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Molecular identification and characterization of filamentous fungi and yeasts isolated in a pharmaceutical industry environment

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ABSTRACT

Microbiological monitoring of pharmaceutical industry aseptic areas is an important procedure to assess the efficiency of contamination control measures in these areas. Once the permitted microbiological level has been exceeded, the microbial contaminant should be identified, and the actions to eliminate this agent should be adopted. The objective of this study was to identify the filamentous fungi and yeasts isolated from the pharmaceutical industry environment by the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) proteomic approach as well as to analyze fungal universal region polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) capacity in discriminating inter- and intraspecies differences in these isolates. The MALDI-TOF method was able to identify 100% of the samples to the genus level; however, only 68.42% of the samples were identified to the species level. *Candida guilliermondii*, *Penicillium* spp., *Rhodosporidium toruloides,* and *Aspergillus* species were the most prevalent microorganisms among the samples. The PCR-RFLP analysis of the fungal universal region revealed a heterogeneous profile in the analyzed samples. The isolates of the same species, collected from different sampling points, presented the same restriction profile. MALDI-TOF and PCR-RFLP methods of the fungal universal region were able to, respectively, identify and characterize the genetic diversity and differentiate interspecifically the yeast and fungi samples analyzed in this study. The genetic profiles established by the PCR-RFLP methods of this study can be used to create a database for in-house identification of yeasts and fungi in the laboratory, where this study was carried out.

INTRODUCTION

The sterility of parenteral products is a key attribute for product safety (Hussong, 2010). An appropriate cleaning standard must be maintained to ensure the sterile production in the controlled environments, where this type of product is manufactured (Xavier *et al*., 2013). Hertroys (1997) addressed the use of cleanrooms as an essential requirement for pharmaceutical production. The cleanliness controls of cleanrooms associated with microbiological monitoring and control are a part of the industry's quality assurance routine (Xavier *et al*., 2017). These control measures aim to maintain the quality and purity required for the parenteral products (Pacheco and Pinto, 2010).

The American (USP, 2012) and Brazilian (Brasil, 2010) Pharmacopoeias establish the requirements for the control and monitoring of controlled environments. The United States Pharmacopoeia also determines that if microorganisms are detected in drug substances, excipients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished drug products in exceeded levels, they must undergo species-level

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characterization. This may include identification and strain typing, as appropriate. This information provides data for investigations on contaminations and the understanding of the area's microbiota.

Sandle (2011) showed two general categories of microbial identification: those that examine phenotypic characteristics and those that examine the genotypic composition, including the nature of the microorganisms' constituent nucleic acids and genes.

One of the methodologies of microbial identification by proteomic methods cited in the United States Pharmacopoeia and described by Arnold and Reilly (1998), Barbosa *et al*. (2019), and Patel (2015) is the MALDI-TOF mass spectrometry (matrix-assisted laser desorption ionization-time of flight). This methodology is based on the protein profile obtained by MALDI-TOF MS, which combines the acquired sequence with a database of known profiles, such as a proteomic fingerprint (Ojima-Kato *et al*., 2014; Porte *et al*., 2017). This technique has already been used for fungal and yeast identification providing a rapid, accurate, and competitive analysis (Patel, 2015; Xavier *et al*., 2019; Zhao *et al*., 2018).

As genotypic methods, the techniques that use the sequences of representative elements based on PCR are widely used for microbial identification. In filamentous fungi and yeasts, 18S rDNA, 5.8S rDNA, and 28S rDNA genic regions are the target regions for these methods. According to Bellemain *et al*. (2010), among these genic regions, there are also conserved DNA intergenic regions, known as internal transcribed spacer (ITS), which can be used for genetic differentiation. As these regions accumulate more mutations when compared to coding regions, they can be used for the infrageneric studies (Baldwin *et al*., 1995; Spier *et al*., 2008).

