibition of ergosterol biosynthesis. In more detail, exposure of fungal species to azoles inhibits the ergosterol enzymatic pathway, especially the enzyme cytochrome P450 sterol 14α-demethylase. This inhibition promotes the disruption of the structure of the membrane as well its functions in nutrient transport and chitin synthesis, reducing the fungal growth (Mathew and Nath 2009).

There are two azole groups in clinical use. The first azole compounds explored are the imidazole-based drugs, such as clorotrimazole, miconazole, ketoconazole and econazole. However, this group is only efficient in superficial treatment. Later, triazole-based drugs, including fluconazole, itraconazole, voriconazole, posaconazole and ravuconazole were developed, which are used as superficial and systemic fungicidal agents (Chen and Sorrell 2007). The differences in the structure of the different azoles are responsible for their variation on antifungal potency, bioavailability, drug interaction and toxicity (Mathew and Nath 2009).

The introduction of fluconazole as antifungal agent of choice in the treatment of superficial candidiasis in the early 1990s triggered the appearance of azoles resistant strains. Moreover, this increasing azole resistance may be also explained by the appearance of species intrinsically resistant to fluconazole, such as *C. glabrata* or *C. krusei* (Chen and Sorrell 2007). The mechanism of resistance to azoles in *Candida* species has been studied, and distinct mechanisms for the acquisition of resistance have been described. These mechanisms include

decreased accumulation of the drug from enhanced efflux interference of their action on the target enzyme, alterations in other enzymes of the biosynthetic pathway of ergosterol and decreased permeability of the fungal membrane to the drug (Masia Canuto and Gutierrez Rodero 2002).

1.4.3. Echinocandins

The increasing incidence of infection caused by fluconazole resistant species required the development of a therapeutic alternative and echinocandins have become an important group in the treatment of these infections. The echinocandins are lipopeptide molecules that act as inhibitors of the synthesis of β -1,3- D-glucan, which is an important component of the fungal cell wall, by blocking the action of a pathway enzyme, β -1,3- D-glucan synthase (Perlin 2007). The absence of β -1,3- D-glucan destabilizes the integrity of the fungal cell wall and promotes the osmotic instability and cell death (Kofla and Ruhnke 2011).

The echinocandins drugs used in antifungal treatment are caspofungin, micafungin and anidulafungin. These agents have broad-spectrum antifungal activity against *Candida* and *Aspergillus* species, however, are not active against *C. neoformans* and *non-Aspergillus* moulds (Perlin 2007). Moreover, echinocandins drugs are effective against azole-resistant species, since their target is the cell wall. Another vantage of these drugs is that the toxicity is infrequent since glucans are not found in mammalian cells (Chen and Sorrell 2007). The echinocandins resistance is unusual, however, some case reports have illustrated the potential for resistance development (Kofla and Ruhnke 2011).

1.4.4. Other antifungal agents

Although the polyenes, azoles and echinocandins are the three major classes of antifungal agents, other compounds, including allylamines, flucytosines, griseofluvins, sordarins, nikkomycins, ciclopiroxolamines, amog others, have been also used (Mathew and Nath 2009).

The flucytosine (5-fluorocytosine or 5-FC) is one of the oldest antifungal agents and its mechanism of action is based in the conversion into 5-fluorouracil within target cells. Fluorouracil is incorporated into RNA, where it causes premature chain termination, and also inhibits DNA synthesis through effects on thymidylate synthase (Vermes *et al.* 2000). This drug

is selectively toxic to fungi because mammalian cells lack cytosine permease and do not convert flucytosine into 5-fluorouracil (Mathew and Nath 2009).

Although this agent shows antifungal activity against *Candida* species in cases of systemic candidiasis, the development of resistance is frequent. In order to overcome the development of resistance, the use of monotherapy is not recommended and this agent must be combined with azoles. The mechanisms of resistance proposed are (1) the development of mutations that result in a deficiency in the enzymes necessary for cellular transports and uptake of 5-FC or for its metabolism, and (2) the increase in the synthesis of pyrimidines, which compete with the fluorinated antimetabolites of 5-FC and thus diminish its antimycotics activity (Vermes *et al.* 2000).

Although in the last years a number of antifungal agents have been developed, the selection of the most appropriate drug is imperative. As stated above, the susceptibility of the different species to the different antifungal agents varies considerable. Thus, the correct identification of infectious agents represents an important tool in reducing the mortality rate.

2. Identification of *Candida* species

The rapid and correct identification of infecting species is crucial for several reasons. The main reason is the use of appropriate antifungal treatment, since *Candida* species differ in their susceptibility to antifungal agents. For instance, *C. krusei* is intrinsically resistant to azoles and *C. glabrata* easily acquires resistance to fluconazole (Parkinson *et al.* 1995; Orozco *et al.* 1998). Moreover, species identification is also important for epidemiological purposes, for example, repeated identification of a particular species in a given hospital ward or locate may indicate a point source outbreak (Denning *et al.* 2003; Sabino *et al.* 2010). An additional reason to explain the significance of correct diagnosis is the fact that the risk of developing deep organ involvement, and the severity of clinical manifestations, differs depending on the infecting species (Rabkin *et al.* 2000).

Clinical microbiology laboratory methodologies for the identification of pathogenic fungal species are based on the morphological, physiological and biochemical tests. However, new serological and molecular tests have also been developed for the differential identification of the fungal species. These tests are classified as conventional, serological and molecular.

2.1. Conventional methods

The light microscopy analysis of biological products is the first methodology used in clinical laboratory practice, and is used to observe the presence, shape and size of blastopores as well as the hyphae/pseudohyphae formation. This method only allows the presumptive identification, since some species can present specific microscopic characteristics (Lee *et al.* 1999; Pinoni *et al.* 2007). For example, the presence of true hyphae in *C. albicans* or the shape of the blastopores that in *C. krusei* is elongated, whereas in *C. albicans* or *C. parapsilosis* is oval and spherical (Ellepola and Morrison 2005).

The growth and isolation of species present in clinical samples is an important method used in microbiology laboratories. The media selected should sustain the growth of all the *Candida* ssp., inhibit the growth of bacteria and should facilitate the identification of clinical specimens, however, should not interfere with the viability of the organisms (Sullivan *et al.* 1996; Alvarez-Perez *et al.* 2011). Several chromogenic media have been developed in order to distinguish *Candida* species. These culture media incorporates substrates linked to chemical dyes in a solid medium to differentiate *Candida* species by the colour and texture of the colonies (Okulicz *et al.* 2008; Ozcan *et al.* 2010). However, these media only allows the presumptive identification of some *Candida* species, especially *C. albicans, C. tropicalis* and *C. krusei* (Ghelardi *et al.* 2008; Okulicz *et al.* 2008). Examples of commercial chromogenic media are ChromIDCandida (BioMerieux®), CandiSelect4 (BioRad®) or CHROMagar Candida (BD®) (Sendid *et al.* 2007; Guzel *et al.* 2011).

For *Candida* species differentiation the physiological and biochemical methods are the most commonly used. The biochemical identification consists in carbohydrate and nitrogen assimilation, such as glucose, xylose, urease, trehalose, saccharose, nitrates, among others; fermentation tests and enzymes detection (Lopez *et al.* 2001; Ellepola and Khan 2012). However, these tests can have a number of problems associated with the results interpretation. The results obtained may be inconsistent since different isolates from the same species could yield different profiles or genetically diverse species can yield similar profiles (Campbell *et al.* 1999; Cardenes-Perera *et al.* 2004). For example, *C. parapsilosis, C. metapsilosis* and *C. orthopsilosis* or *C. albicans* and *C. dubliniensis* have similar biochemical profiles, however, are genetically different (Pasligh *et al.* 2010). Auxacolor (BioRad®),

APICandida (BioMerieux®), API20CAux (BioMerieux®) or Vitek Yeast Biochemical Card (BioMerieux®) are examples of commercial kits for biochemical *Candida* spp. Identification.

Although conventional methods are the most commonly used in clinical microbiology laboratories, there are several limitations, such as inaccuracy, high cost and the long time required for identification (Ellepola and Morrison 2005). Therefore, the application of alternative methodologies is needed in order to overcome these limitations.

2.2. Serological methods

The species identification based in serological methods consists in the detection of specific antigens, antibodies or metabolites (such as D-anabinitol) in clinical samples. For this purpose several methods are used, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), latex agglutination (LA) or reverse passive latex agglutination (RPLA) (Ellepola and Morrison 2005).

Numerous antigens have been used as potential targets for the diagnosis of disseminated candidiasis, including secreted aspartyl proteinases, 1,3-β-D-glucans and mannans. Mannan is an abundant cell wall polysaccharide of *Candida* spp. and is the most used and studied antigen (Guery *et al.* 2009). However, the detection of mannan in clinical samples depends on the frequency of sampling, the underlying disease, the degree of immunosuppression, the *Candida* species involved, the specificity and titer of the capture antibodies and the method used. Another important limitation of this method is the rapid clearance of the antigen from the patient sera (Poulain *et al.* 1997; Ellepola and Morrison 2005).

A number of *Candida* antigens are highly immunogenic for humans and the detection of antibodies against them in clinical samples may represent an important diagnostic method for invasive candidiasis (Quindos *et al.* 2004). The detection of anti-mannan antibodies is the most common used, however, the detection of antibodies against antigens with enzymatic activity (enolase or aspartyl proteinase) and antibodies against proteins of *C. albicans* germ tubes are also options (Quindos *et al.* 1987; Ponton *et al.* 1994). The limitations of this technique are the possibility of false-negative results in immunocompromised patients that produce low levels of antibody, false-positive results in patients with superficial colonization and the fact that antibody production may occur only at an advanced stage of disease. Nevertheless, it is

possible to overcome these limitations since the specificity of the tests has been improved by selecting the appropriate antigens (purified molecules, recombinant antigens, among others) (Quindos *et al.* 2004; Ellepola and Morrison 2005).

Several commercial kits have been developed for the diagnosis of *Candida* spp. based on detection of antigens or antibodies. For example, Fungiter-G MK® or Glucatell®, which detects the presence of 1,3-β-D-glucans, PlateliaCandida Ag Plus (BioRad®), which detects the presence of mannans in blood samples in ELISA format, or PlateliaCandida Antibody Plus (BioRad®), which is an ELISA-based test for of anti-mannan antibodies (Sendid *et al.* 2003). Recent studies have suggested that the combined detection of mannan and anti-mannan antibodies considerably improves the diagnosis of candidiasis (Alam *et al.* 2007).

2.3. Molecular methods

Molecular methodologies, especially based in the analysis of DNA sequences, are characterized by their high specificity, sensibility and reproducibility. To overcome the limitations of conventional methods several molecular approaches have been developed in molecular research laboratories for *Candida* species identification.

2.3.1. Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) analysis consists in the digestion of total chromosomal or plasmid DNA as well as PCR products with one or more restriction endonucleases. The endonucleases selected (*Eco*R1 is the most frequent) recognize specific nucleotide sequences, breaking the DNA into small fragments. The fragments are finally separated by agarose gel electrophoresis and the number and sizes of the restriction fragments depend on recognition sequence of the enzyme as well as the composition of the DNA. The different RFLP patterns obtained allow the species or strains differentiation (Sullivan *et al.* 1996).

Several RFLP studies have been performed to differentiate individual *Candida* species or *Candida* strains, especially *Candida albicans* strains (Xu *et al.* 1999; Isik *et al.* 2003). Williams et. al (Williams *et al.* 1995) demonstrated the possibility to distinguish eight medically important *Candida* species (*C. guilliermondii, C. glabrata, C. pseudotropicalis, C. albicans,*

C.tropicalis, C. stellatoidea, C. parapsilosis, and *C. krusei*) using three restriction enzymes, *BfaI*, *DdeI* and *HaeIII*. Pinto et al. (Pinto *et al.* 2004) also demonstrated this possibility, using several enzymes, and the identification of six *Candida* species using only one enzyme, *MspI,* was confirmed by Mirhendi et al. (Mirhendi *et al.* 2006).

The RFLP analysis presents several advantages, including high reproducibility and accuracy. However, this is a time-consuming technique, the RFLP patterns obtained from *Candida* spp. can contain a limited number of bands hampering the interpretation, and the same species can present different patterns (Sullivan *et al.* 1996).

2.3.2. Polymerase Chain Reaction (PCR) based methods

The polymerase chain reaction (PCR) based methodologies are sensitive, specific and rapid assays that have been accepted as the standard method for detecting nucleic acids from a number of microorganisms in clinical samples, including *Streptococcus agalactiae* (de Zoysa *et al.* 2012), *Treponema pallidum* spp. (Leslie *et al.* 2007), *Aspergillus* species (Walsh *et al.* 2011) or *Candida* species (Correia *et al.* 2004). The conventional PCR methodology was developed by Kary Mullis in 1983 (Mullis *et al.* 1986) to amplify target DNA sequences derived from dead or living cells by thermostable DNA polymerase-mediated extension of specific oligonucleotide primers. The PCR amplification is followed by PCR products detection or analysis, and the most common methods are the agarose or polyacrylamide gel electrophoresis, sequencing or pyrosequencing.

The design of specific oligonucleotide primers, complementary to DNA sequences unique to the organisms, is important since it can provide identification of an organism to the species level. For this purpose specific sequences need to be selected as DNA targets. For *Candida* species identification the most commonly used target is the ribosomal DNA (rDNA), which encodes three subunits, 18S, 5.8S and 28S, and is largely distributed in *Candida* genome (Sullivan *et al.* 1996; Ramos *et al.* 2006). Although the rRNA genes are highly conserved, the internal transcribed spacer (ITS) is variable and species specific. The ITS region is located between the 18S and 26S rRNA genes and is subdivided into the ITS1 region, between the 18S and 5.8S rRNA genes, and the ITS2 region, between the 5.8S and 26S rRNA genes (Figure 1.3). PCR with specific primers (ITS1 and ITS4), targeting the conserved sequences of 5.8S and 28S rDNAs, results in the amplification of the species-specific ITS1 and ITS2 regions,

which vary in amplicon length and sequence according to species (Ellepola *et al.* 2003; Coignard *et al.* 2004).

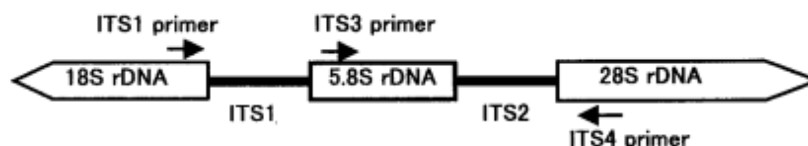

Figure 1.3. Constitution of rRNA gene operon.

The tRNA genes can also be used and DNA target to species identification into a specific genus (Baele *et al.* 2000). Although these genes are highly conserved, the lengths of tRNA intergenic spacers vary considerably and primers design in the highly conserved flanking tRNA genes can be used to amplify the polymorphic region in any organism that is sufficiently closely related. For inter-tRNA gene amplification several primers pairs have been developed and the length of the resultant PCR products, rather than its presence or absence, is characteristic of the species. The amplification of this region was firstly described by Welsh and McClelland (Welsh and McClelland 1991) in *Staphylococcus* strains, demonstrating the potential of investigation of tRNA gene intergenic lengh polymorphism in species differentiation. For *Candida* species identification, T3B primer pair, previously described in the identification of *Staphylococcus* species (McClelland *et al.* 1992), has been successfully used in species differentiation (Correia *et al.* 2004).

In research laboratories, the most commonly used PCR based methodology for species or strains identification is RAPD (Randomly Amplified Polymorphic DNA) (Novak *et al.* 2004; Valerio *et al.* 2006). This method uses short primers, typically 9 to 10 nucleotides in length, which anneal at multiple genomic loci since it does not depend on prior knowledge of species-specific sequences. Following the amplification, the PCR products obtained are analysed by agarose gel electrophoresis and visualized after specific staining. The RAPD patterns obtained allow the species or strains differentiation. This methodology has been successfully used to identify *Candida* species, including *C. albicans, C. tropicalis, C. parapsilosis, C. glabrata* and *C. krusei* (Bautista-Munoz *et al.* 2003; Valerio *et al.* 2006). The RAPD-PCR has a high discriminatory power, its easy to perform, does not require radiolabelled probes and it is applicable to several microorganisms. However, presents some limitations, such as the necessity of fastidious conditions for reproducible PCR and the inter-laboratory reproducibility is very low (Tang *et al.* 1997).

