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Systematic review and new insights into the molecular characterization of the *Candida rugosa* species complex ☆

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ABSTRACT

Recently, *Candida rugosa* was characterized as a species complex comprising four taxa: *C. rugosa sensu stricto*, *Candida pseudorugosa*, *Candida neorugosa* and *Candida mesorugosa*. Although considered relatively rare, several clusters of candidemia due to *C. rugosa* complex had been reported presenting mortality rates close to 70%. In this work we discuss the systematization, phenotyping and molecular methods based on internal transcribed spacer region (ITS) sequencing and proteomic analyses for species identification, as well as clinical aspects of the *C. rugosa* complex. We performed a Bayesian phylogenetic analysis using 72 ITS sequences representative of *C. rugosa* complex isolates and related species within the genus. Biochemical, morphological and MALDI-TOF MS analyses were processed with *C. rugosa* complex type strains and related species isolates. We described that the phylogeny showed four distinct clades inferred with high posterior probabilities, corresponding to the four species within the *C. rugosa* complex, excluding *C. pararugosa*. Biochemical and morphological aspects distinguished only *C. rugosa sensu stricto* but were not sufficient to accurately identify species within the rest of the complex. Protein spectrum profiles differentiated all reference strains from different species analyzed. To our knowledge, we presented the first phylogenetic analysis using a large collection of ITS sequences as well as proteomic profiles generated from isolates of the *C. rugosa* complex and related species that can enlighten systematics, diagnostics and clinical research fields.

1. Introduction

Candida rugosa is an ascomycetous yeast organism that appears to be emerging as an etiological agent of human infectious diseases in different parts of the world (Pfaller et al., 2006). This species is most frequently found in environmental sources and is a cause of bovine mastitis, which is one of the most important diseases in dairy cows (Crawshaw et al., 2005; Scaccabarozzi et al., 2011). However, in recent years, *C. rugosa* has been described as a cause of candidemia in critically ill trauma patients (Behera et al., 2010; Pfaller et al., 2006; Singh et al., 2011), with the isolation frequency estimated at 0.6% worldwide, but a higher prevalence in South America (2.7%) (Pfaller et al., 2006).

Candida rugosa is phenotypically characterized by the formation of macroscopic wrinkled colonies that vary in color from white to cream and microscopic blastoconidia and pseudohyphae (De Hoog et al., 2000). This species is an anamorph yeast without a described sexual cycle (Calderone and Clancy, 2012). It has been extensively studied as a source of extracellular lipases that can be used in the production of several compounds, such as fatty acids and esters (Benjamin and Pandey, 1998; Dominguez de Maria et al., 2006).

Historically, *C. rugosa* was first named *Mycoderma rugosa* by Anderson after isolation from human feces in 1917 (Moretti et al., 2000). It possessed species synonymies in the botanical field for decades, known as *Azymocandida rugosa*, *Mycotorula rugosa* and *Torula rugosa*. Finally, in 1942, *C. rugosa* was reclassified by Diddens and Lodder (Meyer et al., 1998).

In recent years, with the knowledge gained from molecular studies, systematic and taxonomic classifications of molds and yeasts have been dramatically revised. *Candida rugosa* is now addressed as a complex of different species that encompasses *C. rugosa*, *C. pseudorugosa*, *C. neorugosa* and *C. mesorugosa* (Chaves et al., 2013; Paredes et al., 2012).

Due to the novelty of the molecular characterization of cryptic species within the *C. rugosa* complex, there is a lack of robust and consistent data on the putative clinical peculiarities and antifungal susceptibility of all four species. Consequently, most of the publications on human infections caused by strains of the *C. rugosa* complex had not provided accurate identification of species by gene sequencing (Colombo et al., 2003; Minces et al., 2009).

In the three largest series of candidemia due to the *C. rugosa* complex, crude mortality rates ranged from 44% to 68% (Behera et al., 2010; Colombo et al., 2003; Dube et al., 1994). It remains

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unclear whether high mortality rates reported by patients with hematogenous infections are mostly related to the severity of illness and comorbidities present at the time of the diagnosis of fungemia or whether this finding may be related to the limited antifungal susceptibility of *C. rugosa* to amphotericin B and fluconazole (Behera et al., 2010; Colombo et al., 2003).

In addition to hematogenous candidiasis, yeasts of the *C. rugosa* complex have been rarely isolated from the respiratory tract, urine, feces, skin and peritonitis (Kocyigit et al., 2010; Li et al., 2006; Paredes et al., 2012; Tay et al., 2011).

Authors had little success in demonstrating that *C. rugosa* isolates produce virulence factors, *e.g.*, secreted enzymes. Among different species of *Candida* spp. tested, *C. rugosa* isolates were negative for phospholipase and protease activities (Kantarcioglu and Yucel, 2002), and could only produce alpha hemolysis (Luo et al., 2001). Lipase secretion by *C. rugosa* strains has been extensively investigated, and these enzymes have many applications in biotechnological processes (Benjamin and Pandey, 1998; Dominguez de Maria et al., 2006). However, it remains unclear whether *C. rugosa* secreted lipases have any relevance to colonization or infection in human hosts, suggesting that *C. rugosa* complex is one of the less virulent of the genus.

The focus of the present work is to review taxonomic and diagnostic aspects of the *C. rugosa sensu lato* providing new data related to proteomic profiles as well as a phylogenetic approach to enlighten the systematics of the species within this complex.

