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AFLP genotyping of *Candida metapsilosis* clinical isolates: Evidence for recombination

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ABSTRACT

In a collection of 395 independent clinical isolates classified as *Candida parapsilosis* on a biochemical profile basis, 20 *Candida metapsilosis* strains were identified by molecular tests with an isolation frequency of 5%. Isolates were screened for their susceptibility to conventionally used antifungals and for virulence determinants, such as biofilm formation and protease production. Molecular characterization of *C. metapsilosis* independent isolates by amplified fragment length polymorphism (AFLP) revealed a high percentage of polymorphic bands. Statistical analysis of the pairwise genetic distances and bootstrapping revealed that recombination occurs and significantly contributes to *C. metapsilosis* genetic population variability. No association between specific AFLP markers and drug resistance or other phenotypes was observed.

1. Introduction

Candida orthopsilosis and *Candida metapsilosis*, formerly considered intraspecific variants of *Candida parapsilosis*, have recently been classified as separate species on the basis of extensive genomic differences and molecular phylogenetics analysis (Tavanti et al., 2005). Evidence for the genetic differences within the psilosis complex was collected over the years and includes: high level of dissimilarity in *ITS1* sequence (Tavanti et al., 2005); DNA reassociation studies (Roy and Meyer, 1998); different hybridization patterns with Cp13 probe (Enger et al., 2001); ribosomal and mitochondrial DNA sequence analysis (Kurtzman and Robnett, 1997; Nosek et al., 2002). In addition, karyotype and amplification fragment length polymorphism (AFLP) analyses indicate a complete different profile for each species (unpublished results and Tavanti et al., 2007). Sequence data indicated the presence of heterozygous loci in all three species (Tavanti et al., 2005), suggesting that diploidy or aneuploidy characterizes the psilosis complex.

C. metapsilosis and *C. orthopsilosis* are still not identified by routine biochemical laboratory tests, but with the aid of DNA-based techniques, including PCR-RFLP of secondary alcohol dehydrogenase gene (*SADH*) fragment, ITS sequencing, AFLP and a DNA-microarray based system (Pryce et al., 2006; Tavanti et al., 2005, 2007; Campa et al., 2008), thus hindering a thorough epidemiological evaluation of their clinical relevance. While *C. parapsilosis* has now been assessed as a major fungal pathogen of humans, ranking as second/third most frequently occurring bloodstream infection in Europe, Canada, and Latin America (Almirante et al., 2006; Fridkin et al., 2006; Peman et al., 2005; Pfaller et al., 2008; Swinne et al., 2004), data on the isolation frequency of *C. orthopsilosis* and *C. metapsilosis* are just starting to be released (Gomez-Lopez et al., 2008; Kocsubè et al., 2007; Lockhart et al., 2008; Odds et al., 2007a; Tavanti et al., 2007).

By screening a large collection of *C. parapsilosis* isolates obtained in various hospitals in Italy, we have previously shown that 4.5% of the infections/colonizations attributed to *C. parapsilosis*, was instead caused by *C. orthopsilosis* (Tavanti et al., 2007).

Koksubé and co-workers provided the first report on the identification of two isolates of *C. metapsilosis* from bloodstream infections (Kocsubè et al., 2007). A recent survey on the distribution of a worldwide collection of the "psilosis" complex indicates that *C. metapsilosis* was less represented (1.8%) than *C. orthopsilosis* (6.8%) in the presumed *C. parapsilosis* collection screened (Lockhart et al., 2008). However, the relative geographic distribution of these species varies greatly, with *C. metapsilosis* (6.8%) overturning *C. orthopsilosis* prevalence in Australia, and reaching the highest percentage in Poland (13.8%). Others report the isolation frequency of the two new species in bloodstream infection as 6.5% for *C. metapsilosis* and 5.7% for *C. orthopsilosis* in Spain (Gomez-Lopez et al., 2008), and zero for both species in Scotland (Odds et al., 2007a).

In this study BanI digestion profile analysis of the *SADH* gene fragment and AFLP were used to evaluate the frequency of *C. metapsilosis* in a collection of 395 independent isolates previously

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classified as *C. parapsilosis* on the basis of conventional biochemical tests. The data obtained in this report confirms the recent literature on the occurrence of *C. metapsilosis* in the clinical setting and its association with colonizations/infections in humans, thus overcoming the initial hypothesis of a species rarely isolated from human mycoses. The ability of *C. metapsilosis* isolates to produce biofilm and to secrete extracellular proteases was evaluated, together with susceptibility to the commonly used antifungal drugs. It has been shown that the three species have marked differences in drug susceptibility, potentially interfering with therapeutic strategies and clinical outcomes (Garcia-Effron et al., 2008; van Asbeck et al., 2008). AFLP was used to perform a molecular characterization of *C. metapsilosis* isolates to evaluate their genetic diversity. Indeed, very little is known on the population genetics of *C. metapsilosis* and on its genome organization. Furthermore, no evidence for a sexual reproductive cycle has been yet reported.