In clinical laboratories, only Real Time PCR is performed using a variety of commercial kits, including Septifast (Roche Diagnostics®) or Quantifast Pathogen (Quiagen®). The commercial kit Septifast (Roche Diagnostics®) is the most used and allows the identification of twenty-five clinically important microorganisms, including *Candida* species, directly from blood samples (Vince *et al.* 2008). This methodology uses the internal transcribed spacer (ITS) region as the target region for fungal (18S–5.8S) species identification and the diagnosis is based in melting curves differences (Ellepola and Morrison 2005; Wellinghausen *et al.* 2009). Although Real Time PCR using the commercial kit Septifast (Roche Diagnostics®) is an alternative to conventional and serological methods, these techniques has several disadvantages, namely the use of nonspecific targets that can increase the appearance of nonspecific signals from environmental microorganisms from laboratory contamination, the presence of large amounts of host nucleic acid in blood samples that can interfere with primer hybridization and amplification or the presence of inhibitors of *Taq* DNA polymerase (Bravo *et al.* 2011). These limitations can be overcome by the prior DNA extraction and purification, which is not always simple due to the difficulty to lyse the complex fungal cell wall. Regarding to *Candida* species identification, this methodology has low analytical sensitivity in *C. glabrata* identification, probably due to the reduced efficiency of the amplification reaction owing to the larger genomic target flanked by the primers designed for the ITS region in this specific organism (Lehmann *et al.* 2008).

3. DNA microsatellite

The genomic DNA of all living organisms, including eukaryotes and prokaryotes, demonstrates a considerable number of repetitive sequences, namely transposons, which move around the genome and satellite DNAs. Satellite DNAs are tandemly repeated sequences, which can be subdivided into two classes according to the size of the repetitive motif, namely minisatellite DNA and microsatellite DNA (Richard and Paques 2000). Therefore, minisatellite DNAs are tandem arrays of longer units (10-100 bp), while microsatellite DNAs, also designated as simple sequence repeats (SSRs) or short tandem repeats (STRs), are tandem arrays of short units (1-6 bp). The STRs are stably inherited being unique to an individual and the same in all cells from the same individual. However, the special interest of these repetitive sequences is

due to their high level of polymorphism among individuals since the numbers of repeats within specific STRs tend to be variable (Tautz 1989). According to the length of the major repeat motif, STRs may be classified into mono-, di-, tri-, tetra-, penta- and hexanucleotide repeats, being the mono- and dinucleotide repeats the most frequently found. The total number of each type decreases as the size of the repeat unit increases. Moreover, STRs can also be classified into different groups according to the repeat structure, namely perfect repeats when it contains only one repetitive unit and imperfect repeats when it contain different repeat compositions (Fan and Chu 2007).

The STRs exhibit a strong level of instability, which lead to great polymorphism. This characteristic is explained by the high mutation rates in STR sequences comparing to unique DNA sequences, which can vary between 10^{-6} and 10^{-2} per locus per generation. Regarding to yeasts, the STRs mutation rate is estimated in 10^{-5} per locus per generation. The STR mutation rate can be influenced by species, environmental conditions, repeat unit, repeat structure, base composition of repeat unit, flanking sequence, recombination and the interruptions in STRs (Fan and Chu 2007).

Three mechanisms have been purposed to explain the STR mutation (addition or deletion of repeated units), including unequal crossing over in meiosis, retrotransposition mechanism and strand-slippage replication. However, the last appears to be the main explanation of STR mutation. The slippage replication, also called DNA slippage, polymerase slippage or slipped strand mispairing, was initially proposed by Kornberg and co-workers (Kornberg *et al.* 1964) and occurs during DNA replication. This process is a consequence of dissociation between the nascent and template DNA strands followed by their misaligned reassociation, resulting in unpaired repeat units (hairpins) on either the nascent or on the template strain. The majority of these errors are recognized and corrected by the mismatch repair system, however, occasionally can escape to DNA repair and the repeat number of this STR is altered (Li *et al.* 2002). If the loop was on the primer strand the number of repeats will be greater than the original number of repeats whereas if the loop was on the template strand the number of repeats will be smaller (Wierdl *et al.* 1997). Moreover, the rate of the slippage is the highest in dinucleotidic STRs and the lowest in tetranucleotidic, demonstrating the reason why the total number of each repetition decreases as the size of the STRs repeat unit increases (Kruglyak *et al.* 1998).

The STRs are widely distributed in the genome and are most frequently found in non-coding regions. However, STRs can also be located in coding regions, such as protein-coding genes or expressed sequence tags, even with relatively small repeat numbers and total lengths (Li *et al.* 2004). STRs are usually considered as evolutionary neutral DNA, and most of them are thought to have no biological uses. However, some STRs can play an important role in chromatin organization, regulation of DNA metabolic processes, regulation of gene activity, recombination, DNA replication, cell cycle, mismatch repair system, among others. This is supported by the involvement of trinucleotidic repeats in the development of human diseases, such as fragile X syndrome, Huntington's disease, myotonic dystrophy, among others (Li *et al.* 2002).

Although the most common types of STRs are mono- and dinucleotide repeats, they are relatively rare in coding regions, since give rise to frameshifts and are therefore strongly selected against. However, trinucleotide repeats, are overexpressed in coding regions, since they can be accommodated more readily: changes in their length simply result in gain or loss of a single amino acid in the protein sequence (Hancock and Simon 2005). Moreover, the repetitive structure (perfect or imperfect STRs) can also influence the stability of microsatellites in coding regions, since imperfect microsatellites would be expected to undergo less replication slippage and therefore be more stable during evolution.

3.1. Microsatellites described in *Candida* species

In the last years, a number of microsatellites loci have been described in several organisms, including yeast species. The interest in the study of microsatellites loci is related to their high polymorphism and co-dominant character, being potential markers for strains identification and characterization (Tautz 1989). As molecular markers, STRs are stable, easy to assay, less time consume, inexpensive, adaptable to a large series and discriminatory enough to investigate clinical issues. Therefore, the study of microsatellite markers by PCR based methodologies may be an excellent alternative to differentiate species or strains.

Several microsatellite markers have been described and studied to differentiate strains from genus *Candida*, including *C. albicans, C. parapsilosis, C. glabrata, C. tropicalis* and *C. krusei* strains. The first microsatellite markers used for *C. albicans* typing were described by Field and

co-workers (Field *et al.* 1996) in 1996. In this work eight trinucleotide *C. albicans* microsatellites loci located in coding regions were studied, namely ERK1, ZNF1, CCN1, CCN2, MNT2, CPH1, EFG1 and EFG2, however, these presented low polymorphism. In 1997 a novel *C. albicans* microsatellite loci, CEF3, was described. This microsatellite composed by trinucleotide repeats is also located in a coding region, namely in the upstream sequence of the elongation factor 3 gene (EF3) (Bretagne *et al.* 1997). The high discriminatory power obtained (comparing to previously described microsatellites markers) leads to its use in posterior studies, including the differentiation of independent *C. albicans* isolates from two geographic regions (Lott *et al.* 1999), the comparison of genotypes of *C. albicans* strains isolated from bloodstream and non-bloodstream infection (Dalle *et al.* 2000) or the comparison of genotypes of *C. albicans* strains isolated from oral mucosa (Dalle *et al.* 2003). Until to 2001, the best discriminatory power, 0.97, was obtained by Bretagne and co-workers (Botterel *et al.* 2001) when described a rapid genotyping method of *C. albicans* using two new microsatellite markers located in coding regions, CDC3 and HIS3, and the previously described CEF3 in multiplex reaction. However, in 2003, Sampaio and co-workers (Sampaio *et al.* 2003) described a new microsatellite locus, CAI, located in RLM1 gene, which presents an individual discriminatory power of 0.97. This microsatellite marker has been used in several studies (Sampaio *et al.* 2005; Li *et al.* 2008; Ge *et al.* 2010), and shown to be highly efficient, reproducible and able to differentiate a large variety of strains. The same group also described five new microsatellite loci, CAIII, CAIV, CAVI and CAVII, located in non-coding regions, and used CAI, CAIII and CAVI to developed a multiplex strategy, obtaining the higher discriminatory power ever reported (0.99) (Sampaio *et al.* 2005).

Regarding to *C. parapsilosis,* two major works were performed in order to describe and test microsatellite loci. The first study was performed in 2006 by Lasker and co-workers (Lasker *et al.* 2006), which described seven microsatellite markers, A to G, with discriminatory powers ranging from 0.341 to 0.876. These microsatellite markers were also studied using a multiplex strategy, presenting a discriminatory power of 0.971. In 2010, Sabino and co-workers (Sabino *et al.* 2010) described three new microsatellite loci located in non-coding regions, Cp1, Cp4 and Cp6. They developed a multiplex, including microsatellite B from Lasker' study, resulting in discriminatory power of 0.99. Moreover, this multiplex strategy have been largely used to type *C. parapsilosis* strains isolated from outbreaks in infants hospitalized (Vaz *et al.* 2011; Reiss *et al.* 2012; Romeo *et al.* 2012).

The first *C. glabrata* microsatellite loci were described in 2005 by Foulet and co-workers (Foulet *et al.* 2005), namely RPM2, ERG3 and MTI. These microsatellites are located in coding regions and the discriminatory powers obtained ranged from 0.521 to 0.757, multiplexing these three microsatellite markers the discriminatory power increased to 0.84. This multiplex was also used to test the genotypic variability of invasive *C. glabrata* isolates over a period of six years presenting a total of 12 genotypes (Abbes *et al.* 2011). In 2007, Grenouillet and co-workers (Grenouillet *et al.* 2007) described six new *C. glabrata* microsatellite loci, three of them composed by dinucleotide repeats and located in non-coding regions (Cg4, Cg5, Cg6), and the remaining composed by imperfect trinucleotidic motifs and located in coding regions (Cg7, Cg10 Cg11). The discriminatory powers obtained ranged from 0.64 (Cg7) to 0.79 (Cg10). However, in the multiplex analysis, using the six microsatellite markers, the discriminatory power was only 0.902. The higher discriminatory power for this species, 0.96, was obtained by Brisse and co-workers (Brisse *et al.* 2009) in 2009, testing nine microsatellite markers in multiplex PCR. The nine microsatellite loci were composed by five trinucleotide microsatellites located in coding regions, three trinucleotide loci located in non-coding regions and a tetranucleotide located in a non-coding region. In 2010, this multiplex strategy was used to type a large panel of both blood culture and digestive tract isolates, obtaining a discriminatory power of 0.97 (Enache-Angoulvant *et al.* 2010).

A few microsatellites were observed in *C. tropicalis* and *C. krusei* genome. Desnos-Ollivier and co-workers (Desnos-Ollivier *et al.* 2008) described two *C. tropicalis* microsatellite loci, URA3, which is located in a coding region and CT14, which is located in a non-coding region. In *C. krusei* genome only one microsatellite, CKTNR, was described so far (Shemer *et al.* 2001).

4. Objectives

Candida species are the most common and clinically important pathogens representing the major fungal agents causing serious infection in Europe (Tortorano *et al.* 2004). Cancer chemotherapy, organ transplantation, antimicrobial therapy and abdominal surgery are among the main risk factors predisposing for *Candida* infections (Perlroth *et al.* 2007). Although *Candida albicans* is the most frequently isolated species other species such as *Candida tropicalis, Candida glabrata, Candida parapsilosis* and *Candida krusei* have been increasingly

recognized as pathogens with a wide distribution (Tortorano *et al.* 2004).

Microsatellite DNAs are tandem arrays of short units (1-6bp), stably inherited, present high polymorphism and co-dominant character. They are widely distributed in the genome and are most frequently found in non-coding regions, although can also be located in coding regions, such as protein-coding genes or expressed sequence tags. Microsatellite sequences are potential markers for strains identification and characterization and have been largely used as molecular targets to differentiate and characterize strains from several species, including *Candida* spp. However, no studies have been performed using microsatellite DNA for *Candida* species identification. Thus, the main objectives of this study were the evaluation of the potential of microsatellite markers for species differentiations and for identification of specific *C. albicans* lineages.

To fulfil these objectives the present work was organized into three main chapters. Chapter I encompasses a brief review on fungal infections and their significance, especially the ones caused by *Candida* species. This chapter discusses the epidemiology and clinical manifestations of candidiasis as well as the virulence factors needed for the development of infection. The methodologies commonly used in research and clinical laboratories for *Candida* species identification are also described due to their significance in the selection of appropriate antifungal treatment. The last parameter discussed in this chapter is a brief review of strains identification methods with emphasis in microsatellite loci since it is commonly used for *Candida* strains characterization and is the object of this thesis.

The second chapter describes the development of a new multiplex PCR based methodology to discriminate clinically important *Candida* species and it encompasses the selection of specific microsatellite loci for the most common pathogenic *Candida* species (*C. albicans, C. parapsilosis, C. glabrata, C. krusei* and *C. tropicalis*), the optimization of the multiplex PCR conditions and the evaluation of the specificity, sensibility and reproducibility of the proposed method.

The third chapter describes the characterization of new *C. albicans* microsatellite marker, denominated by CAVIII, as well as the evaluation of the specificity and polymorphism. This chapter also describes the use of CAVIII and CAI to genotype *C. albicans* strains, and the ability of these markers to differentiate *C. albicans* lineages.

CHAPTER II

New multiplex PCR based methodology to discriminate clinically important *Candida* species

1. Introduction

Fungal pathogens represent the major eukaryotic agents of serious infection in Europe, in which infections due to *Candida* species are the most common and clinically important (Tortorano *et al.* 2004). The development of medicine has caused a dramatic increase in the number of immunocompromised individuals. Cancer chemotherapy, organ transplantation, antimicrobial therapy and abdominal surgery are among the main risk factors predisposing for *Candida* infections (Perlroth *et al.* 2007). Thus, the incidence of *Candida* bloodstream infections has increased dramatically in the last years and *Candida* spp is now the third on the list of nosocomial agents of sepsis, being associated with high morbidity and mortality rates as well as high hospital costs (Pappas *et al.* 2003; Tortorano *et al.* 2006).

There are about 150 species of *Candida*, but only a small number are human pathogens. Although *Candida albicans* is the most frequently isolated species other species such as *Candida tropicalis, Candida glabrata, Candida parapsilosis* and *Candida krusei* have been increasingly recognized as pathogens with a wide distribution (Tortorano *et al.* 2004). The incidence rates reported are dependent on the type of hospital/patients studied and on the geographic region (Van Asbeck *et al.* 2008). Sabino and co-workers (Sabino *et al.* 2010) studied the incidence of candidaemia in a Portuguese oncology hospital for a period of six years and demonstrated that although *C. albicans* was more frequently isolated from patients with solid tumours, non-*albicans* species were most frequently found in haematological patients.

Clinical microbiology laboratory methodologies for the identification of pathogenic fungal species are based on the morphological, physiological and biochemical tests, which requires three or more days and may be inaccurate. In order to overcome these drawbacks a variety of molecular based methods, particularly PCR-based methods, have been developed, presenting high simplicity, specificity and sensitivity (Ellepola and Morrison 2005).

Microsatellites are extremely common in *Candida* spp. genome and may be located in coding and non-coding regions. These repetitive regions may represent an important target for species identification due to the high specificity as well as for strains characterization due to the high polymorphism (Sampaio *et al.* 2005; Li *et al.* 2008; Sabino *et al.* 2010). This study describes the development of a sensitive and specific method, based in microsatellite multiplex PCR

analysis, for the identification of the most clinically important pathogenic *Candida* species, namely *C. albicans, C. parapsilosis, C. glabrata, C. tropicalis* and *C. krusei*.

2. Materials and methods

2.1. Yeast Strains

A total of fifty-six previously identified isolates, including eight *C. albicans*, five *C. parapsilosis,* nineteen *C. glabrata,* six *C. tropicalis,* four *C. krusei,* five *C. metapsilosis,* five *C. orthopsilosis,* three *C. bracarensis* and one *L. elongisporus*, isolated from different sources, such as saliva, vagina, respiratory tract, faeces, skin, blood culture, urine, catheter and wine, were selected for this study (Table II.I). The fifty-six strains, provided from collection of Biology Department of Minho University, have been identified by the use ID 32C strips or VITEK YBC identification cards (BioMérieux, SA, Marcy-l'Étoile, France) and by PCR fingerprinting with primer T3B (Correia *et al.* 2004). The reference strains *C. albicans* (PYCC 3436), *C. tropicalis* (PYCC 3097), *C. krusei* (PYCC 3341), *C. glabrata* (PYCC 2418) and *C. bracarensis* (153M) were also included in this study. The reference strains were obtained from the Portuguese Yeast Culture Collection (PYCC), New University of Lisbon, Lisbon, Portugal. As the reference strains of *C. parapsilosis* were not available, strains already described as *C. parapsilosis* were used (Vaz *et al.* 2011). Strains previously sequenced, namely *C. metapsilosis* HSM CAN155 (GQ152299.1) and *C. orthopsilosis* HSM CAN138 (GQ152298.1) were used as references. To test the specificity of PCR multiplex methodology, twenty isolates without previous identification, isolated from hands of healthy volunteers and saliva of patients with oral infection, were also tested (Table II.I).

All isolates were stored in 40% glycerol at − 80 °C, and grown at 30°C for 48 hours on YPD agar medium (Yeast extract 1%, Bactopeptone 1%, Glucose 2% and Agar 2%) whenever needed.

Table II.I. Isolates used in the study and respective sources.