2. Material and methods

2.1. Phylogenetic analysis

To perform the phylogenetic analysis, ITS sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) after BLASTn searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the sequences from type strains of each species within the *C. rugosa* complex and *C. pararugosa* as queries: *C. rugosa* CBS 613^T or ATCC 10571^T (AY500374), *C. mesorugosa* CBS 12656^T (FJ768909), *C. pseudorugosa* CBS 10433^T (DQ234792), *C. neorugosa* CBS 12627^T (HE716762) and *C. pararugosa* ATCC 38774^T (AF421856). Only sequences that presented identity and coverage of $\geqslant$80% with query sequences with low levels of gaps and/or ambiguities were considered in the analysis, to include a more representative sample of interspecies and intraspecies variations (Ciardo et al., 2006; Nilsson et al., 2008). Additionally, the ITS sequences of *C. albicans* (FJ662406), *C. dubliniensis* (AB369916), *C. parapsilosis* (EU564205), *C. metapsilosis* (EU484054) and *C. orthopsilosis* (EU557373), that represent well-known species complexes were added for comparison. All sequences were aligned using the muscle algorithm implemented by SEAVIEW 4.2.12 (Gouy et al., 2010) and adjusted by eye before phylogenetic analysis. An unconstrained consensus phylogeny was inferred with MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with default priors as input. The number of generations was 2.5 million, where the average standard deviation of split frequencies was <0.01, with data saved every 100 generations and run in four chains and two runs. During the runs, the GTR model and shape of the gamma distribution parameters, as well as the proportions of invariant sites, were estimated.

2.2. Phenotypic and biochemical characterization of the C. rugosa species complex and related species

The colony morphology of *C. rugosa* ATCC 10571^T, *C. mesorugosa* CBS 12656^T, *C. pseudorugosa* CBS 10433^T, *C. neorugosa* CBS 12627^T, and *C. rugosa* (*pararugosa*) CBS 1948^T was determined on Sabouraud Dextrose Agar (SDA) (BD Difco, USA) plates after 48 h of incubation at 35 °C. CHROMagar Candida (BD, USA) plates were incubated at 35 °C for 72 h before colony color observation. For micromorphological observation, cells were cultured in Yeast Peptone Dextrose (YPD) broth and incubated overnight at 30 °C and also in cornmeal agar plates incubated at 35 °C for 72 h. For biochemical and assimilation analyses, ID32C (BioMerieux, France) was used according to the manufacturer's instructions.

2.3. Mass spectrometry analysis of the Candida rugosa complex and related species

All tested *Candida* sp. isolates were recovered from frozen stock onto SDA and incubated for 3 days at 35 °C before experiments. To obtain the proteins from the isolates, we first tested the protocol described by Stevenson et al. (2010), but we found that *C. rugosa* ATCC 10571^T, which formed very wrinkled colonies, and *C. rugosa* (*C. pararugosa*) CBS 1948^T did not yield reproducible results. Therefore, we adapted the protocol by growing the isolates in YPD broth overnight at 30 °C before proceeding with the analysis. One milliliter of cultured cells was pelleted and washed twice with ultrapure water, and the excess of water was removed. A small loop of cells was directly spotted onto each position of a Micro Scout Plate (MSP) 96 polished steel target (Bruker Daltonics GmbH, Germany). Then, 25% formic acid was immediately poured onto the samples. After drying at room temperature, each sample was overlaid with matrix solution, which consisted of a saturated solution of alpha-cyano-4-hydroxy-cinnamic acid (Sigma, USA) in 50% acetonitrile/2.5% trifluoroacetic acid (Sigma, USA), and the matrix/sample was co-crystallized by air-drying at room temperature. Each isolate was spotted in triplicate. Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonics) using FlexControl software (version 3.0, BrukerDaltonics). Spectra were recorded in the positive linear mode (laser frequency, 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.6 kV; lens voltage, 7.5 kV; mass range, 2000–20,000 Da). For each spectrum, 100 shots in 50-shot steps from different positions of the target spot (automatic mode) were collected and analyzed. Spectra were internally calibrated by using *Escherichia coli* ribosomal proteins. MALDI Biotyper software 3.0 (Bruker Daltonik GmbH) was used to visualize mass spectra and generate the MSP dendrogram.

3. Results and discussion

3.1. Taxonomy, systematics and geographic trends of different species within the C. rugosa complex

In the past years, the high genetic heterogeneity of the *C. rugosa* taxon has gained attention (Dib et al., 1996; Redkar et al., 1996; Scaccabarozzi et al., 2011; Tay et al., 2011), and more studies on the morphology, biochemical patterns and sequencing analysis, mostly based on ribosomal RNA genes, have impacted the taxonomy of *C. rugosa*.

The novel species within the *C. rugosa* complex have been identified based on 26S D1/D2 rDNA sequences, complemented with ITS sequences (Butinar et al., 2011; Chaves et al., 2013; Li et al., 2006; Paredes et al., 2012; Taverna et al., 2012; Tay et al., 2011). Of note, only *C. mesorugosa* was characterized based on sequences of rDNA regions as well as housekeeping genes (Chaves et al., 2013).

To our knowledge, no complete genome of any species within the *C. rugosa* complex is available. Chaves et al. (2013) succeeded in amplifying fragments of *ACT1* and *COX2* of *C. rugosa*, *C. mesorugosa* and *C. pseudorugosa*, but the *RBP1* gene fragment could not be amplified from one of the *C. pseudorugosa*-like isolates, and none of