In the genotypic method of restriction fragment length polymorphism analysis using polymerase chain reaction (PCR-RFLP), the PCR-amplified fragments are digested with one or more specific restriction endonucleases, followed by agarose gel electrophoresis (Bai, 2014; Farber *et al*., 2001; Gandra *et al*., 2008), whereas evaluation of the obtained fragments may be used to detect intra- and interspecific polymorphisms.

The PCR-RFLP method has been described in the literature as a fast and efficient method capable of discriminating fungal and yeast species in routine laboratory identification (Fatima *et al*., 2017; Gharaghani *et al*., 2018; Mirhendi *et al*., 2006).

The objective of this study was to identify the filamentous fungi and yeasts isolated from an industrial environment by the MALDI-TOF MS proteomic approach and to analyze the PCR-RFLP capacity in discriminating inter- and intraspecific differences in these isolates.

MATERIALS AND METHODS

Sampling and identification of filamentous fungi and yeasts

The fungal and yeast samples used in this study came from the environmental monitoring and surfaces of drug production areas and microbiological quality control laboratories of a pharmaceutical industry located in southeastern Minas Gerais, Brazil. The isolates in question were selected from the available samples stored from January 2018 to January 2019. The sampling method consisted of active and passive air sampling and surface sampling. All the isolates had been previously collected to irradiated tryptic soy agar culture medium plates (BioMérieux) incubated at 30°C–35°C for 3 days. After this period, the microorganisms were kept in a cold (temp) chamber and were periodically transferred according to the internal procedures of the industry where they were isolated. Besides the isolates, a standard strain of *Aspergillus brasiliensis* ATCC16404 and two strains of *Candida albicans* ATCC10231 were used in this study.

Identification of isolates by the MALDI-TOF MS method

The standard strains and selected microorganisms presumably identified by microscopic methods such as filamentous fungi and yeasts were reactivated in irradiated tryptic soy agar culture medium (BioMérieux) incubated at 30°C–35°C for 3 days. After the incubation period, they were subjected to species-level identification procedures by the MALDI-TOF proteomic approach.

Since they relate to fungal/yeast samples, which present a more resistant cell wall as compared to bacteria, the samples were prepared through the Bruker Daltonics 70% formic acid ribosomal protein extraction method, for later identification by the MALDI-TOF MS methodology. The reactivated colonies were individually added to tubes containing tryptic soy broth, which were kept under constant rotation at the room temperature until the demonstration of enough biological material to perform extraction. After growth in the tubes, they were rested on a bench for approximately 10 minutes for the fungi to settle to the bottom of the tubes. About 1.5 ml of the supernatant from each tube was transferred to microtubes, which were centrifuged for 2 minutes at 13,000 rpm. After centrifugation, all supernatants from the microtubes were removed, and 1 ml of sterile deionized water was added to each pellet formed on centrifugation, which was then subjected to vortex agitation and further centrifugation, whereas such steps were repeated twice. To each microtube, 300 µl of sterile deionized water and 900 µl of EtOH were then added, followed by vortex agitation and centrifugation for 2 minutes at 13,000 rpm. All supernatants were removed, and the pellets were oven-dried in an inverted position at 37°C for 10 minutes. After drying, the amount of 70% formic acid needed to cover and suspend the pellets was added. The same amount of acetonitrile was added to the microtubes which were subjected to agitation and centrifugation. Finally, 1 µl of the supernatant of each tube was added to the spots of a Microflex LT equipment stainless steel plate. After the material was dried on the plate, 1 µl of HCCA matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid from supplier Bruker Daltonics) was applied to the spots. It was necessary to wait for the complete drying of the spots on the plate before placing it into the analysis equipment.