Isolate code	Species	Origin	Isolate code	Species	Origin
		Strains previously identified			
S085	*C. albicans*	Saliva	Cipo43	*C. tropicalis*	RT
S092	*C. albicans*	Saliva	176C	*C. tropicalis*	Blood culture
S104	*C. albicans*	Saliva	10F4	*C. tropicalis*	Urine

S040	*C. albicans*	Saliva	2D	*C. tropicalis*	Saliva
S038	*C. albicans*	Saliva	3D	*C. tropicalis*	Saliva
OB15V	*C. albicans*	Vagina	4D	*C. tropicalis*	Saliva
Cipo72	*C. albicans*	RT	109/RN0000.001	*C. krusei*	Unknown
1V	*C. albicans*	Rectum	H11	*C. krusei*	Unknown
2251	*C. parapsilosis*	Unknown	535	*C. krusei*	Unknown
2252	*C. parapsilosis*	Unknown	Cipo 94	*C. krusei*	RT
2253	*C. parapsilosis*	Unknown	IPO A911012	*C. metapsilosis*	Unknown
2256	*C. parapsilosis*	Unknown	960161	*C. metapsilosis*	Unknown
2257	*C. parapsilosis*	Unknown	Ana R.	*C. metapsilosis*	Unknown
M2	*C. glabrata*	Unknown	0113	*C. metapsilosis*	Unknown
177	*C. glabrata*	Blood culture	HSM CAN 155	*C. metapsilosis*	Unknown
H38	*C. glabrata*	Vagina	J981226	*C. orthopsilosis*	Unknown
70V	*C. glabrata*	Unknown	HSM CAN 138	*C. orthopsilosis*	Unknown
24/9-10	*C. glabrata*	Vagina	H10 USA	*C. orthopsilosis*	Unknown
14666a	*C. glabrata*	Vagina	154a	*C. orthopsilosis*	Blood culture
14666b	*C. glabrata*	Vagina	1 AII	*C. orthopsilosis*	RT
14408	*C. glabrata*	Vagina	CL-7030	*C. bracarensis*	Unknown
24/9-4	*C. glabrata*	Vagina	246188	*C. bracarensis*	Vagina
7/5-17	*C. glabrata*	Vagina	NCYC 3133	*C. bracarensis*	Catheter
21/9-26	*C. glabrata*	Vagina	ISA 1421	*L. elongisporus*	Wine
21/9-20	*C. glabrata*	Vagina			
1/4-22a	*C. glabrata*	Vagina		**Type strains**	
23/9-24	*C. glabrata*	Vagina			
6/9-17	*C. glabrata*	Vagina	IGC 3436T	*C. albicans*	Skin
27/5-16	*C. glabrata*	Vagina	IGC 2418T	*C. glabrata*	Faeces
17/3-3	*C. glabrata*	Vagina	IGC 3097T	*C. tropicalis*	RT
14573	*C. glabrata*	Vagina	153MT	*C. bracarensis*	Vagina
14735	*C. glabrata*	Vagina	IGC 3341T	*C. krusei*	RT
			Strains without previous identification		
CDQN5	-	Saliva	APC4	-	Skin
CDQN10	-	Saliva	APC5	-	Skin
CD1	-	Saliva	APC6	-	Skin
CD2	-	Saliva	APC7	-	Skin
S152	-	Saliva	C1	-	Saliva
S153	-	Saliva	C2	-	Saliva
D1	-	Skin	C3	-	Saliva
APC1	-	Skin	C4	-	Saliva
APC2	-	Skin	C5	-	Saliva
APC3	-	Skin	1432	-	Saliva

RT-Respiratory tract; - no previous identification

2.2. Primers selection

A search for previously described and studied microsatellites for *Candida* species was performed in order to identify specific microsatellites loci for *C. albicans, C. parapsilosis, C. glabrata, C. tropicalis* and *C. krusei*. Primers selection was based on (1) annealing temperature (above 54°C), to ensure reproducibility and specificity, (2) size of DNA fragments, to allow multiplexing and differentiation of species (different species would have PCR products with different molecular weights) and (3) number of nucleotides of the repetitive motif (trinucleotidic repeats), to facilitate alleles differentiation.

The microsatellites selected for locus specific amplification, and the respective primers pairs (forward and reverse) are present in Table II.II. The selected reverse primers were fluorescently labelled with hexachlorofluorescein (HEX), 6-carboxyfluorescein (FAM) or ortetrachloro-6-carboxyfluorescein (TET) for DNA size determination in automatic sequencer ABI 310 (Applied Biosystems).

Table II.II. Sequences and characteristics of the microsatellite loci selected.

Specie	STR	Motif	Primer Sequence (5' to 3')	Size (bp)	Ta (ºC)	Ref.
C. albicans	CAIII	$(GAA)_n$	[a]F-TTG GAA TCA CTT CAC CAG GA R-TTT CCG TGG CAT CAG TAT CA	95-110	60°C	(Sampaio *et al.* 2005)
C. parapsilosis	Cp1	$(AAG)_{27}$	F-AAA GTG CTA CAC ACG CAT CG R-GGC TTG CAA TTT CAT TTC CT	207-270	62°C	(Sabino *et al.* 2010)
C. glabrata	2bis	$(AAC)_6$	F-ACA CCT ACG AGA AAC CAA CA R-TAG CGG TCA TCC AGC ATC A	127-138	65°C	(Brisse *et al.* 2009)
C. tropicalis	CT14	$(CAT)_n$	F-GTA AAT CTT GTA TAC CGT GGA R-TAG CCC ATT TTC TAG TTT TGC	140-160	55°C	(Desnos-Ollivier *et al.* 2008)
C. krusei	CKTNR	-	F-ACA GCA GTC GCA GGC CC R-GTC GGA GAC ATA ACC GC	200-260	58°C	(Shemer *et al.* 2001)

a) In the start of each primer, F means the forward primer and R the reverse primer.

2.3. Colony-PCR

For microsatellite amplification in PCR reaction colony-PCR was performed. Colony-PCR is a simple and fast methodology that uses directly the colony in the PCR tube to obtain the DNA template. To perform this technique, a single colony was picked with a micropipette tip and added to PCR microtube. For DNA release, the cells were lysed by thermal shock during 90

seconds in the microwave, and the microtubes were immediately placed in the ice to inhibit DNA degradation (Vaz *et al.* 2011). A volume of 10 µl of the PCR reaction mix was added and the PCR performed as described below.

2.3.1. PCR amplification conditions

(1) Singleplex amplification

In order to evaluate the locus specific amplification and compare DNA fragment sizes with previously described results, singleplex amplification was performed for all selected microsatellite loci. A total of five *C. albicans* strains (S038, S040, S085, S090 and IGC 3436T), five *C. parapsilosis* (2551, 2552, 2554, 2556 and 2557), five *C. krusei* (109/RN0000.001, H11, 535, Cipo 94 and IGC 3341T), five *C. glabrata* (M2, 177, H38, 70V and IGC 2418T) and five *C. tropicalis* 2D, 2D, 4D, Cipo 43 and IGC 3097T) were used. The singleplex amplification was performed according to the conditions described in the literature (Shemer *et al.* 2001; Sampaio *et al.* 2005; Desnos-Ollivier *et al.* 2008; Brisse *et al.* 2009; Sabino *et al.* 2010). Briefly, the PCR reaction mix was performed by combining 1x PCR Buffer (20mM TrisHCl [pH 8.4], 50mM KCl), 0.2mM of each of the four deoxynucleotide triphosphates (dNTPs), 2mM $MgCl_2$, 1 U of *Taq* polymerase and the respective concentration of each primer carried in a 10 µl final volume. The samples were amplified in UNO II thermocycler (Biometra®) and the PCR program consisted of an initial denaturation step for 5 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at respective annealing temperature, 1 min at 72°C for, and with a final extension step of 10 min at 72°C.

(2) Multiplex amplification

Initially the multiplex PCR was tested using the same strains amplified by singleplex PCR. Multiplex PCR reaction mix was performed by combining 1x PCR Buffer (20mM TrisHCl [pH 8.4], 50mM KCl), 0.2mM of each of the four deoxynucleotide triphosphates (dNTPs), 2mM $MgCl_2$, 1 U of *Taq* polymerase and 0.2 µM of each primer carried in a 10 µl final volume. The samples were amplified in iCycler Thermal Cycler (BIO-RAD) and the PCR program consisted of an initial denaturation step for 7 min at 95°C, followed by 30 cycles of 45 s at 94°C, 30 s at 64°C, 1 min at 72°C for, and with a final extension step of 10 min at 72°C.

2.4. DNA Sequence Analysis and Fragment Size Determination

For DNA sequence analysis, 2.5 µl of each PCR product was added to 12.5µl of mixture of formamide and the internal size standard (GeneScan 500 6-carboxytetramethylrhodamine [TAMRA]; Applied Biosystems Inc.), and PCR fragments were separated by capillary electrophoresis with POP4 polymer in the automatic sequencer ABI 310 (Applied Biosystems Inc.). The results were analysed using the GeneScan 3.7 Analysis Software and fragment sizes of the PCR products were determined automatically using the same software.

2.5. Multiplex PCR optimization

Following PCR multiplex evaluation, the PCR conditions were optimized and the results obtained were always compared with the ones observed in the singleplex assay. The parameters optimized include the amplification cycles, annealing temperature (55°C, 58°C, 60°C, 62°C and 64°C), magnesium chloride ($MgCl_2$) concentration (2mM, 2.5mM and 3mM), primers concentration (from 1.5µ to 4.0µ), *Taq* polymerase and PCR adjuvants (Tween 20, BSA and DMSO). After optimization the PCR conditions, multiplex PCR was tested using the strains present in Table II.I.

3. Results and discussion

3.1. Microsatellite selection

In the present study a new multiplex PCR protocol for the rapid and simultaneous identification of the most clinically important *Candida* species: *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei* was developed. For this purpose primer pairs previously designed, for specific amplification of *C. albicans, C. parapsilosis, C. glabrata, C. krusei* and *C. tropicalis* microsatellites loci, were selected.

The microsatellite polymorphism analysis is a commonly used methodology for strains differentiation. However, this work entails species identification and the microsatellite selection was based on the characteristic of these markers that is its species specificity. Microsatellite markers are designed to be species specific and the majority are located on non-coding

regions. Since this regions of the genome are more prone to accumulate mutations, the discrimination of closely related species its even more probable.

Thus, we selected microsatellite marker of each species according to three main criteria (1) specificity, (2) size of the expected amplified fragments and (3) the repeated motif should be at least a trinucleotide. The specificity is obviously important as stated before. The DNA fragments size is crucial in planning a multiplex strategy, since differences in the size of the alleles amplified at each of selected loci and the possibility of combining different fluorescent dyes will make possible the simultaneous amplification. Ideally, all the primer pairs in a multiplex PCR should enable similar amplification efficiencies for their respective target. This may be achieved through the utilization of primers with nearly identical optimum annealing temperatures (Henegariu *et al.* 1997).

Several microsatellite loci have been identified in the *C. albicans* genome, such as EF3 (Bretagne *et al.* 1997), HIS3 or CDC3 (Botterel *et al.* 2001), however, in the last years our work group identified and described new microsatellite loci with great specificity and stability (CAI, CAIII, CAIV, CAV, CAVI, CAVII and CAVIII) (Sampaio *et al.* 2003; Sampaio *et al.* 2005).

In order to select the ideal microsatellite locus for *C. albicans* identification, four primer pairs were tested by singleplex amplification, namely CAI, CAIII, CAIV and CAVIII. The most studied repetitive region is CAI, which is an imperfect trinucleotidic microsatellite ($(CAA/G)_n$) located in RLM1 gene ((Sampaio *et al.* 2009). The repetitive region CAVIII is composed by trinucleotidic repeats ($(CAA/G)_n$) and is also located in a coding region, which encodes an extracellular secreted aspartyl proteinase (SAP8) with unknown function. On the other hand CAIII and CAIV are composed by perfect trinucleotidic motifs and are located in a non-coding region (Sampaio *et al.* 2005).

The microsatellite loci amplification was performed using previously described conditions (Sampaio *et al.* 2005). CAI, CAIII and CAIV forward primers were labelled with 6-carboxyfluorescein (FAM) and CAVIII forward primer was labelled with hexachlorofluorescein (HEX). An example of GeneScan profile obtained with amplification of the four loci is shown in Figure 2.1.

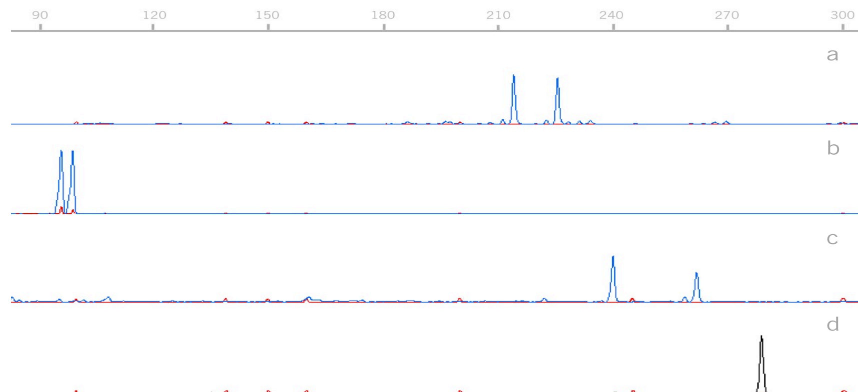

Figure 2.1. GeneScan profiles obtained in a singleplex analysis using the markers (a) CAI, (b) CAIII, (c) CAIV and (d) CAVIII for *C. albicans.*

After this analysis the microsatellite marker selected for *C. albicans* identification was CAIII. The criteria defined for this selection were the species specific amplification demonstrated in previous studies, the small size range (95 to 110 bp) as well as the small molecular weight (Sampaio *et al.* 2005). Another selection factor was the high annealing temperature above 60°C, inhibiting the development of secondary structures. CAI presented a great polymorphism being ideal for strains differentiation, and it was not selected due to the large size range of the alleles, between 189-303 base pairs (Sampaio *et al.* 2003). CAIV and CAVIII were also not selected since their size range overlaps with the other markers (Cp1 and CKTNR) size ranges (described ahead) (Sampaio *et al.* 2005).

Although several microsatellites of *C. parapsilosis* have been described (Lasker *et al.* 2006; Pulcrano *et al.* 2012), the microsatellite selected for this study was Cp1. This repetitive region was described by our study group and is located in a non-coding region (Sabino *et al.* 2010). The main criteria for primer selection were its great specificity and reproducibility as well as the high annealing temperature (62°C).

For other *Candida* species, the microsatellite selection was based on the literature. Some of the microsatellites of *C. glabrata* described are RPM2, ERG3, MTI (Foulet *et al.* 2005), Cg4, Cg10 (Abbes *et al.* 2012), among others. The repetitive region selected for specific amplification was 2bis described by Brisse and co-authors (Brisse *et al.* 2009). The criteria defined for this selection was the specificity as well as the molecular weight and range of PCR products (between 127 and 138), differing from the products obtained in other species. Another criteria for 2bis selection was the annealing temperature, of 65°C that allows the multiplex to be carried out at a Ta above 60°C.

A few microsatellites were described in the literature for *C. tropicalis* and *C. krusei* species. Desnos-Ollivier and co-workers (Desnos-Ollivier *et al.* 2008) described two *C. tropicalis* microsatellite loci, URA3 (located in a coding region) and CT14 (located in a non-coding region). In this study the locus CT14 was selected due to the fact that was located in a non-coding region. However, the described annealing temperature of CT14 was only 55°C. In *C. krusei* genome only one microsatellite, CKTNR, was described, but no information regarding its genome location is available (Shemer *et al.* 2001). The annealing temperature described for amplification of this microsatellite was 58°C.

After the microsatellite loci selection the reverse primers for each species was fluorescently labelled with different dyes to allow simultaneous amplification and identification of the specific PCR products. The reverse primers of *C. albicans* and *C. krusei* were labelled with FAM, *C. parapsilosis* and *C. glabrata* with TET and *C. tropicalis* with HEX (Figure 2.2).

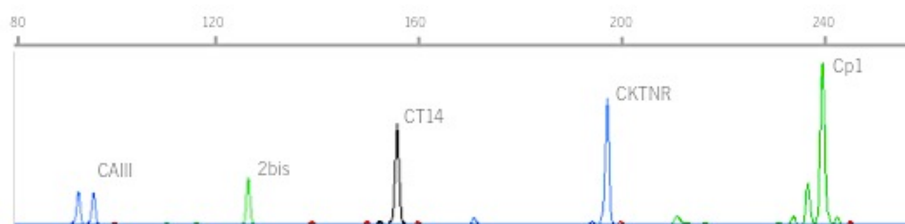

Figure 2.2. GeneScan profile of PCR products amplified with different primer pairs.

3.2. Singleplex amplification

All selected microsatellite were used to amplify, in singleplex, five strains of each species, in order to evaluate the locus-specific amplification.