2. Materials and methods

2.1. Strains

The *C. parapsilosis* strain collection screened included 395 isolates obtained from 395 different individuals. The majority of the Italian isolates ($n = 234$) were provided by the Unità Operativa di Microbiologia, Ospedale Universitario, Pisa, 88 isolates were from different Italian hospitals (Table 1). Other *C. parapsilosis* isolates ($n = 73$) were generously given by Dr. Arlo Upton, Auckland City Hospital, New Zealand. The isolates used in this study (Table 1) were originally identified as *C. parapsilosis* according to their biochemical profile on API32 ID and a Vitek 2 advanced colorimetric semi automated system (bioMérieux, Marcy l'Etoile, France). Strain CP286 (originally named J960161) was a gift from Prof. F.C. Odds. *C. metapsilosis* ATCC 96143 and ATCC 96144, *C. parapsilosis* ATCC 22019 and CBS604; *C. orthopsilosis* ATCC 96139 and ATCC 96140, and *Candida albicans* SC5314 and DSM1386 were also included in the study as reference strains. All the isolates were maintained on Sabouraud agar (Liofilchem S.r.l., TE, Italy) for the duration of the study.

2.2. DNA extraction and molecular identification of C. metapsilosis

Genomic DNA was extracted from yeast samples grown in Sabouraud broth (Liofilchem S.r.l., TE, Italy), as previously described (Tavanti et al., 2007).

BanI digestion patterns of the secondary alcohol dehydrogenase PCR products were used to identify the three species (Tavanti et al., 2005). Briefly, a 716 bp fragment of the *SADH* gene was amplified, purified and digested with BanI. Digestion patterns were used to discriminate the three species, since the *C. parapsilosis, C. metapsilosis* and *C. orthopsilosis SADH* amplicons contain 1, 3 and 0 BanI restriction sites, respectively.

AFLP analysis was used to confirm species identification and to evaluate genetic relatedness of *C. metapsilosis* isolates. AFLP was performed on 50 ng of genomic DNA as previously described (Tavanti et al., 2007). Besides the restriction-enzyme combination EcoRI/MseI, the combination EcoRI/HindIII was used in the first restriction/ligation step. The concentration of the HindIII adaptor was equal to the EcoRI (0.45 μM). Sequences of the adapters and pre-selective primers used for AFLP analysis are shown in Table 2. Pre-selective, selective amplifications and gel electrophoresis conditions were performed as previously described (Tavanti et al., 2007).

2.3. AFLP data analysis

AFLP profiles, ranging from 50 to 800 bases, were exported as a TIFF file and analyzed with the TotalLab TL120 software package (Nonlinear Dynamics Ltd., UK). DNA bands obtained for each isolate were size-matched. AFLP bands were defined by time (Rf value) and by the surface of the fluorescent peak they form. For AFLP band quantification/definition, background was subtracted and the surface of each peak was determined. The total quantity of AFLP fragments present for each isolate was represented by the sum of all peak surfaces for each lane and it was defined as "Lane Volume". This lane volume was set to 100%, and peak surfaces (relative intensities) were expressed as a percentage of the "Lane Volume", thus permitting lane normalization. Only bands which were at least present as 0.5% of the lane volume in at least one of the isolates were included in the analysis. Bands were considered to be absent as the surface of the peak was less than 0.03% of the lane volume. 1/0 matrices representing presence/absence of bands were exported to Microsoft Excel. The matrices obtained from the four gels were combined.

The fact that AFLP markers are dominant can lead to an underestimation of the frequencies of null alleles, potentially misleading population genetic studies. Therefore, Lynch and Milligan (1994) recommended ignoring loci (markers) with fewer than three null homozygotes when dominant markers are used. For this reason, bands which were present in all 22 or 21 or 20 strains were omitted (null allele frequency $\leqslant 3$). The resulting binary similarity file obtained for the *C. metapsilosis* strains was exported to the AFLP-SURV 1.0 program (Vekemans et al., 2002). Nei's genetic distance matrix was calculated after Lynch and Milligan (1994) and an UPGMA dendrogram was generated with the program Treefit and visualized with the Treeview program (Hampl et al., 2001; Page, 1996).

Since the above described band sharing index considers only the presence/absence of bands, the relative intensities of the observed band patterns were also analyzed. The relative intensity of all 418 bands from the four gels (expressed as % of the lane volume) for each strain was exported from the TL120 program to Microsoft Excel®. Data from the four gels were combined and the Pearson correlation coefficient between all isolates was calculated. Isolates with complete identical banding patterns and relative intensities have a correlation of 1, with the correlation distance therefore being: 1-correlation.

To test for clonality within the *C. metapsilosis* species, Nei's genetic distances were calculated after Lynch and Milligan (Nei, 1987; Lynch and Milligan, 1994), using the AFLP-SURV 1.0 program, and the index of association (*Ia*) test was performed as already described (Taylor et al., 1999; Tavanti et al., 2007). In this analysis, the variance of the observed genetic distances between the 22 strains was compared with the distribution of variance calculated as *Ia* for 1000 shuffled data sets. These distance matrices were computed by bootstrapping over the 193 polymorphic AFLP loci by the AFLP-SURV software (Vekemans et al., 2002).

Student's *t* test was used to compare genetic diversities between groups of isolates with different phenotypes, as described by Xu et al. (2000).

2.4. Biofilm formation

Biofilm production by *C. metapsilosis* clinical isolates was evaluated as previously described (Tavanti et al., 2007). One set of plates was incubated at 37 °C and another at 30 °C without agitation. After 24 h, plates were washed and the optical density was measured (OD $\lambda_{450\ nm}$). Biofilm production was considered as absent, when the OD was lower than 0.03 (Tavanti et al., 2007).