The mass spectra of the samples were obtained and classified using FlexControl 3.4 and MBT-RTC 4.0 software programs, respectively, on Bruker Daltonics GmbH's Microflex LT mass spectrometer, equipped with an N2 laser operating at a wavelength of 337 nm and frequency of 60 Hz. The positively charged intact proteins and peptides were electrostatically accelerated (at about 20 kV) on a 96-cm time-of-flight analyzer. The ions were measured in a positive linear model with instrument parameters optimized in the range of 1,900–20,000 m/z. Before sample acquisition, a calibrator named BTS— Bacterial Test

Standard (*E. coli* DH5 alpha, Bruker Daltonics)—was acquired and compared to a standard spectrum, with a standard deviation result <300 ppm. All spectra acquired in the analysis were compared to the system database, and a score representing the degree of similarity to the standard was given to each result. The scores ≥2,000 indicate a reliable identification to the species level, scores ≥1,700 and <2,000 indicate reliable identification to the genus level, and scores <1,700 do not represent the reliable results for release.

DNA extraction from isolates for genetic analysis

The isolates previously identified by MALDI-TOF were added to microtubes, and their DNA was extracted using PrepMan™ Ultra Sample Preparation Reagent Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The quality of the DNA extracted for subsequent PCR analysis was verified in 1.0% agarose gel stained with ethidium bromide.

Characterization of genetic profile by PCR-RFLP

To obtain DNA fragments from the samples of approximately 1,000 base pairs, 5'-TTGGTCATTTAGAGGAAGTAA-3' and 5'-CCGTGTTTCAAGACGG-3' oligonucleotide sequences, named OligoITS1F and OligoLR3, described by Raja *et al*. (2017), were selected. Primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and 5'- TACTACCACCAAGATCT-3', named OligoITS1 and OligoLR7, respectively, described by Fujita *et al*. (2001) and from Duke University (Vilgalys, 2017), were also used to obtain 2,000 base pair amplicons. Two pairs of primers were defined to obtain two different sizes for more robust results.

Fragments obtained with the oligonucleotides selected for the study comprise fungal universal gene regions 18S rDNA, 5.8S rDNA, 28S rDNA, ITS1, and ITS2, as shown in Figure 1.

Reactions were performed in a mix containing 2× Platinum™ HotStart Green Master Mix (Thermo Fisher Scientific, USA), $MgCl_2$ (2.5 mM), 10 µM of each primer, and 50 ng of yeast DNA in a final reaction volume of 50 µl. The PCR was performed on an Applied Biosystems Veriti Thermal Cycler. The amplification conditions followed the following parameters: an initial denaturation cycle at 98°C for 30 seconds, followed by 30 denaturation cycles at 98°C for 10 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes. The amplicons were visualized in 1.5% agarose gel stained with ethidium bromide and photodocumented.

For restriction analysis of approximately 2,000-bp PCR products, 15 µl of PCR product (containing approximately 500 ng of DNA), 10U of *Bsu*RI enzyme (Thermo Fisher Scientific), 2.5 µl of digestion buffer (10×), 1 µl of bovine serum albumin (0.2 µg/µl), and 5.5 µl of sterile water were used in a digestion system. The digestion system was incubated in a dry bath in an Applied Biosystems Veriti Thermal Cycler at 37ºC for 2 hours. After the incubation period, 15 µl of the restriction enzyme-digested PCR product was electrophoresed in 3% agarose gel stained with ethidium bromide, visualized, and photodocumented.

For restriction analysis of approximately 1,000-bp PCR products, 15 µl of PCR product (containing approximately 500 ng of DNA), 10U of *Taq*$^{\alpha}$I enzyme (Promega Corporation), 2.5 µl of digestion buffer (10×), 1 µl of bovine serum albumin (0.2 µg/µl), and 5.5 µl of sterile water were used in a digestion system. The digestion system was incubated in a dry bath in an Applied Biosystems Veriti Thermal Cycler at 65ºC for 2 hours. After the incubation period, 15 µl of the restriction enzyme-digested PCR product was electrophoresed in 3% agarose gel stained with ethidium bromide, visualized, and photodocumented.