The singleplex PCR was performed in a UNO II thermocycler (Biometra®) and the amplification of CAIII, Cp1, 2bis and CKTNR loci was successful. However, no GeneScan profiles were obtained with the CT14 locus in any of the analysed strain. In order to observe if the absence of GeneScan profiles was due to the fluorescent dye ineffectiveness an agarose gel electrophoresis 1.2% was performed and no amplification products were found in the gel, confirming the absence of amplification.

The singleplex PCR was performed again with the same conditions, however, using the iCycler (Bio-Rad™), and the amplification of all selected loci was observed (Figure 2.3).

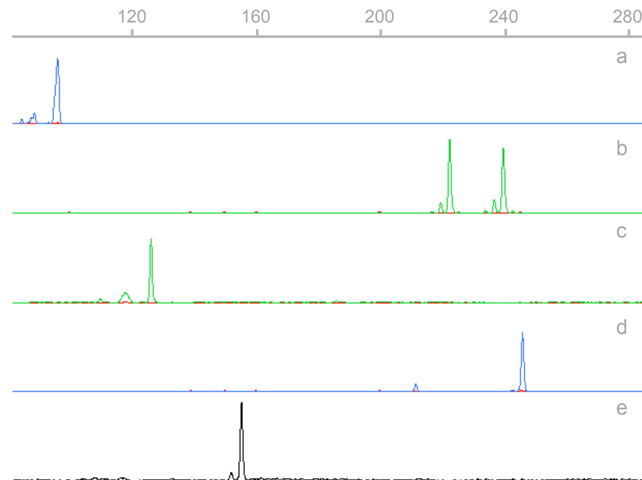

Figure 2.3. GeneScan profiles obtained with (a) CAIII, (b) Cp1, (c) 2bis, (d) CKTNR and (e) CT14 in strains S038 (*C. albicans*), 2257 (*C. parapsilosis*), 70V (*C. glabrata*), 109/RN0000.001 (*C. krusei*) and 2D (*C. tropicalis*), respectively, by singleplex PCR amplification.

The main reason proposed for the differential amplification is the difference in the ramp rates (heating and cooling) of both thermocyclers. The ramp rate consists in the time that thermocycler takes to change the temperature between PCR stages. A quicker temperature change improves PCR results, thus the results obtained suggest that iCycler presents lower ramp rates, improving the CT14 locus amplification.

Molecular weights of PCR products obtained were compared with results previously described in the literature and similar molecular weights were identified in this study, confirming the effectiveness of this methodology. The expected differences between the molecular weights of PCR products obtained in the five different species ensure the possibility of simultaneous amplification and identification.

Although the specificity of these molecular markers was well established, this feature was confirmed since no amplification products were obtained when the primers and PCR conditions described were tested with strains from different species. Not even the closest related species of *C. parapsilosis* amplified with *C. metapsilosis, C. orthopsilosis* or *L. elongisporus* or the primers for *C. glabrata* amplified *C. bracarensis.*

3.3. Multiplex amplification

Amplification of strains from the five *Candida* species was performed by multiplex PCR with the selected primers in order to test the applicability of this methodology in the identification of the

most frequent species isolated from clinical samples. For this step the same strains tested in singleplex amplification were used and the results obtained compared. The amplification was performed using the iCycler (Bio-Rad™).

The PCR products obtained in multiplex amplification of *C. albicans, C. parapsilosis, C. glabrata* and *C. krusei* loci were similar to those obtained in singleplex amplification, confirming the specificity of the method. However, no PCR products were obtained in the analysis of *C. tropicalis* strains (Figure 2.4).

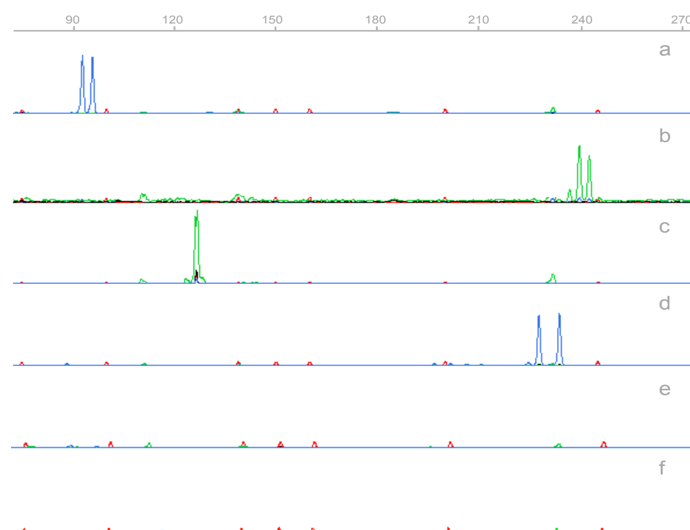

Figure 2.4. GeneScan profiles obtained by multiplex amplification with (a) *C. albicans,* S040, (b) *C. parapsilosis,* 2252, (c) *C. glabrata,* M2, (d) *C. krusei,* H11, (e) *C. tropicalis,* 2D and (f) *L. elongisporus* strains, ISA 1421.

The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining unspecific results, because of the formation of primer dimers. In order to evaluate if the absence of CT14 locus amplification was due to the primer dimer formation, AutoDimer Check 1.0 software was used (Vallone and Butler 2004), however this feature was not observed.

It is thought that several factors can affect the PCR amplification, particularly in multiplex analysis. Some of these factors are the amplification cycles, annealing temperature, magnesium chloride ($MgCl_2$) concentration, primers concentration, *Taq* polymerase and PCR adjuvants (Markoulatos *et al.* 2002). In order to verify the influence of these factors in *C. tropicalis* CT14 locus in the multiplex amplification, an optimization of PCR conditions was performed.

3.4. Optimization of multiplex amplification conditions

The PCR conditions represent an important factor to obtain reliable results particularly when several loci are analysed simultaneously. Adverse multiplex PCR conditions can present several difficulties, including poor sensitivity and specificity, and/or preferential amplification of certain specific targets. In these cases, the optimization of PCR conditions is needed (Markoulatos *et al.* 2002).

In this study, several PCR conditions were tested in order to amplify the locus CT14 in multiplex reaction and also to improve the multiplex methodology. For this optimization step three *C. tropicalis* strains and one strain from *C. albicans, C. parapsilosis, C. glabrata* and *C. krusei* were used.

3.4.1. Annealing temperature

The annealing temperature (Ta) is one of the most important parameters in PCR amplification and depends on length and composition of the primers selected. Comparing with the primer optimal temperature, if the Ta is too high the binding between primers and DNA may not occur, and if Ta is too low the development of nonspecific products can occur (Kramer and Coen 2006).

In order to inhibit the nonspecific products development the Ta used in multiplex amplification was 64°C, however, Desnos-Ollivier and co-authors (Desnos-Ollivier *et al.* 2008) described that the optimal temperature for CT14 primers annealing is 55°C. Thus, the absence of CT14 locus amplification could be due to the higher Ta. The amplification using a gradient temperature in singleplex PCR was performed to verify the ideal temperature for CT14 locus amplification. In this study, five annealing temperatures were tested, namely 55°C, 58°C, 60°C, 62°C and 64°C (Figure 2.5).

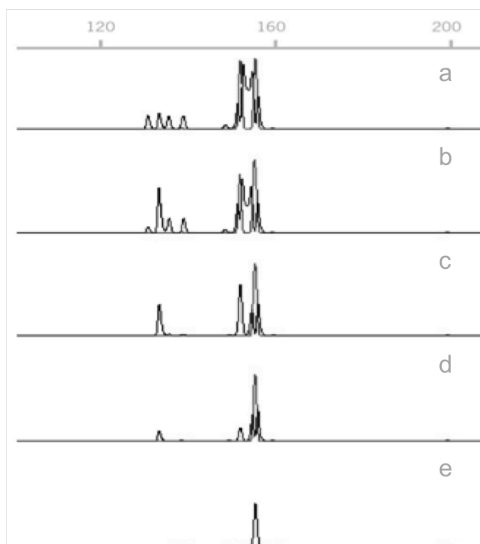

Figure 2.5. GeneScan profile of *C. tropicalis* strain (2D) amplified with CT14 at different annealing temperatures (a) 55°C, (b) 58°C, (c) 60°C, (d) 62°C and (e) 64°C.

The results obtained demonstrated that in singleplex amplification the CT14 locus was observed in all annealing temperatures selected. It was also demonstrated that Ta of 64°C ensures less secondary structures development. These results indicate that the absence of CT14 locus amplification was not due to the annealing temperature, and that 64°C is the optimal temperature for multiplex PCR amplification of selected loci.

No attempt to change the annealing temperature in a multiplex reaction was made since several secondary structures were observed in lower Ta and that would difficult the interpretation of the results.

3.4.2. Amplification cycles

The PCR amplification is composed by 20-40 cycles, and each cycle consists of different amplification stages (denaturation, annealing and extension/elongation). The number of cycles and the time of each stage depend on several parameters such as polymerase, ions and dNTPs concentration and the melting temperature of the primers (Mullis and Faloona 1987). The denaturation step represents an important factor in PCR amplification since it promotes the separation of DNA strands. The denaturation time needs to ensure the total separation of DNA strands, however, a long time can reduce the polymerase half-life. The temperature and the denaturation time depend on the template used, essentially to the amount of guanine and cytosine (Kramer and Coen 2006).

In order to verify if the absence of CT14 locus amplification in multiplex was related with the denaturation step an optimization of this feature was performed. Firstly, the PCR program consisted of an initial denaturation step for 5 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 64°C, 1 min at 72°C, with a final extension step of 10 min at 72°C. The multiplex PCR amplification was then tested changing the initial denaturation step to 7 minutes and the denaturation cycle to 45 seconds.

The results demonstrated that denaturation time was not related with the absence of CT14 locus amplification since no amplification products were found. However, the new denaturation conditions were considered since it improved the profiles obtained in the amplification of the other loci, removing same secondary structures of the PCR products.

3.4.3. Primers concentration

The optimal primers concentration depends essentially on the concentration and complexity of DNA template. The primers concentration should always be adjusted since higher concentrations can lead to secondary structures or primer dimers formation, while lower concentrations can lead to unsatisfactory amplification. Hot start PCR often eliminates nonspecific reactions (production of primer dimers) caused by primer annealing at low temperature (4–25°C) before initiating thermocycling (Koreth *et al.* 1996).

In multiplex amplification the total primer concentration was 2μM. However, in order to optimize primer concentration several concentrations were tested (Table II.III).

Table II.III. Primers concentrations tested in multiplex amplification.

Primers pairs	First conc. (μM)	Second conc. (μM)	Third conc. (μM)
CAIIIF+CAIIIR	0.4	0.8	0.3
Cp1F+Cp1R	0.4	0.8	0.3
2bisF+2bisR	0.4	0.8	0.3
CKTNRF+CKTNRR	0.4	0.8	0.3
CT14F+CT14R	0.4	0.8	0.3
Total	2	4	1,5

No CT14 locus amplification was observed in multiplex when 2, 4 or 1,5μM of total primer concentrations were used. However, with 4μM an increase of secondary structures was observed whereas with 1.5μM the decrease of the CAIII, Cp1, 2bis and CKTNR amplification products was observed. The obtained results demonstrated that ineffectiveness CT14 locus

amplification was not related with the primer concentration. It was also demonstrated that 2μM was the optimal primer concentration for CAIII, Cp1, 2bis and CKTNR amplification.

3.4.4. Magnesium Chloride ($MgCl_2$) concentration

The optimization of $MgCl_2$ concentration is an important procedure for successful PCR reaction since Mg^{2+} is a cofactor required for *Taq* polymerase activity. Each PCR reaction has an optimal concentration of Mg^{2+} and a low concentration of Mg^{2+} does not enable the polymerase activity, reducing the amount of product. Moreover, a high Mg^{2+} concentration stabilizes the DNA double strand, preventing the complete denaturation of DNA, and stabilizes unspecific annealing of primer to incorrect template sites, decreasing specificity. The dNTPs bind to free Mg^{2+} in a 1:1 molar ratio, so the $MgCl_2$ concentration must be proportional to the concentration of dNTPs (Ely *et al.* 1998; 2011).

In order to verify if the absence of CT14 locus amplification was due to the $MgCl_2$ concentration three concentrations were tested, namely 2mM, 2.5mM and 3mM.

No CT14 locus amplification was observed using the three $MgCl_2$ concentrations, demonstrating that the absence of amplification was not related with Mg^{2+} concentration. The results also demonstrated that no significant differences were observed in the amplification of the other selected loci using the three concentrations. Figure 2.6 gives an example of amplification with different $MgCl_2$ concentrations. For this reason, the concentration selected for the multiplex amplification was 2mM.

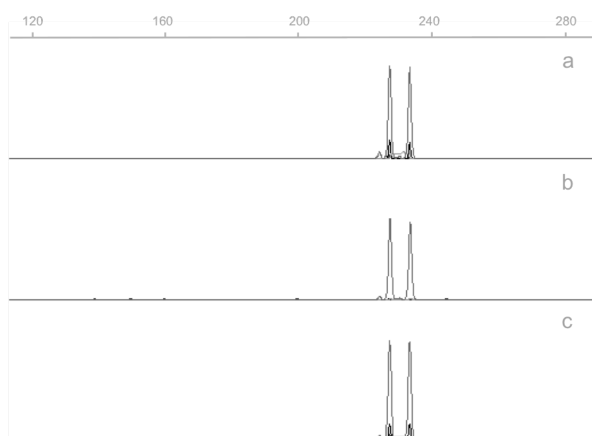

Figure 2.6. GeneScan profiles of *C. krusei* H11 strains obtained with multiplex reaction using (a) 2.0mM, (b) 2.5mM and (c) 3.0mM of $MgCl_2$.

3.4.5. PCR additives

Several studies demonstrated that PCR adjuvants have a beneficial effect on the yield of PCR amplification of purified DNA. The most common additives used are organic co-solvents (dimethyl sulfoxide (DMSO) or formamide), reducing compounds (dithiothreitol (DTT)), non-ionic detergents (Triton X-100, Tween 20), stabilising proteins (Bovine serum albumin (BSA)), and glycerol (Nagai *et al.* 1998; Ralser *et al.* 2006). Three of the most studied adjuvants, DMSO, Tween 20 and BSA were tested to improve the multiplex reaction. DMSO has been used to increase the yield of a PCR reaction on GC rich DNA templates, by preventing the formation of secondary structures. However, more than 10%DMSO can reduce the polymerase activity. BSA has been applied to increase the thermal stability and half-life of the enzymes and neutralize inhibitory contaminants that may be present in the DNA (Farell and G. 2012). Finally, the Tween 20 improves the polymerase stability and suppresses the formation of secondary structures (Ralser *et al.* 2006).

Rasler and co-workers (Ralser *et al.* 2006) also demonstrated that the use of BSA combined with DMSO increases the range of organic solvent effectiveness. The concentrations of DMSO, BSA and Tween 20 used in this study were selected according Rasler and co-authors (Ralser *et al.* 2006), namely 1.2% DMSO, 10μg/μl BSA and 0.01% Tween 20. The three additives and combination of additives tested in our study are presented in Table II.IV.

Table II.IV. Additives and combinations tested.

Additives
0.01% Tween 20
10μg/μl BSA
1.2% DMSO
0.01% Tween 20 + 1.2% DMSO
1.2% DMSO + 10μg/μl BSA
0.01% Tween 20 + 1.2% DMSO + 10μg/μl BSA

No CT14 locus amplification was observed in all conditions tested. No significant differences were also observed in CAI, Cp1, 2bis and CTKNR loci amplification. The results obtained demonstrated that the absence of CT14 locus amplification was independent of the use of PCR additives. Also demonstrated that the adjuvants did not influence the amplification of the other loci analysed in this study.

Several factors are involved in PCR amplification and the optimization of other factors, such as *Taq* polymerase or dNTPs concentration could be performed to promote the CT14 locus amplification, however it seamed more reasonable to design new primers for the CT14 locus. Four new primer pairs were designed in the non-variable flanking regions of CT14 locus using Primer 3 Input 4.0 software. These primers were analysed in AutoDimer Check 1.0 software to observe the primer dimer formation and only one primer pair was selected, namely F-5' CCCCACCAAAAACATACATACAT 3' and R-5' TTACATTCAGCCCGCCACAG 3'. However, due to the lack of time, these primers were not yet tested.

3.5. Multiplex amplification (without CT14 primers pair)

The same strains analysed by singleplex amplification were tested using the multiplex mix without CT14 primers. The molecular weights obtained by multiplex analysis were compared with previous singleplex results and the same peaks were observed in both profiles. Although the molecular weights obtained were the expected ones, the intensity of amplification of the different markers was unbalanced. In these cases, the primer concentration should be optimized since the more efficiently amplified loci would negatively influence the yield of product amplification from the less efficient loci. This feature is due to the fact that PCR has a limited supply of enzyme and nucleotides, and all products compete for the same pool of supplies (Markoulatos *et al.* 2002). The total primers concentration used was 2μM equally divided by all primers pairs, however, a low intensity of amplification of locus CAIII and Cp1 and a high intensity of amplification of locus 2bis was observed.

In order to balance the intensity of amplification of different loci, the primers concentration was adjusted, according to Table II.V.

Table II.V. Concentration of each primer pairs used in multiplex reaction.

Primers pairs	Initial concentration (μM)	Final concentration (μM)
CAIIIF+CAIIIR	0.5	0.6
Cp1F+Cp1R	0.5	0.6
2bisF+2bisR	0.5	0.3
CKTNRF+CKTNRR	0.5	0.5
Total	2	2

Using the new primers concentration an increase of intensity of amplification of CAIII and CP1 loci as well as a decrease of intensity of amplification of 2bis locus was observed (Figure 2.7). The results obtained demonstrated that the use of the new primers concentration improved the quality of amplification of all selected loci.

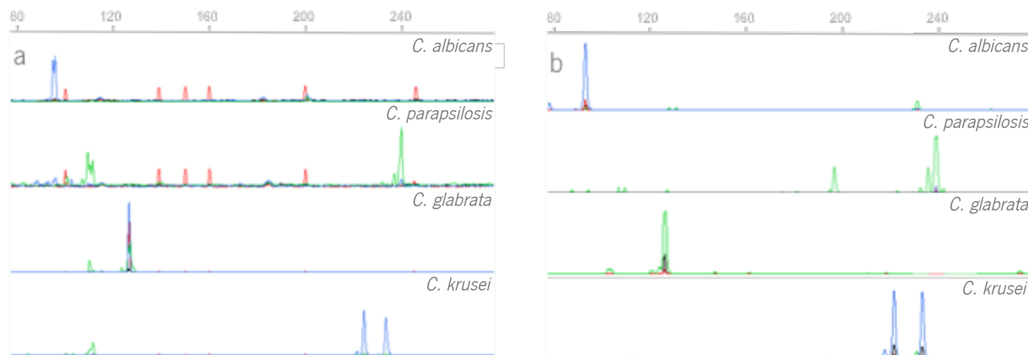

Figure 2.7. GeneScan profiles of *C. albicans, C. parapsilosis, C. glabrata* and *C. krusei,* using the (a) initial primer concentrations and the (b) adjusted primer concentrations.

The multiplex mix with new primer concentrations was then used with the forty-six strains previously identified in order to evaluate their specific amplification. The species identification was essentially based in molecular weights/fluorescent label of PCR products obtained. The results of multiplex analysis showed the same species identification for all strains as the previously reported, confirming the specificity of the method (Table II.VI).

The specificity was also confirmed by simultaneous amplification of two strains in the same reaction, for example *C. albicans* (S085) and *C. glabrata* (M2), as well as the amplification of one strain covered by the multiplex and a second that was not included in the multiplex, for example *C. albicans* (S085) and *C. orthopsilosis* (H10USA).

The mix developed was also tested in the twenty strains without previous identification, isolated from hands and saliva of healthy patients. No amplification products were obtained in ten of the twenty unknown strains, indicating that they did not belonged to range of species covered by the multiplex. The ten remaining strains amplified with primer CAIII were suspected to be *C. albicans* (Table II.VI).

Table II.VI. Isolates tested with new multiplex mix, genotypes obtained e respective identification.

Isolate	Genotype	Dye	Identification	Isolate	Genotype	Dye	Identification
S085	95-95	FAM	*C. albicans*	Cipo 94	227-233	FAM	*C. krusei*
S092	107-107	FAM	*C. albicans*	IPO A911012	-	-	-
S104	95-98	FAM	*C. albicans*	960161	-	-	-

S040	92-95	FAM	*C. albicans*	Ana R.	-	-	-
S038	95-95	FAM	*C. albicans*	O113	-	-	-
OB15V	98-98	FAM	*C. albicans*	HSM CAN 155	-	-	-
Cipo72	92-95	FAM	*C. albicans*	J981226	-	-	-
1V	95-101	FAM	*C. albicans*	HSM CAN 138	-	-	-
2551	238-241	TET	*C. parapsilosis*	H10 USA	-	-	-
2252	223-238	TET	*C. parapsilosis*	154a	-	-	-
2253	238-238	TET	*C. parapsilosis*	1 All	-	-	-
2256	241-241	TET	*C. parapsilosis*	CL-7030	-	-	-
2257	223-238	TET	*C. parapsilosis*	246188	-	-	-
M2	127	TET	*C. glabrata*	NCYC 3133	-	-	-
177	127	TET	*C. glabrata*	ISA 1421	-	-	-
H38	127	TET	*C. glabrata*	IGC 3436T	95-95	FAM	*C. albicans*
70V	127	TET	*C. glabrata*	IGC 2418T	127	TET	*C. glabrata*
24/9-10	127	TET	*C. glabrata*	IGC 3097T	-	-	-
14666a	127	TET	*C. glabrata*	153MT	-	-	-
14666b	127	TET	*C. glabrata*	IGC 3341T	197-209	FAM	*C. krusei*
14408	127	TET	*C. glabrata*	CDQN5	110-110	FAM	*C. albicans*
24/9-4	127	TET	*C. glabrata*	CDQN10	98-98	FAM	*C. albicans*
7/5-17	127	TET	*C. glabrata*	CD1	95-95	FAM	*C. albicans*
21/9-26	127	TET	*C. glabrata*	CD2	95-98	FAM	*C. albicans*
21/9-20	127	TET	*C. glabrata*	S152	95-101	FAM	*C. albicans*
1/4-22a	127	TET	*C. glabrata*	S153	110-110	FAM	*C. albicans*
23/9-24	127	TET	*C. glabrata*	D1	-	-	-
6/9-17	127	TET	*C. glabrata*	APC1	-	-	-
27/5-16	127	TET	*C. glabrata*	APC2	-	-	-
17/3-3	127	TET	*C. glabrata*	APC3	-	-	-
14573	127	TET	*C. glabrata*	APC4	-	-	-
14735	127	TET	*C. glabrata*	APC5	-	-	-
Cipo43	-	-	-	APC6	-	-	-
176C	-	-	-	APC7	-	-	-
10F4	-	-	-	C1	110-110	FAM	*C. albicans*
2D	-	-	-	C2	-	-	-
3D	-	-	-	C3	95-95	FAM	*C. albicans*
4D	-	-	-	C4	95-95	FAM	*C. albicans*
109/RN0000.001	245-245	FAM	*C. krusei*	C5	-	-	-
H11	227-233	FAM	*C. krusei*	1432	95-98	FAM	*C. albicans*
535	224-233	FAM	*C. krusei*				

- no data was obtained for these strains.

In order to confirm the results obtained, a multiplex PCR for *C. albicans* strains differentiation described by Sampaio and co-workers (Sampaio *et al.* 2005) was used. All of the ten strains were amplified using the *C. albicans* multiplex and their identification was confirmed.

The strains that did not showed amplification with the multiplex are being further analysed with other techniques in order to specifically identify then at the species level.

In conclusion, the specific amplification of all selected strains, as well as the ability of simultaneous amplification, by combining different molecular weights and different fluorescent dyes demonstrated that the methodology developed is a fast and accurate alternative in clinical microbiology laboratories. The study also demonstrated that PCR methodologies are dependent of several conditions, and the optimization of the process is essential.

4. Conclusion and final remarks

The rapid and accurate identification of the species involved in *Candida* infections is of extreme relevance to the development and application of the correct therapeutic strategies, once different species present different susceptibility to the antifungal agents (Lass-Florl 2009). In clinical practice fungal species differentiation involves morphological, physiological and biochemical assays, however these techniques requires three or more days and may be inaccurate. The fast and accurate diagnosis can be improved by molecular approaches, such as PCR based methods, which represent an excellent alternative for methodologies used. The PCR based methods have several advantages over the other methodologies used, since PCR is less time-consuming, the results can be easily reproduced and is suitable for screening large number of isolates with reduced workload (Ellepola and Morrison 2005).

In clinical laboratories, the PCR based technique used for *Candida* species diagnosis is the Real Time PCR. The commercial kits developed do not required prior yeast culture and the amplification is performed directly from clinical samples, especially from blood samples (Casalta *et al.* 2009). Although this is a rapid methodology, this technique is mainly based in the melting curves differences of the amplification products, the 18S and 5.8S rRNA sequences are the most used targets, which are less sensitive than the analysis of species-specific markers, such as microsatellite loci. The use of nonspecific targets can also increase the appearance of nonspecific signals from environmental microorganisms from laboratory contamination. This risk is present in several steps of the PCR procedure, from the blood sampling to the performance of the PCR assay (Lehmann *et al.* 2008). Moreover, the use of clinical samples has several disadvantages, including the presence of large amounts of host nucleic acid that can interfere with primer hybridization and amplification or the presence of

inhibitors of *Taq* DNA polymerase, such as EDTA (Peters *et al.* 2004). Microsatellites are found in all genomes and has being designated as excellent molecular markers for fungal species/strains differentiation. Several microsatellites were successfully used to characterize and rapidly type isolates of different fungal species, such as *Penicillium marneffei* (Fisher *et al.* 2004; Lasker and Ran 2004), *Saccharomyces cerevisiae* (Legras *et al.* 2005), *Aspergillus fumigatus* (Vanhee *et al.* 2008; Araujo *et al.* 2009), *C. albicans* (Bretagne *et al.* 1997; Sampaio *et al.* 2005), *C. parapsilosis* (Sabino *et al.* 2010) or *C. glabrata* (Foulet *et al.* 2005; Brisse *et al.* 2009). Their main advantage over the 18S and 5.8S rDNA is the possibility of designing highly specific primers and since these regions are under less tight selection pressure accumulates more mutation, enabling the discrimination of closely related species.

The development of multiplex systems, co-amplifying several STRSs, in order to test rapidly and reproducibly a great number of isolates represents and important tool in biomedical mycology (Rosehart *et al.* 2002; Illnait-Zaragozi *et al.* 2010; L'Ollivier *et al.* 2012). In this work a new multiplex methodology was developed to identify the most clinically important *Candida* species (*C. albicans, C. parapsilosis, C. glabrata, C. tropicalis* and *C.* krusei), and although no CT14 locus (*C. tropicalis*) amplification was observed, this methodology showed a high stability and capacity to discriminate the four different *Candida* species. The Colony PCR overcomes some limitations such as the need for DNA extraction (Mirhendi *et al.* 2007). The methodology developed is easy to perform and can be implemented at relatively low cost for routine identification in hospitals and health centres.

The multiplex system developed showed to be a fast and accurate method, however, several difficulties were found with *C. tropicalis* selected locus. *C. tropicalis* infections have been reported in immunocompromised patients with chronic mucocutaneous candidiasis and have progressively been observed to be the cause of invasive candidiasis in neutropenic patients (Kothavade *et al.* 2010). Thus, it is imperative to include *C. tropicalis* identification in this methodology testing the new primers pair specific for CT14 locus already designed. Given the need of new fast and accurate diagnostic methodologies in microbiology laboratories, the test of clinical samples from different Hospitals and Health Centres would be relevant to determine the applicability of this methodology in clinical practice. Thus, this multiplex system will also be tested directly in biological samples to test for its applicability in clinical laboratories.

CHAPTER III

Genotypic differentiation of *C. albicans* lineages by microsatellite loci analysis

1. Introduction

Candida albicans is commensal yeast present in the mucosal surfaces of genitourinary system, gastrointestinal tract, skin and oral cavity in humans (Guarro *et al.* 1999; Hube 2004). In immunocompromised and intensive care patients is the most common opportunistic yeast pathogen, causing several superficial or systemic candidiasis (Sabino *et al.* 2010; Spiliopoulou *et al.* 2011), with high level of morbidity and mortality (Fridkin and Jarvis 1996; Weinberger *et al.* 2005). One major problem of *C. albicans* infections is the variable virulence of this species attributed to several virulence factor (Romani *et al.* 2003), like adhesion capacity, phenotypic switching, hyphal formation and secretion of extracellular hydrolytic enzymes (Ramage *et al.* 2012), which promotes the flexibility to resist to the immune system defences (Calderone and Fonzi 2001; Yang 2003). Extracellular hydrolytic enzymes include phospholipase A and B (Ghannoum 2000), lipases (Trofa *et al.* 2008) and secreted aspartyl proteinases (Saps), which are the most studied extracellular hydrolytic enzymes in this species (Naglik *et al.* 2003; Abegg *et al.* 2011). *C. albicans* Saps have been implicated in the development of systemic and mucosal infections, influencing adhesion, tissue damage and host immune responses evasion.

The success of pathogenic yeasts depends on their dynamic interactions with the host and the adaptive responses that enable them to escape/adjust to host defences. This is particularly important for commensal organisms, such as *C. albicans*, due to the diverse and polymorphic nature of the colonized host and environments that it can live on (Mavor *et al.* 2005). Microorganisms evolved mechanisms for increasing genetic variations in loci that are involved in critical interaction with the host. These alterations can be achieved by intergenomic or intragenomic events but the contribution of intergenomic in a clonal reproducing organism as *C. albicans* is limited. Within intragenomic mechanisms that generate hypervariability the addition or deletion of repeat units during replication, through slipped-strand mispairing or gene conversion is the best characterized.

The genome of the human pathogen *Candida albicans* contains approximately 2600 repeat-containing ORFs, three and ten times more, respectively, than those of the ascomycete yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Braun *et al.* 2005). Comparative genomic analyses of *C. albicans* strains suggest that repeat-containing ORFs may be important *C. albicans* fitness determinants (Zhang *et al.* 2009). To date, only a few of these genes have been characterized, including EAP1, PIR1 CEK1, HYR1, HYR2, HWP1, and the ALS (agglutinin

like sequence) family of adhesins (Hoyer 2001; Staab *et al.* 2004; Sumita *et al.* 2005; Li and Palecek 2008). RLM1 and SAP8 are two repeat-containing ORFs. Rlm1, this is one of the transcription factors of the cell wall integrity (CWI) pathway. Sampaio and co-workers (Sampaio *et al.* 2003; Sampaio *et al.* 2009) have demonstrated that Rlm1 presents a great variability at its C-terminus, conferred by the CAI microsatellite with more than 35 alleles identified. Phenotypic analysis of strains harbouring CAI alleles with higher number of (CAA/G) repetitions showed that they displayed a higher tolerance to cell wall stress agents, indicating that CAI repetitive region confers a high genetic variability to RLM1 gene, which is reflected in strain susceptibility to different stress conditions (Sampaio *et al.* 2009). SAP8 gene, encoding an extracellular SAP with a C-terminal consensus sequence typical for glycosylphosphotidylinositol (GPI) with unknown function, is transiently overexpressed in cases of oral and vaginal infection (Wu and Samaranayake 1999; Ripeau *et al.* 2002). This ORF also contains a microsatellite consisting of (CAA/G) repeat units at the C-terminus of the protein that has not been characterized.

In this context, we addressed the question of a possible correlation between the pathogenicity of lineages of *C. albicans* strains with regard to their genotype at these two repeat-containing ORFs.

2. Materials and methods

2.1. Yeast Strains

A total of 244 clinical isolates of *C. albicans,* obtained from Hospitals, Health Centres and Oral Clinics with different geographical origins were analysed in this study (Table III.I). Fifty-one clinical strains were isolated from saliva of patients diagnosed with oral infection, before antifungal treatment, 51 from the oral cavity of healthy volunteers, 42 from vagina, 27 from urine, 43 from upper respiratory tract, and 30 from blood cultures. Two *C. albicans* reference strains (WO-1 and PYCC 3436 (ATCC 18804)) and reference strains of *C. krusei* PYCC 3343 (ATCC 6358), *C. tropicalis* PYCC 3097 (ATCC 750), *C. lusitaniae* PYCC 2705 (ATCC 34449), *C. guilliermondi* PYCC 2730 (ATCC 6260) and *C. dubliniensis,* CBS 7987 (ATCC MYA-646) were also included in this study. The reference strains were obtained from the Portuguese

Yeast Culture Collection (PYCC), New University of Lisbon, Lisbon, Portugal, and from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

All strains were previously identified by their assimilation patterns on ID32C strips (BioMérieux, SA, Marcy-L'Étoile, France) and by PCR fingerprinting with primer T3B (Correia *et al.* 2004).

2.2. Growth conditions and PCR amplification

Yeast cells were grown at 30°C for 48 hours on YPD-agar medium (Yeast extract 1%, Bactopeptone 1%, Glucose 2% and Agar 2%). For microsatellite amplification, colony-PCR was performed. A single colony was picked with a micropipette tip, added to a microtube and the cells lysed by thermal shock during 90 seconds in microwaves (Ward 1992; Vaz *et al.* 2011). The microtubes were immediately placed in the ice, and 10µl of PCR reaction mix was added. This mixture included 1x PCR Buffer (20mM Tris HCl [pH 8.4], 50mM KCl), 0.2mM of each of the four deoxynucleoside triphosphates (dNTPs), 1.5mM $MgCl_2$, 0.25µM of each primer and 1 U of *Taq* polymerase. The primers for CAI locus amplification in *RLM1* locus are described in Sampaio et al. (Sampaio *et al.* 2003), while the specific primers used for SAP8 locus amplification were CAVIII-F:5'- TCCCTGAAGACATTGATAAAAGAGC-3' and CAVIII-R:5'-AGAATCAACCACCCATAAATCAGAA-3'. For automatic allele size determination, the CAVIII forward primer was 5' fluorescently labelled with hexachlorofluorescein (HEX) and CAI with 6-carboxyfluorescein (FAM).