RESULTS AND DISCUSSION

In this study, the MALDI-TOF method could identify only 68.42% (13/19) of the samples to the species level (MALDI-TOF real-time identification score ≥2,000). However, this methodology enabled the genus-level identification of all isolates (19/19) and the three standard strains included in this study (Figure 2). The most prevalent microorganisms among the isolated yeast samples were *Candida guilliermondii* (04/07), followed by *Rhodosporidium toruloides* (02/07) and *Rhodotorula mucilaginosa* (01/07). Among the filamentous fungi samples, the genus *Aspergillus* was the most common (03/09), followed by *Penicillium* (02/09). The remaining fungi and yeasts identified and their frequencies are shown in Figure 2.

The MALDI-TOF MS methodology is a technique that has recently been used worldwide to perform the inexpensive and reliable real-time identification of different types of microorganisms (Lau *et al*., 2019; Patel, 2015; Schubert and Kostrzewa, 2017; Xavier *et al*., 2019; Zhao *et al*., 2018). The use of solvents, such as formic acid, ethanol, and acetonitrile, in the sample preparation facilitates cell disruption and, consequently,

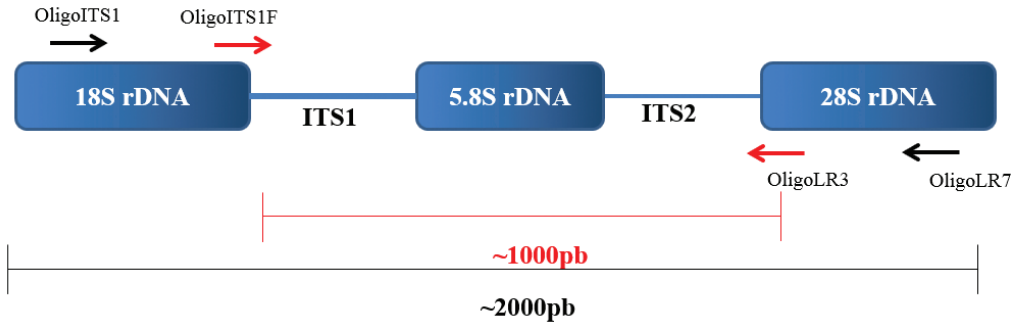

Figure 1. Schematic representation of the fungal and yeast ribosomal genes containing the target sites of oligonucleotide annealing and expected PCR amplification sizes used in this study.