The samples were amplified in UNOII Thermocycler (Biometra) with a pre-incubation step for 5 min at 95°C, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min, and with a final extension step of 10 min at 72°C.

2.3. Fragment Size Determination and DNA Sequence Analysis

Following amplification, 2.5 µl of each PCR product was added to 12.5µl of mixture of internal size standard (GeneScan 500 6-carboxytetramethylrhodamine [TAMRA]; Applied Biosystems Inc.), formamide, and PCR fragments separated in an ABI 310 Genetic Analyzer (Applied Biosystems Inc.). Fragment sizes were determined automatically using the GeneScan 3.5 Analysis Software.

2.4. Statistical analysis

The discriminatory power of the microsatellites markers CAI and CAVIII was expressed as a numerical index based on the probability that two unrelated isolates will be placed into different typing groups and calculated according to the method of Hunter and Gaston (Hunter and Gaston 1988).

All data were subjected to statistical analysis with the use of Statistical Package for Social Science (SPSS®) vers. 11.0, Chicago, IL, EUA. P values were calculated by analysis of variance, chi-square test or McNemar's correlation analysis with 95% of confidence interval (CI) and 5% significance level, using the software Genepop version 4.1.3 and allelic frequency was calculated use Populations version 1.2.28 (http://www.cnrs-gif.fr/pge).

Genetic distance between *C. albicans* strains was calculated using the Cavalli-Forza method with the Populations 1.2.30 software (Populations 1.2.30, Oliver Langella, Boston, MA. http://bioinformatics.org/~tryphon/population/). Clustering of the isolates was performed with NTSys software version 2.0 software (Applied Biostatistics Inc), by using the unweighted pair group method with arithmetic mean (UPGMA).

3. Results and discussion

3.1. Microsatellite locus analysis

In this work, the ability of CAVIII to differentiate strains was initially studied. The specific amplification and the polymorphism of CAVIII microsatellite in *SAP8* locus was tested using two reference strains and 181 unrelated *C. albicans* strains, including 51 from saliva of patients diagnosed with oral infection, 51 from the oral cavity of healthy volunteers, 42 from vagina, 20 from urine and 17 from upper respiratory tract (Table III.IV). It is known that *C. albicans* is diploid, and each obtained fragment was considered one allele, so the presence of two different fragments represents heterozigozity and the presence of only one fragment homozygozity. Less intense stutter bands were frequently present, reflecting polymerase slippage during the PCR, but they did not present any problem in the identification of the correct fragment since they were of a lower intensity (Fig. 3.1).

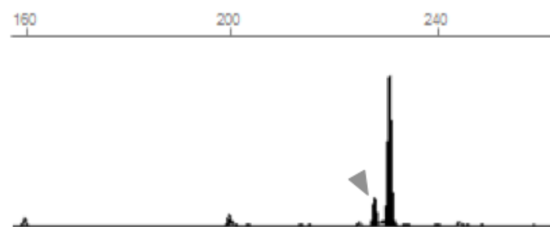

Figure 3.1. GeneScan profile demonstrating a less intense stutter band ( ).

In vitro stability of CAVIII microsatellite was analysed by growing four independent strains over 300 generations. For the four strains we observed that genotypes were the same after 300 generations, suggesting an expected mutation rate less than 3.33×10^{-3}. CAVIII microsatellite also revealed to be species specific, since no amplification products were obtained when using the described primers in the amplification conditions with DNA from other pathogenic *Candida* species, namely *C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, C. guilliermondi, C. lusitaniae and C. dubliniensis.* It is noteworthy to mention the specificity regarding *C. dubliniensis*, which is very closely related to *C. albicans* (Sullivan *et al.* 1995).

Eight different alleles and 15 genotypes were identified in this survey. The CAVIII fragments obtained in this analysis were sequenced in order to determine the nature of the polymorphisms observed and the number of repeated units. The number of repeats of the obtained PCR products varied between 7 and 14 (CAA/G) repeats, thus the alleles were designated by their number of trinucleotidic repeats instead of the molecular weight (Table III.I).

Table III.I Alleles structure of CAVIII locus. The consensus sequence, obtained from data base sequence for SC5314 strain is indicated and contain 10 repetitive units.

CAVIII – Consensus sequence:		
P1(25bp)tgaaaaagttgtctcattagattttaccgttaccagaaaaccttttaatgctactgctcatggacaacatca tcaatccCAA(CAG)$_3$(CAA)$_6$ccagctcaaaaaagaggaactgttcaaacaagtttgattaatgaaggtccatcat atgctgctaccatcactgttggttcaaacaaacaacaacaaactgttattgttgacacaggttc-P2(25bp)		
Allele (bp):		
7	(269)	Data not analysed
8	(272)	(79bp) ————(CAA)$_8$————(119bp)
9	(275)	(79bp) ————(CAA)$_9$————(119bp)
10a	(278)	(79bp) CAA(CAG)$_3$(CAA)$_6$————(119bp)
10b	(278)	(79bp) CAA(CAG)$_4$(CAA)$_5$————(119bp)
11	(281)	Data not analysed
12a	(284)	(79bp) CAA(CAG)$_3$(CAA)$_8$————(119bp)

12b	(284)	(79bp) CAA$(CAG)_2(CAA)_5$CAG$(CAA)_3$–(119bp)
13	(287)	(79bp) CAA$(CAG)_3(CAA)_9$————(119bp)
14	(290)	Data not analysed

The most frequent CAVIII genotypes were 10-10 (84 strains, 46.4%) and 8-10 (37 strains, 20.4%) (Figure 3.2).

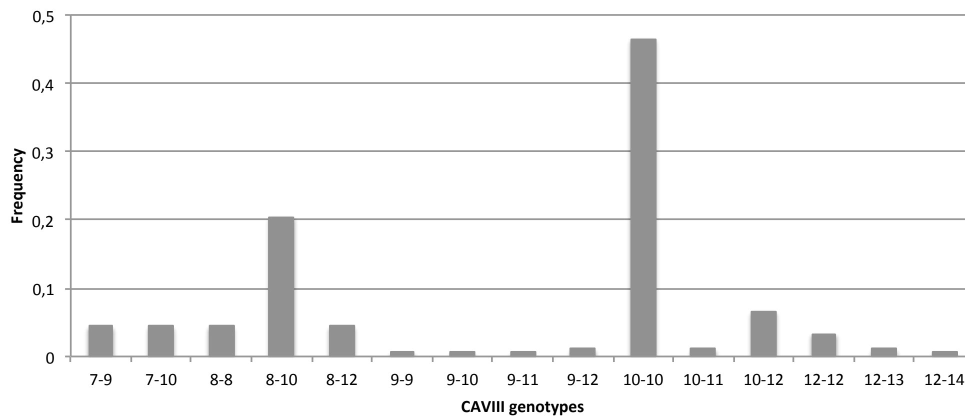

Figure 3.2. Genotypes and respective frequencies obtained in CAVIII analysis of all *C. albicans* strains.

The discriminatory power was calculated according to the Simpson index of diversity:

$$DP = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

where *N* is the number of strains, *s* is the total number of different genotypes, and *nj* is the number of strains of *j* genotype (Hunter and Gaston 1988). The results indicated that CAVIII presented a DP value of 0.72.

CAI microsatellite presented a great genetic variability even though it is present in the coding region of a gene, the transcription factor RLM1. Amplification of the same *C. albicans* isolates with the CAI microsatellite resulted in a total of 80 different genotypes with fragments varying from 11 to 49 repeat units. The most frequent CAI genotypes were 21-25 (22 strains, 12.2%), 25-25 (11 strains, 6.1%) and 21-22 (9 strains, 5.0%), and the frequency of the other genotypes ranges between 0,6% (1 strain) and 4.4% (8 strains) (Figure 3.3). The discriminatory power, calculated according to the Simpson index of diversity for CAI in these same strains was 0.97.

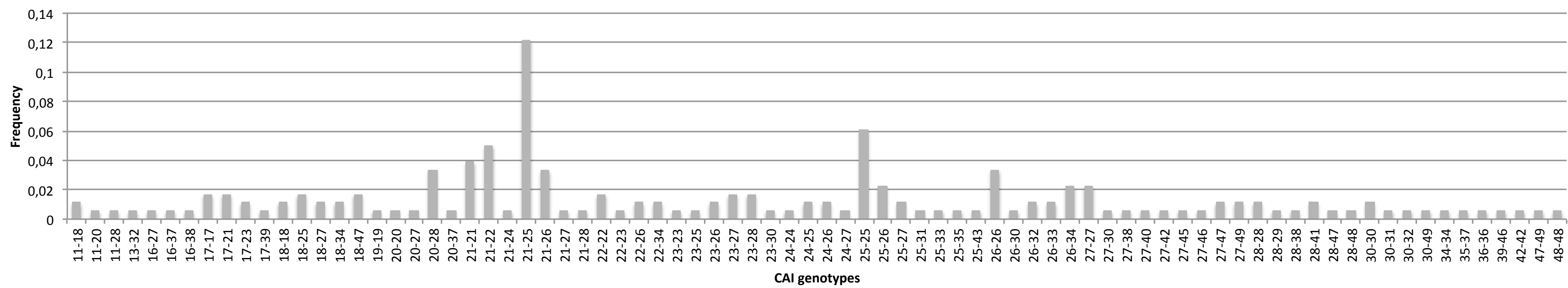

Figure 3.3 Genotypes and respective frequencies obtained in CAI analysis of all *C. albicans* strains.

3.2. Use of the microsatellites CAVIII and CAI for strains differentiation

Sampaio and co-workers (Sampaio *et al.* 2009) showed a relationship between higher molecular weight of CAI genotypes and the ability to survive in different stress conditions, and Liu and co-workers (Liu *et al.* 2009) confirmed a relationship in strains isolated from vulvovaginitis with CAI genotypes with higher molecular weight alleles in comparison with strains from vagina of asymptomatic women. In this context, we addressed the question of a possible correlation between the pathogenicity of lineages of *C. albicans* strains with regard to their genotype at these two repeat-containing ORFs. For this purpose, the strains analysed in this study were grouped according to their origin of isolation and population differentiation tests were performed concerning allelic and genotypic distribution in the different groups by testing the null hypothesis Ho: “the allelic/genotype distribution is identical across populations”.

Our analysis indicated that the null hypothesis could not be rejected (P>0.05) in the comparison of strains form oral infections and from oral asymptomatic individuals, considering genetic and genotypic data (Table III.II).

Table III.II. Unbiased P-values of the probability test estimated by the Fisher method and obtained for each population pair considering the combination of CAI and CAVIII microsatellite data.

Genetic \ Genotypic	Oral Infection	Oral Commensal	Vulvovaginitis	URT Infection	Urinary Infection
Oral Infection		0.536	0.002	0.125	0.003
Oral Commensal	0.232		0.000	0.003	0.002
Vulvovaginitis	0,00000	0,000		0.236	0.000
URT Infection	0.033	0.000	0.071		0.200
Urine	0.000	0.000	0.000	0.070	

This result suggests that the development of an oral infection is mainly dependent on the host conditions rather than on the strain. Additionally, no significant differences were observed in the genetic and genotypic distributions between strains from the URT and urine. In this view, it was decided to analyse the strains only in three groups. All strains from the oral cavity were considered into an oral group, the vaginal strains were incorporated into the vaginal group, and the strains from the URT and urine into the extra-mucosal group. The population differentiation tests were performed once again and results are showed in Table III.III.

Table III.III. Significance of unbiased *P*-values of the probability test estimated by the Fisher method and obtained for each population pair considering microsatellite data. (+ when *P*<0.05 and - *P*>0.05). **A-** Results obtained with CAVIII. **B-** Results obtained with CAI.

A

Genotypic / Genetic	Oral	Vaginal	Extra-mucosal
Oral		+	+
Vaginal	+		+
Extra-mucosal	+	+	

B

Genotypic / Genetic	Oral	Vaginal	Extra-mucosal
Oral		-	+
Vaginal	-		+
Extra-mucosal	+	+	

The results obtained with CAVIII demonstrated significant allelic and genotypic differences (P<0.05) in the comparison between the three groups, showing that the polymorphism in SAP8 microsatellite could contribute to differentiate strains isolated from the three different groups. However, with CAI no significant differences (P>0.05) between strains from the oral and vaginal groups were observed. A significant difference (P<0.05) remained between extra-mucosal group and the other groups, regarding genetic and genotypic data. Combining the two microsatellites, significant differences (P<0.05) were observed in the comparison between all groups.

To confirm this tendency it was necessary to balance the number of strains analysed in each group. Therefore, we decided to address again this question increasing the number of strains isolated from extra-mucosal infections. With this purpose we used CAI genotypes of strains described in previous studies, namely 7 from urine, 26 from upper respiratory tract and 30 from blood stream, making a total of 102 strains in oral group, 42 in vaginal group and 100 in extra-mucosal group (Table III.IV).

Table III.IV – *C. albicans* strains used and respective CAI and CAVIII genotypes.

Strains	Source	Country	CAI Genotype	CAVIII Genotype	Strains	Source	Country	CAI Genotype	CAVIII Genotype
S008	Saliva	Portugal	23/30	9/9	46C	VE	Portugal	27/27	10/10
S020	Saliva	Portugal	18/34	12/13	49C	VE	Portugal	21/26	7/10
S038	Saliva	Portugal	21/25	10/10	51C	VE	Portugal	22/34	7/10
S040	Saliva	Portugal	26/34	8/10	52C	VE	Portugal	22/34	7/10
S046	Saliva	Portugal	22/26	10/10	53C	VE	Portugal	27/47	7/10
S073b	Saliva	Portugal	22/26	10/10	55C	VE	Portugal	27/42	7/10
S078	Saliva	Portugal	26/26	10/10	57C	VE	Portugal	11/28	7/10
S085	Saliva	Portugal	25/25	10/10	58C	VE	Portugal	28/47	7/10
S092	Saliva	Portugal	21/28	8/10	3J	VE	Portugal	17/23	10/10
S094b	Saliva	Portugal	22/22	10/10	7J	VE	Portugal	30/30	10/10
S104	Saliva	Portugal	21/25	10/10	12J	VE	Portugal	18/25	10/10

S110	Saliva	Portugal	24/25	10/10
S111	Saliva	Portugal	26/27	8/8
S117	Saliva	Portugal	18/25	10/10
S128a	Saliva	Portugal	21/25	10/10
S133	Saliva	Portugal	18/27	10/10
S134	Saliva	Portugal	25/26	10/10
S140	Saliva	Portugal	24/26	10/10
S141	Saliva	Portugal	21/25	10/10
S142	Saliva	Portugal	27/38	8/10
S143	Saliva	Portugal	23/28	7/9
S144	Saliva	Portugal	23/25	10/10
S145	Saliva	Portugal	25/26	10/10
S148	Saliva	Portugal	21/26	8/10
DM-1	Saliva	UK	26/32	10/10
DM-2	Saliva	UK	25/31	10/10
ND-1	Saliva	UK	17/39	10/12
ND-2	Saliva	UK	16/37	10/12
ND-5	Saliva	UK	16/38	10/12
RB-1	Saliva	UK	28/41	8/10
RB-2	Saliva	UK	28/41	8/10
RB-3	Saliva	UK	27/40	8/10
SAR1	Saliva	UK	23/27	10/10
S001	Saliva	Portugal	25/33	12/12
S005	Saliva	Portugal	25/25	12/12
S006	Saliva	Portugal	21/21	10/10
S009	Saliva	Portugal	28/38	8/10
S013	Saliva	Portugal	26/26	10/10
S017a	Saliva	Portugal	21/21	10/10
S019	Saliva	Portugal	21/25	10/10
S031	Saliva	Portugal	25/25	10/10
S032	Saliva	Portugal	27/27	8/10
S034	Saliva	Portugal	26/34	8/12
S036b	Saliva	Portugal	26/32	8/12
S045a	Saliva	Portugal	25/25	10/10
S052a	Saliva	Portugal	26/26	12/12
S076	Saliva	Portugal	23/23	10/12
S108	Saliva	Portugal	24/25	10/10
S151	Saliva	Portugal	30/49	8/10
S152	Saliva	Portugal	17/17	8/10
S153	Saliva	Portugal	28/48	8/10
2247	Saliva	Brazil	25/43	8/10
2248	Saliva	Brazil	25/25	10/10
2249	Saliva	Brazil	21/25	10/10
2250	Saliva	Brazil	47/49	8/10
2251	Saliva	Brazil	11/20	8/10
2252	Saliva	Brazil	26/33	9/10
2253	Saliva	Brazil	27/30	8/10
2254	Saliva	Brazil	27/49	8/10
2255	Saliva	Brazil	25/25	10/10
17J	VE	Portugal	21/21	10/10
20J	VE	Portugal	26/26	10/10
22J	VE	Portugal	23/27	7/9
27J	VE	Portugal	22/23	7/9
29J	VE	Portugal	20/20	10/10
31J	VE	Portugal	21/26	10/10
35J	VE	Portugal	25/25	10/10
37J	VE	Portugal	18/27	10/10
39J	VE	Portugal	21/21	10/12
41J	VE	Portugal	23/27	7/9
45J	VE	Portugal	25/25	10/10
1M	Urine	Portugal	21/25	10/10
2M	Urine	Portugal	21/25	10/10
31M	Urine	Portugal	21/25	10/10
8M	Urine	Portugal	21/25	10/10
13M	Urine	Portugal	21/25	10/10
37M	Urine	Portugal	17/23	8/10
41M	Urine	Portugal	21/22	10/12
43M	Urine	Portugal	21/22	10/12
45M	Urine	Portugal	21/22	10/12
47M	Urine	Portugal	21/22	10/12
48M	Urine	Portugal	21/22	10/12
49M	Urine	Portugal	21/22	12/12
51M	Urine	Portugal	36/36	12/12
52M	Urine	Portugal	21/21	9/12
55M	Urine	Portugal	21/21	9/12
63M	Urine	Portugal	25/27	10/10
74M	Urine	Portugal	20/28	10/10
88M	Urine	Portugal	20/28	10/10
82M	Urine	Portugal	18/47	8/10
84M	Urine	Portugal	18/47	8/10
CIPO 34	Urine	Portugal	19/28	-
CIPO 47	Urine	Portugal	22/22	-
CIPO 45	Urine	Portugal	25/26	-
CIPO 90	Urine	Portugal	17/40	-
CIPO 26	Urine	Portugal	22/22	-
CIPO 64	Urine	Portugal	23/27	-
CIPO 92	Urine	Portugal	21/23	-
H37	RT	Portugal	25/27	7/9
5M	RT	Portugal	13/32	12/12
10M	RT	Portugal	17/17	8/10
12M	RT	Portugal	17/17	8/10
26M	RT	Portugal	20/28	10/10
35M	RT	Portugal	24/27	7/9
39M	RT	Portugal	18/18	10/10
61M	RT	Portugal	17/21	8/10
62M	RT	Portugal	28/28	8/10
64M	RT	Portugal	22/22	10/11
67M	RT	Portugal	22/22	10/11

2256	Saliva	Brazil	25/25	10/10
2257	Saliva	Brazil	25/25	10/10
Guy331-1	Saliva	UK	48/48	8/10
Guy775-1	Saliva	UK	42/42	8/10
Guy778-w1	Saliva	UK	30/32	8/10
AX	Saliva	Portugal	26/34	8/12
O	Saliva	Portugal	27/27	8/10
AR	Saliva	Portugal	20/28	10/10
F	Saliva	Portugal	23/26	8/8
J	Saliva	Portugal	26/34	8/12
AF	Saliva	Portugal	21/21	10/10
AC	Saliva	Portugal	21/25	10/10
Aa	Saliva	Portugal	21/25	10/10
S	Saliva	Portugal	21/25	8/10
AA	Saliva	Portugal	21/22	8/12
R	Saliva	Portugal	21/26	10/10
Q	Saliva	Portugal	30/31	10/10
BB	Saliva	Portugal	26/26	10/10
AH	Saliva	Portugal	27/45	8/10
AL	Saliva	Portugal	18/25	10/10
L	Saliva	Portugal	21/22	10/12
M	Saliva	Portugal	28/28	8/10
BC	Saliva	Portugal	21/25	10/10
AP	Saliva	Portugal	23/28	10/10
AN	Saliva	Portugal	35/37	10/10
CDQN5	Saliva	Portugal	23/26	8/8
CDQN10	Saliva	Portugal	23/28	10/10
CD1	Saliva	Portugal	19/19	10/10
BE1	Saliva	Portugal	21/25	10/10
BH	Saliva	Portugal	21/24	10/10
BI	Saliva	Portugal	21/22	8/12
BJota	Saliva	Portugal	20/28	10/10
B1	Saliva	Portugal	25/25	10/10
N1	Saliva	Portugal	27/47	8/10
O1	Saliva	Portugal	27/46	8/10
Bx	Saliva	Portugal	25/26	10/10
AZ1	Saliva	Portugal	21/21	10/10
V1	Saliva	Portugal	30/30	10/10
BL	Saliva	Portugal	28/29	8/10
BP	Saliva	Portugal	25/35	10/10
BQ	Saliva	Portugal	34/34	8/12
BV	Saliva	Portugal	26/26	8/12
1C	VE	Portugal	17/21	8/8
2C	VE	Portugal	26/33	8/8
3C	VE	Portugal	20/37	12/14
5C	VE	Portugal	18/18	8/8
6C	VE	Portugal	21/26	10/10
7C	VE	Portugal	25/26	10/10
9C	VE	Portugal	24/26	8/8

69M	RT	Portugal	21/25	10/10
75M	RT	Portugal	21/25	10/10
86M	RT	Portugal	21/25	10/10
79M	RT	Portugal	18/34	12/13
90M	RT	Portugal	24/24	10/12
91M	RT	Portugal	21/27	10/10
CIPO 93	RT	Portugal	26/34	-
CIPO 54	RT	Portugal	20/28	-
CIPO 14	RT	Portugal	21/21	-
CIPO 3	RT	Portugal	19/27	-
CIPO 84	RT	Portugal	23/27	-
CIPO 16	RT	Portugal	18/18	-
CIPO 99	RT	Portugal	21/22	-
CIPO 8	RT	Portugal	21/30	-
CIPO 25	RT	Portugal	18/18	-
CIPO 76	RT	Portugal	21/25	-
CIPO 15	RT	Portugal	17/21	-
CIPO 7	RT	Portugal	25/33	-
CIPO 49	RT	Portugal	25/25	-
CIPO 66	RT	Portugal	26/30	-
CIPO 31	RT	Portugal	20/28	-
CIPO 21	RT	Portugal	21/21	-
CIPO 19	RT	Portugal	13/25	-
CIPO 13	RT	Portugal	21/25	-
CIPO 40	RT	Portugal	17/17	-
CIPO 36	RT	Portugal	18/25	-
CIPO 27	RT	Portugal	18/27	-
CIPO 41	RT	Portugal	20/26	-
CIPO 74	RT	Portugal	26/26	-
CIPO 32	RT	Portugal	24/26	-
CIPO 85	RT	Portugal	18/25	-
CIPO 63	RT	Portugal	21/22	-
HSJ 63	BC	Portugal	16/25	-
HSJ 69	BC	Portugal	16/25	-
HSJ 90	BC	Portugal	21/25	-
HSJ 93	BC	Portugal	21/25	-
HSJ 114	BC	Portugal	12/12	-
HSJ 124a	BC	Portugal	18/34	-
HSJ 140	BC	Portugal	18/34	-
HSJ 144	BC	Portugal	18/34	-
HSJ 130	BC	Portugal	12/12	-
HSJ 141	BC	Portugal	12/17	-
HSJ 143	BC	Portugal	26/26	-
HSJ 150	BC	Portugal	26/26	-
HSJ 154	BC	Portugal	26/26	-
HSJ 155	BC	Portugal	29/29	-
HSJ 164	BC	Portugal	26/28	-
HSJ 165	BC	Portugal	21/25	-
HSJ 168	BC	Portugal	21/26	-

10C	VE	Portugal	27/49	8/10	IPOL 1	BC	Portugal	25/25	-
11C	VE	Portugal	21/25	10/10	IPOL 2	BC	Portugal	25/25	-
13C	VE	Portugal	20/27	10/10	IPOL7	BC	Portugal	26/26	-
14C	VE	Portugal	39/46	8/10	IPOL 10	BC	Portugal	12/12	-
16C	VE	Portugal	21/26	10/10	IPOL 11	BC	Portugal	23/27	-
19C	VE	Portugal	27/27	8/10	IPOL 13	BC	Portugal	40/40	-
24C	VE	Portugal	17/21	8/8	IPOL 14	BC	Portugal	20/28	-
27C	VE	Portugal	16/27	7/10	IPOL 15	BC	Portugal	35/44	-
31C	VE	Portugal	21/25	10/10	IPOL 17	BC	Portugal	16/38	-
35C	VE	Portugal	21/25	9/11	IPOL 19	BC	Portugal	18/28	-
36C	VE	Portugal	11/18	7/9	IPOL 20	BC	Portugal	21/25	-
39C	VE	Portugal	20/18	7/9	IPOL 21	BC	Portugal	19/34	-
45C	VE	Portugal	11/18	10/10	IPOL 22	BC	Portugal	21/26	-

- no data was obtained for these strains, VE vaginal exsudate, RT respiratory tract, BC blood culture

A total of 97 CAI genotypes were obtained in the amplification of all selected strains. The number of different genotypes identified in each group was 59 to the oral group, 31 to the vaginal group and 46 to the extra-mucosal group. The genotype 21-25 remained the most common genotype in all groups, as well as 25-25 in strains from oral group, 21-26 in strains from vaginal group and 21-22 in strains from extra-mucosal group (Figure 3.4).

In order to confirm the population differences observed, population differentiation tests were performed concerning allelic and genotypic distribution with higher number of strains. No significant differences were found between strains from oral and vaginal groups, considering the genotypic and genetic distribution ($P>0.05$). The extra-mucosal group remained significant different ($P<0.05$) from strains from the other groups, comparing the genetic data (Table III.V).

Table III.V. Unbiased P-values of the probability test estimated by the Fisher method and obtained for each population pair considering CAI microsatellite data.

Genotypic / **Genetic**	Oral	Vaginal	Extra-mucosal
Oral		0.609	0.008
Vaginal	0.367		0.166
Extra-mucosal	0.000	0.025	

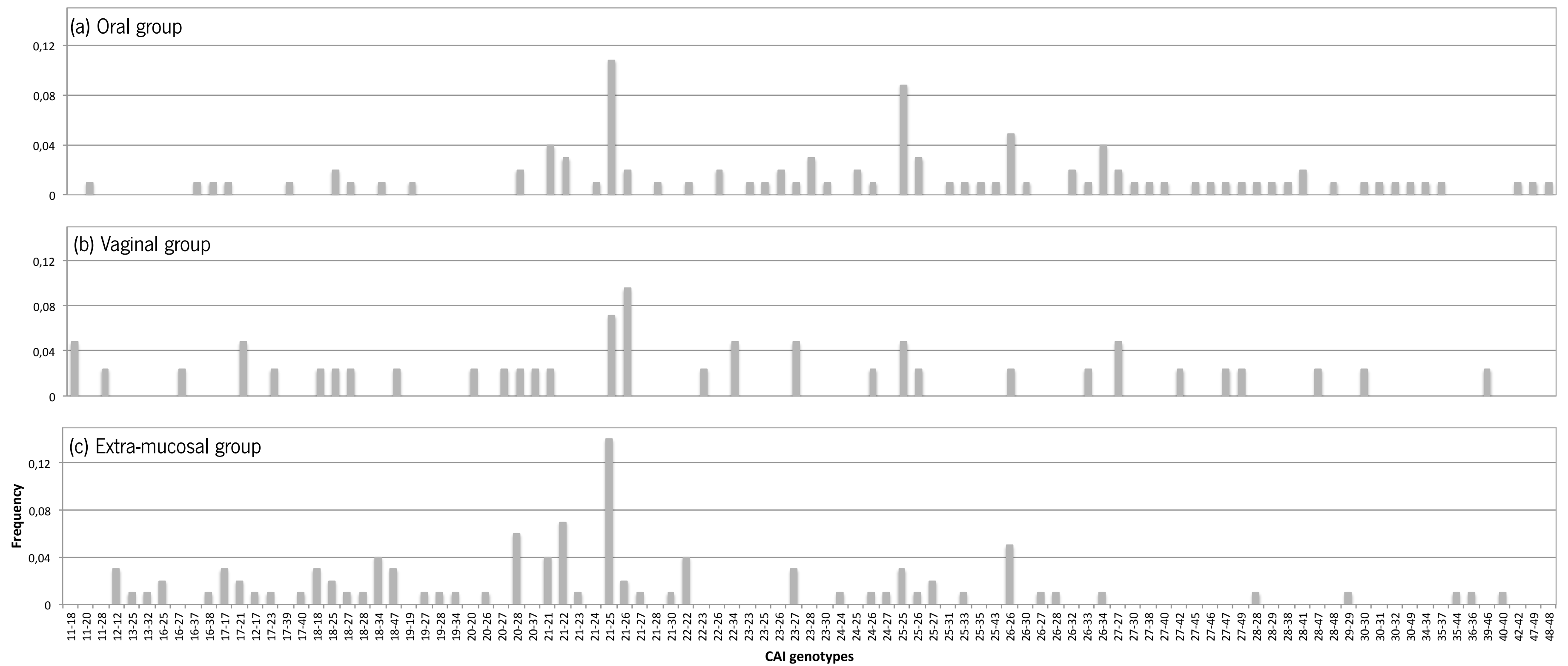

Figure 3.4. Genotypic frequencies based on CAI microsatellite analysis of *Candida albicans* strains from (a) oral group, (b) vaginal group and (c) extra-mucosal group.

These results demonstrated a similarity between the genotypes of oral and vulvovaginal isolates, strains isolated from mucosal surfaces. Therefore, a final analysis was performed comparing strains from superficial infections and strains from invasive infections, regarding CAI genotype. The superficial group (total of 144 strains) contains strains isolated from oral infection, strains from saliva of healthy volunteers and strains from vulvovaginal candidiasis. On the other hand, the invasive group (total of 100 strains) contains strains isolated from upper respiratory tract, urinary and blood stream infection.

The genotypic frequency of both groups demonstrated that the genotype 21-25 remains the most common. These results also demonstrated that strains from the superficial group presented an increase of genotypes with higher molecular weight, comparing to the invasive group (Figure 3.5). Moreover, the superficial group had 51 specific genotypes, which represents 46.5% (67 strains) of all superficial strains, whereas the invasive group had 23 specific genotypes, which represents 27.0% (27 strains) of all invasive strains (Figure 3.6).

Li and co-authors (Li *et al.* 2008) described that CAI genotyping presented a biased distribution in which, strains isolated from vulvovaginal candidiasis (VVC) and *Candida* balanoposthitis showed CAI alleles with high molecular weight, with emphasis in four genotypes, when compared with strains isolated from asymptomatic women. The specific CAI genotypes described were 30-45, 32-46, 30-36 and 30-47, however, these genotypes were not found in strains of vaginal group studied in this work. Nevertheless, strains from the superficial group presented an increase of genotypes with higher molecular weight, comparing with invasive group, such as 42-42, 47-49 and 48-48 (Figure 3.5). These results confirm a biased distribution of CAI genotypes in strains isolated from superficial infections comparing with strains isolated from invasive infections.

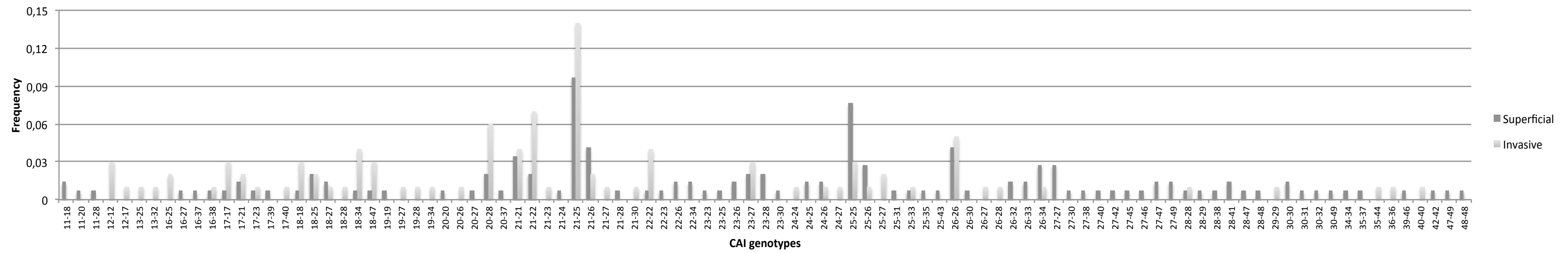

Figure 3.5. Genotypic frequencies based on CAI microsatellite analysis of *Candida albicans* strains from (n) superficial group and (n) invasive group.