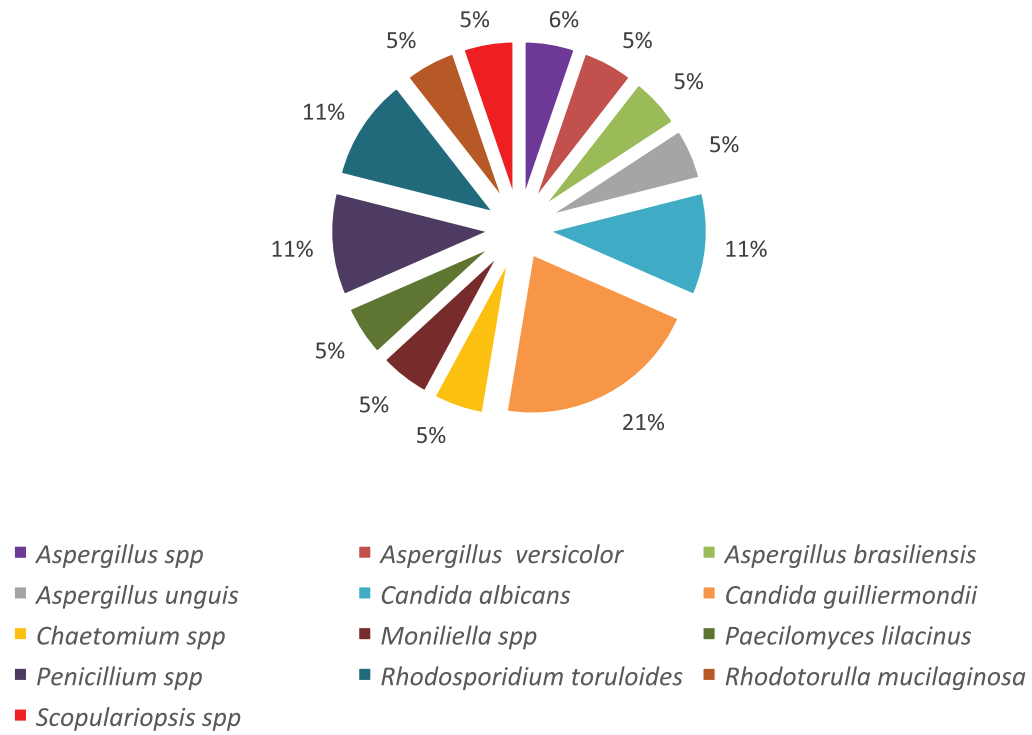

Figure 2. Identification and frequencies of filamentous fungi and yeasts by the MALDI-TOF method.

the release of ribosomal proteins, leading to more accurate measurements by this technique (Intra *et al*., 2018). As reviewed by Angeletti (2017), the MALDI-TOF MS is a technological innovation that allows rapid and accurate microbial identification. The provision of a comprehensive reference spectra database for MALDI-TOF MS is essential to obtain results through this analysis (Freiwald and Sauer, 2009; Normand *et al*., 2017; Zhao *et al*., 2018).

Among the microorganisms identified in our study, isolated from the environmental monitoring programs of a pharmaceutical industry, collected according to Table 1, we observed the prevalence of *C. guilliermondii*, *Aspergillus*, *Penicillium,* and *R. toruloides* (Fig. 2). Despite the difficulties in finding published data to compare with our results, mainly because pharmaceutical companies usually do not disclose the microbiota of their production areas, but Xavier *et al.* (2017), by sampling an industrial environment, identified the genera *Penicillium* spp., *Scopulariopsis* spp., and *Chaetomium* spp., as we have also did here. The identification results show the occurrence of fungi and yeasts that are commonly found in the environment or the human microbiota. The yeast *C. guilliermondii* is found in the microbiota of human skin and mucous membranes, whereas its isolation as a pathogenic microorganism is not common, and it lives in symbiosis (Belkaid and Segre, 2014; Marcos-Zambrano *et al*., 2017; Pasqualotto *et al*., 2006). The filamentous fungi genera *Aspergillus* and *Penicillium* are commonly found on earth (in the soil), with more than 300 accepted species (Bennett, 2010; Park *et al*., 2017; Perrone and Susca, 2017). They are responsible for decomposing various organic substances in nature and are also largely used in industrial production (Cairns *et al*., 2018; Houbraken *et al*., 2014; Park *et al*., 2017; Perrone and Susca, 2017). The yeast *R. toruloides* is known for its lipid and chemical production (Singh *et al*., 2018), presenting a great bioengineering potential (Otoupal *et al*., 2019).

Table 1. Isolation site and sampling method of filamentous fungi and yeasts isolated from industrial environment selected for the study.

Designation	Microorganism	Sampling method	Sampling site
Iso1	Yeast	Surface	Production area surface
Iso2	Yeast	Surface	Production area surface
Iso3	Filamentous fungus	Air passive	Laboratory air
Iso4	Filamentous fungus	Air passive	Laboratory air
Iso5	Yeast	Air active	Production area air
CP1	Yeast	ATCC strain	-
CP2	Yeast	ATCC strain	-
Iso8	Yeast	Surface	Production area surface
Iso9	Filamentous fungus	Surface	Production area surface
CP3	Filamentous fungus	ATCC strain	-
Iso11	Yeast	Air active	Production area air
Iso12	Filamentous fungus	Surface	Production area surface
Iso13	Filamentous fungus	Air active	Production area air
Iso14	Filamentous fungus	Air active	Production area air
Iso15	Filamentous fungus	Surface	Production area surface
Iso16	Filamentous fungus	Surface	Production area surface
Iso17	Filamentous fungus	Surface	Production area surface
Iso18	Yeast	Air active	Production area air
Iso19	Yeast	Air active	Production area air

Besides the characterization by MALDI-TOF MS, the ITS regions of the samples were analyzed by the PCR-RFPL method, to assess genetic diversity among the isolates, which is performed by PCR amplification followed by restriction analysis of the amplified fragments (Hierro *et al*., 2004). For the PCR-RFLP analysis, a pilot experiment was performed, and two samples of *C. guilliermondii* (Iso18 and Iso19) isolated from different plates and sampling points were selected (Table 1). The PCR method allowed amplification of an expected fragment of approximately 2,000 bp corresponding to a fungal universal genic region (18S rDNA, 5.8S rDNA, 28S rDNA, ITS1, and ITS2) (Fig. 3, panel A). The restriction profile analysis of the 2,000-bp region digested with *Bsu*RI revealed a homogeneous profile among the *C. guilliermondii* isolates tested (120, 140, 400, 500, and 610 bp) (Fig. 3, panel B; Table 2). Subsequently, we sought to analyze all the isolates and strains of this study (Table 1) by PCR-RFLP. Figure 4 shows the amplification profile of the isolates and strains by the PCRs to detect the 2,000- and 1,000-bp genic universal regions (Fig. 1). Some samples revealed weak or no amplification of the expected 2,000-bp fragment (Iso2, Iso7, Iso10, Iso12, Iso13, and Iso14) (Fig. 4, panel A). In the PCR performed to obtain the 1,000-bp fragment, only two samples did not show any amplification pattern (Iso5 and Iso10) (Fig. 4, panel B). This result can be attributed to problems that occurred in the DNA extraction step, for instance, low DNA yield. However, it was decided to proceed with the hydrolysis of the 2,000 and 1,000 bp fragments of the amplified samples.

The 2,000-bp PCR products were digested with *Bsu*RI and *Taq*$^{\alpha}$1 restriction enzymes to verify the ability of PCR-RFLP methods to characterize inter- and intraspecific genetic variability among the ATCC strains and isolates. The restriction analysis of the 2,000-bp PCR digested with both enzymes revealed a heterogeneous profile among the samples (Fig. 5, panels A and B). However, some isolates showed profiles, whose digestion products could not be clearly defined in 3% agarose gel. Bands presenting higher intensity in the gel were considered for reading, whereas those with poor definition were disregarded. The restriction profile analysis of the 2,000-bp fragment digested with *Bsu*RI enzyme generated eight different profiles for the 13 microorganisms tested. The isolates such as Iso8, Iso9, Iso18, and Iso19—corresponding to *C. guilliermondii* species—presented the same restriction profile (120, 140, 400, 500, and 610 bp). Two isolates identified as *Penicillium* spp. also presented the same restriction profile, sharing the 250, 390, and 500 bp bands (Fig. 5, panel A; Table 2).

Although the PCR-RFLP technique was used here only for genetic diversity analysis, the profile of Iso9, identified as *Aspergillus versicolor,* was the same as that presented by *Aspergillus* spp./Iso17 (identified only to the genus level by MALDI-TOF MS). However, the poor definition of the bands of Iso17 digested with *Taq*$^{\alpha}$1 enzyme does not allow us to infer that the *Aspergillus* spp. could correspond to *A versicolor* species. The restriction profile analysis of the 2,000-bp fragment digested with *Taq*$^{\alpha}$1 enzyme generated nine different profiles for the 11 microorganisms tested. The isolates such as Iso8, Iso9, Iso18, and Iso19, corresponding to *C. guilliermondii* species, presented the same restriction profile (140, 200, 300, 400, and 500 bp). Two isolates identified as *Penicillium* spp. also presented the same restriction profile (90, 140, 150, 350, 400, and 500 bp) sharing 250, 390, and 500 bp bands (Fig. 5, panel B; Table 2).