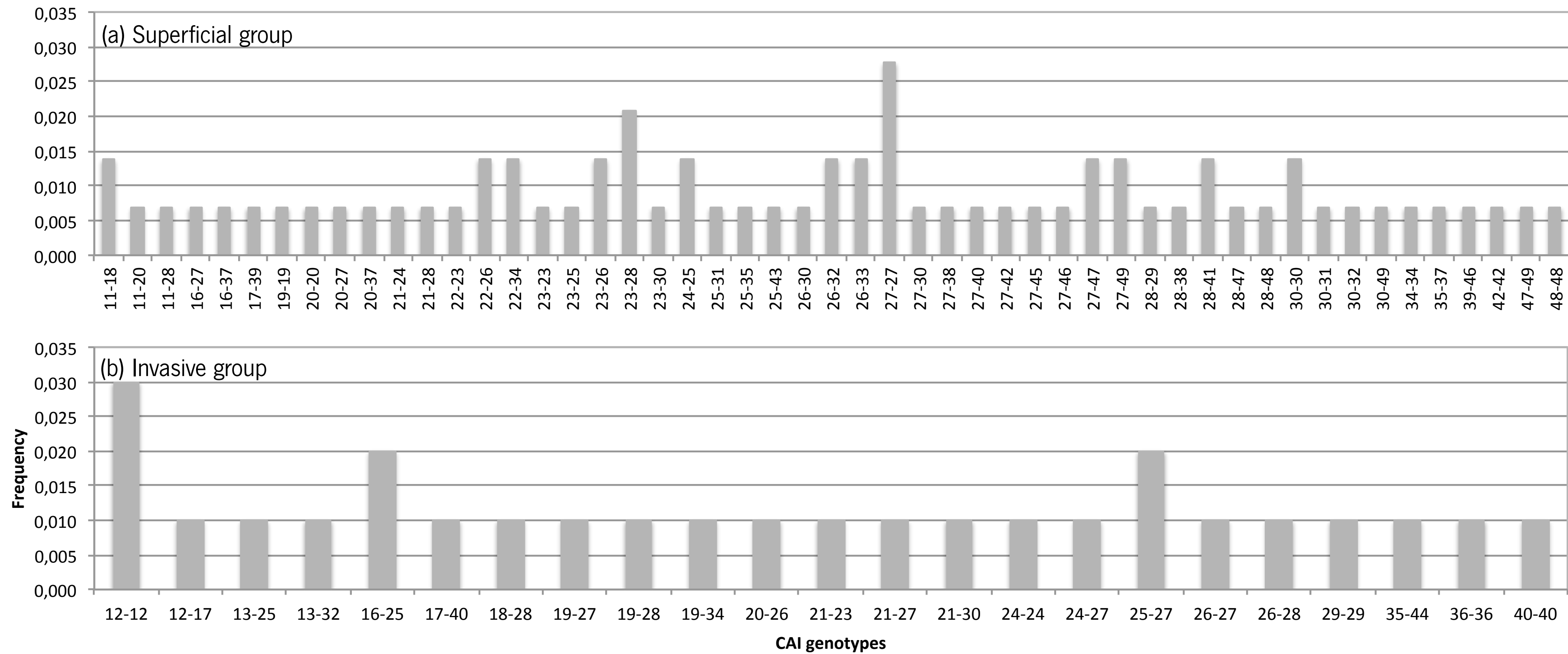

Figure 3.6. Specific genotypes and respective frequencies obtained with CAVI analysis of *C. albicans* strains from (a) Superficial group and (b) Invasive group.

Sampaio and co-authors (Sampaio *et al.* 2009) classified CAI alleles according to its number of CAA/G repetitions, namely alleles with less than 16 repetitions were type I, alleles with repetitions from 17 to 28, type II; and alleles with more than 28 repetitions, type group III. In order to group strains in this study according to this allele classification, genotypes were codified accordingly and the cluster analysis performed (Figure 3.7).

It is possible to distinguish three major groups of isolates. Group A includes 24,18% (59 strains) of all strains, and represents genotypes with high molecular weight alleles, type III; the group B includes 71,31% (174 strains) of the strains representing genotypes with alleles type II; and group C includes 4,51% of all strains (11 strains) and represents genotypes with low molecular weight alleles, type I. In groups B and C an equally distribution of superficial and invasive strains (approximately 50% of each) was observed. However, in group A, the majority of the strains were isolated from superficial infection (76,27%, 45 strains). This result confirms Li and co-authors (Li *et al.* 2008) observation that CAI genotyping presents a biased distribution in which, strains isolated from superficial infection showed CAI alleles with high molecular weight comparing with strains isolated from invasive infections.

To confirm this hypothesis population differentiation tests were performed concerning allelic and genotypic distribution with these two different groups. Regarding genetic ($P=0.000$) and genotypic ($P=0.006$) data, a significant difference ($P<0.05$) was observed in comparison between strains isolated from superficial infection/commensalism and strains isolated from invasive infections, supporting the results presented above.

In conclusion, the presence of specific genotypes in superficial and invasive groups demonstrates the ability of CAI to differentiate strains isolated from different body locations. Moreover, the increasing incidence of CAI genotypes with high molecular weight in superficial strains can explain the influence of this microsatellite in the adaptation to mucosal conditions.

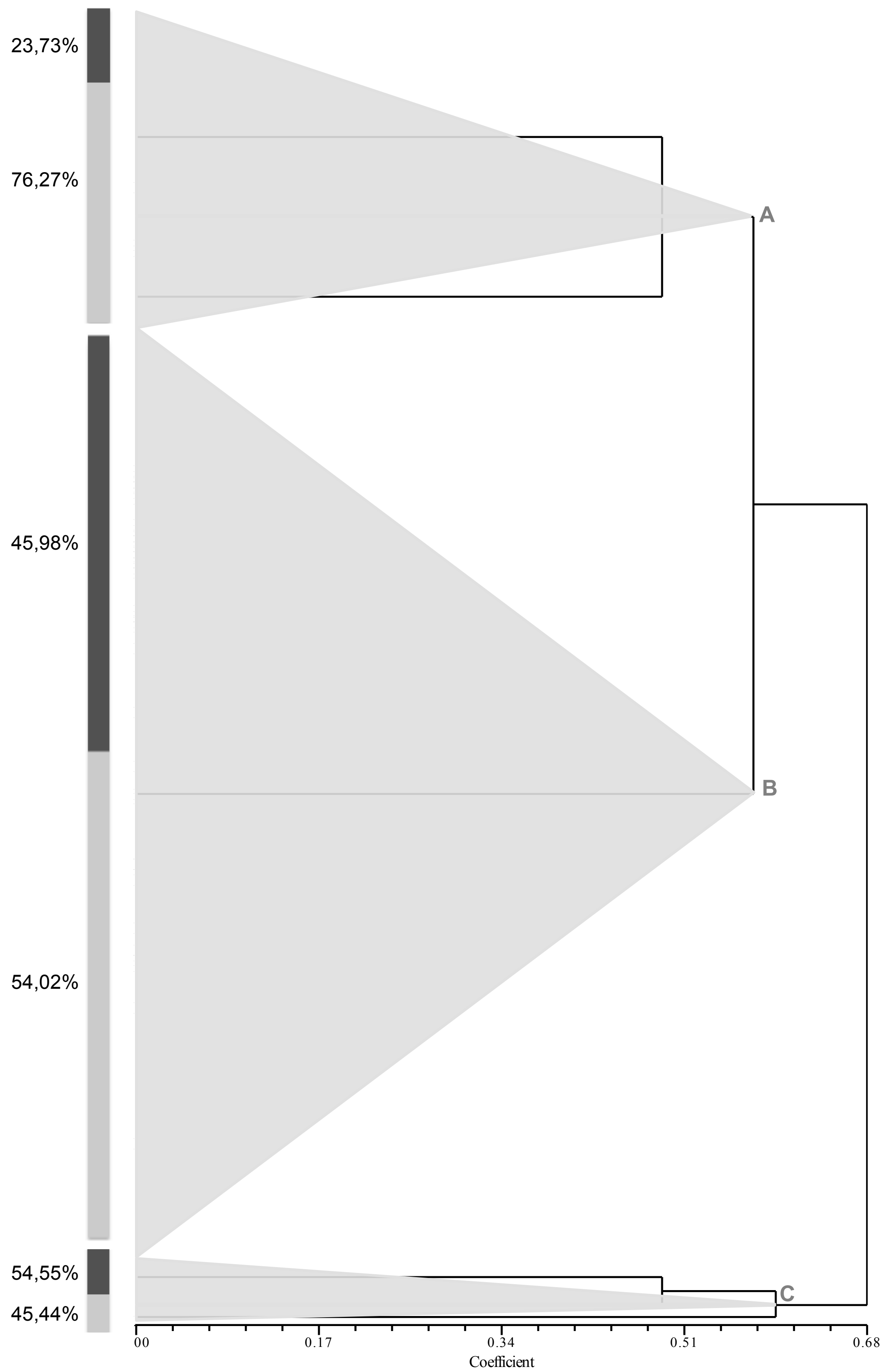

Figure 3.7. UPGMA clustering of 244 *C. albicans* isolates based on the genotypes, showing three phylogenetic groups (A, B and C). The percentage of strains with different origins in each group is represented by different shades: black, invasive infection; grey, superficial infections.

4. Conclusion and final remarks

The commensal yeast *Candida albicans* is the most common opportunistic pathogen, causing a number of superficial and invasive infections in humans (Hube 2004). It is thought that the pathogenicity and antifungal susceptibility of this yeast vary among strains, thus, correlation between the pathogenicity of lineages of *C. albicans* strains with variation at molecular markers can be important. Moreover, the identification of different strains can be essential for addressing medical questions such as the origin of the infective strains.

The genome of *C. albicans* contains a large number of repeat-containing ORFs, including SAP8 and RLM1 (Braun *et al.* 2005). SAP8 gene encoding an extracellular SAP with unknown function and contains an uncharacterized microsatellite consisting of (CAA/G) repeat units (Wu and Samaranayake 1999; Ripeau *et al.* 2002). Several molecular methods have been used to type *C. albicans* strains and microsatellite markers have assumed increasing importance due to their high level of polymorphism and stability. In this work was developed an SAP8 microsatellite marker, designated by CAVIII, in order to observe its ability to differentiate *C. albicans* strains. The results obtained suggested that SAP8 microsatellite loci has a reasonable discriminatory power of 0.72 and presents great specificity.

Although a number of polymorphic microsatellite markers have been reported in *C. albicans* (Botterel *et al.* 2001; Sampaio *et al.* 2005), CAI, which is specific for RLM1 microsatellite loci, appears to be more polymorphic than other STRs (Sampaio *et al.* 2003). Thus, same strains used in CAVIII analysis were tested using CAI microsatellite marker. The results obtained demonstrated the great specificity of CAI marker, which presents a discriminatory power of 0.97.

Previous studies demonstrated the relationship between higher molecular weight of CAI genotypes and the ability to survive in different stress conditions (Liu *et al.* 2009; Sampaio *et al.* 2009). Therefore, we consider important verify the ability of this marker to distinguish strains isolated from different body sources. Since SAP8 microsatellite marker is also located in a coding region, differences in SAP8 genotypes were also studied. The results suggested that CAVII can contribute significantly to differentiate *C. albicans* strains isolated from different body locations, namely from oral cavity, vaginal infection and extra-mucosal infections, however, CAI was not able to differentiate strains isolated from oral cavity and vaginal infection.

The results obtained also suggested that the ability to differentiate strains increased when CAI and CAVIII microsatellite markers were combined.

In order to balance the number of strains isolated from the different groups, 63 strains from extra-mucosal infections were added to study, however, only CAI genotypes were analysed. The results obtained suggested that the CAI microsatellite marker remains unable to differentiate strains isolated from oral cavity and strains from vulvovaginal infection. Therefore, the strains were organized into two groups, namely superficial infections and invasive infections, and the results obtained in this division demonstrated that CAI microsatellite marker is able to differentiate strains isolated from the two selected groups.

Although the CAI microsatellite marker was able to differentiate *C. albicans* strains isolated from superficial and invasive infections, it was observed that the combination of both CAI and CAVIII microsatellite markers is able to differentiate strains into three groups (oral group, vaginal group and extra-mucosal group). Thus, it seems important test all strains used with both microsatellite markers and verify if the tendencies observed with CAI microsatellites remains. The increase of the number of vaginal strains, in the differentiation of oral group, vaginal group and extra-mucosal group, is also important.

CHAPTER IV

Final Considerations

In the last two decades an alarming increase in the number of fungal infections has been observed. The use of more aggressive therapeutic methods, such as chemotherapeutic agents, bone marrow or solid-organ transplants, immunomodulatory agents, broad-spectrum antibiotics and more aggressive surgeries are some of the reasons proposed to explain this increased incidence (Peres-Bota *et al.* 2004; Benjamin *et al.* 2010). A number of ethological agents may be involved in the development of fungal infections, such as *Aspergillus* spp., *Cryptococcus* spp., *Malasezzia* spp., *Fusarium* spp. or *Trichosporon* spp., however, *Candida* species are the most frequently found (Fridkin and Jarvis 1996).

There are about 150 species of *Candida*, but only a small number are human pathogens. *Candida albicans* is considered the most frequently species isolated from *Candida* infections, however, other species such as *Candida tropicalis, Candida glabrata, Candida parapsilosis* and *Candida krusei* have been increasingly recognized as pathogens with a wide distribution (Tortorano *et al.* 2004).

The rapid and correct identification of infecting species is crucial since *Candida* species differ in their susceptibility to antifungal agents. Clinical microbiology laboratory methodologies for the identification of pathogenic fungal species are based on morphological, physiological and biochemical tests, which requires three or more days and may be inaccurate. (Ellepola and Morrison 2005). Molecular methodologies, especially based in the analysis of DNA sequences, are characterized by their high specificity, sensibility and reproducibility. Microsatellite sequences have been largely used as molecular targets to differentiate and characterize strains. However, no studies have been performed using microsatellite DNA for *Candida* species identification. Therefore, the main objectives of this work were the evaluation of the potential of microsatellite markers for species differentiation and for identification of specific *C. albicans* lineages.

Initially a new multiplex methodology based in microsatellite loci analysis was developed to identify the most clinically important *Candida* species. In this work a new multiplex-PCR methodology was developed to identify the most clinically important *Candida* species (*C. albicans, C. parapsilosis, C. glabrata, C. tropicalis* and *C.* krusei). This methodology showed a high stability and capacity to discriminate the different *Candida* species. However, although performing well in singleplex- PCR, the marker design to identify *C. tropicalis* isolates was unable to amplify in multiplex-PCR. *C. tropicalis* infections have been reported in

immunocompromised patients with chronic mucocutaneous candidiasis and its incidence in invasive candidiasis in neutropenic patients has been increasing (Kothavade *et al.* 2010). Thus, in the future is imperative to include *C. tropicalis* identification in this methodology and new primers for CT14 locus has already been designed. Given the need for fast and accurate diagnostic methodologies in microbiology laboratories, it would also be important in the future to test this multiplex directly in clinical samples from different Hospitals and Health Centres The methodology developed is easy to perform and can be implemented at relatively low cost for routine identification in Hospitals and Health Centres.

Microorganisms evolved mechanisms for increasing genetic variations in loci that are involved in critical interaction with the host. These alterations can be achieved by several mechanisms, including mechanisms that generate hypervariability in repeat-containing ORFs, the addition or deletion of repeat units during replication, through slipped-strand mispairing or gene conversion is the best characterized. Thus, it has been suggested that repeat-containing ORFs may be important *C. albicans* fitness determinants. In this view, the applicability of two repeat-containing ORFs, RLM1 and SAP8, in the discrimination of *C. albicans* lineages was also addressed in this study. Rlm1, this is one of the transcription factors of the cell wall integrity (CWI) pathway and it has been demonstrated that Rlm1 presents a great variability at its C-terminus, conferred by the CAI microsatellite with more than 35 alleles identified and that strains with high molecular weight alleles display higher tolerance to cell wall stress agents (Sampaio *et al.* 2009). SAP8 gene, encoding an extracellular Secreted Aspartyl Proteinase contains a microsatellite consisting of (CAA/G) repeat units, named CAVIII, at the C-terminus of the protein that has not been characterized. After the characterization of CAVIII, the genotypes obtained were combined with CAI results to test 144 unrelated *C. albicans* strains isolated from different body locations. The combination of genotypes form these two repeat-containing ORFs allowed the clear statistical differentiation of strains from superficial (oral and vagina) and invasive (respiratory tract, urine and blood) infections, in a universe of 224 strains. This differentiation may reflect adaptation of strains to different host environments since these ORFs express proteins that are important in the direct contact with the host. However, to confirm this capacity to differentiate strains according to its habitat of isolation, genotypes from a few strains from this study must be completed and strains from different geographic areas (different countries) included.

The results obtained with this work allowed concluding that the microsatellite loci analysis can be used to differentiate the most common *Candida* species, being an alternative in clinical diagnosis. Moreover, it was also possible observe that analysis of repeat-containing ORFs, such as RLM1 and SAP8 is able to differentiate lineages of *C. albicans.*

CHAPTER V

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