The 1,000-bp PCR products were also digested with *Bsu*RI and *Taq*$^{\alpha}$1 restriction enzymes to verify the ability of PCR-RFLP methods to analyze inter- and intraspecific genetic variability among the ATCC strains and isolates. The restriction analysis of the 1,000-bp PCR digested with *Bsu*RI and *Taq*$^{\alpha}$1 enzymes revealed a heterogeneous profile among the samples (Fig. 6, panels A and B; Table 3). A digestion of the 1,000-bp fragment with *Bsu*RI

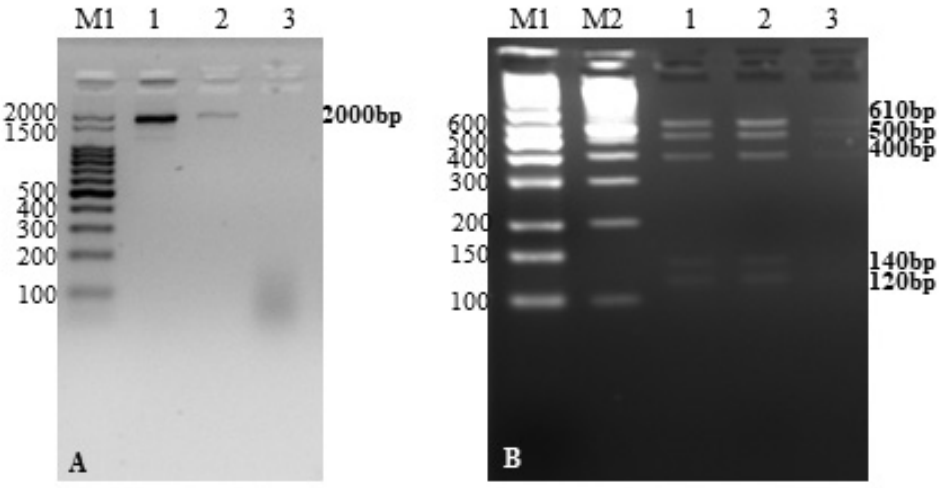

Figure 3. Results of PCR-RFLP optimization of the fungal Universal region. Panel A: PCR of the fungal universal region. M1: 100 base pair molecular mass marker (Ludwig biotechnologies). 1) Iso18 (*C. guilliermondii*). 2) Iso19 (*C. guilliermondii*). 3) Iso18 (*C. guilliermondii*). The 2,000-bp amplified fragment is indicated to the right of the 1.8% agarose gel. Panel B: Restriction profile of PCR products digested with *Bsu*RI enzyme. M1: mid-range molecular mass marker (Cellco); M2: 100 base pair molecular mass marker (Invitrogen). 1) Iso18 (*C. guilliermondii*). 2) Iso19 (*C. guilliermondii*). 3) Iso18 (*C. guilliermondii*). Restriction profiles of 120, 140, 400, 500, and 610 base pairs are indicated to the right of the 3% agarose gel.

resulted in nine different profiles for 15 microorganisms tested. The isolates of the same species of *C. guilliermondii* (Iso8 and Iso11) presented the same band profile (150, 350, and 500 bp). The homogeneous profiles were also observed among *Penicillium* spp., *Rhodosporidium* spp., and *C. albicans* species (Fig. 6, panel A; Table 3). Digestion of the 1,000-bp fragment with *Taq*$^{\alpha}$1 resulted in 11 profiles for 15 microorganisms tested. The homogeneous profiles were also observed among *C. guilliermondii*, *Penicillium* spp., *R. toruloides,* and *C. albicans* species (Fig. 6, panel B; Table 3).

According to Raja *et al*. (2017) and Schoch *et al*. (2012), the ITS region is one of the markers that present a higher probability of correct identification for a broad group of fungi.

Table 2. Size in base pairs obtained by PCR-RFLP (2,000-bp fragment digested with *Bsu*RI and *Taq*$^{\alpha}$1).

Microorganism	Designation	*Bsu*RI profile (bp)	*Taq*$^{\alpha}$1 profile (bp)
***Aspergillus* spp.**	Iso17	450, 600	310, 400, 500
A. versicolor	Iso9	450, 600	140, 200, 310, 350, 400, 500
C. albicans	CP1	225, 300, 400, 550	140, 200, 500
C. guilliermondii	Iso8	120, 140, 400, 500, 610	140, 200, 300, 400, 500
Candida guililermondii	Iso11	120, 140, 400, 500, 610	140, 200, 300, 400, 500
Candida guililermondii	Iso18	120, 140, 400, 500, 610	NT
Candida guillermondii	Iso19	120, 140, 400, 500, 610	NT
***Chaetomium* spp.**	Iso16	550	250, 300, 400
***Penicillium* spp.**	Iso3	250, 390, 500	90, 140, 150, 350, 400, 500
***Penicillium* spp.**	Iso4	250, 390, 500	90, 140, 150, 350, 400, 500
R. toruloides	Iso1	400, 550, 610	140, 160, 210, 390, 500, 600
R. mucilaginosa	Iso5	610, 1500	140, 200, 550
***Scopulariopsis* spp.**	Iso15	500, 610	200, 350, 500

Iso = isolate; CP = standard strain; NT = not tested. Fragments shared among strains of the same species with the same profile are underlined.

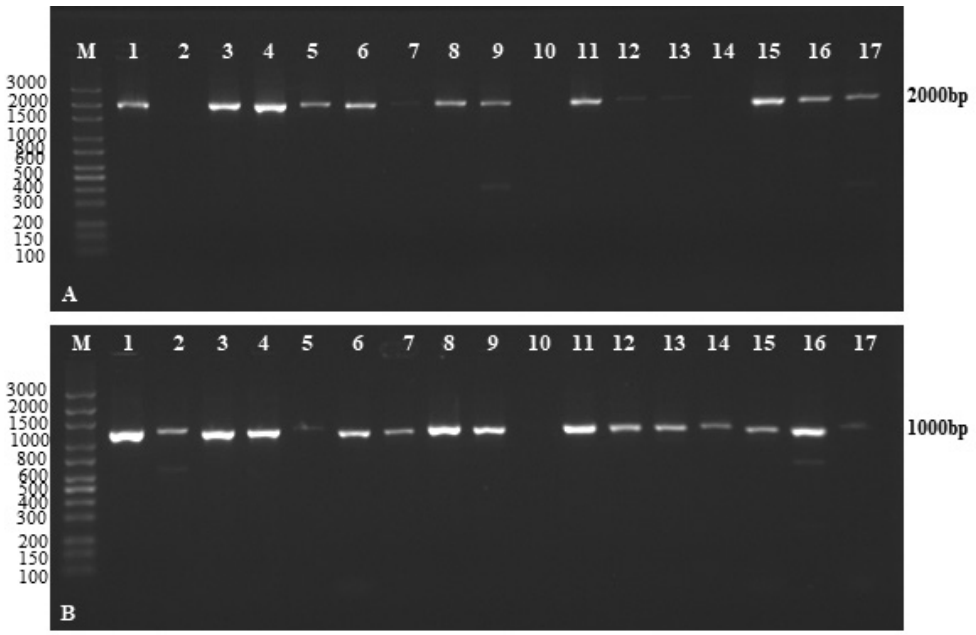

Figure 4. PCR of the fungal universal region. Panel A: Amplification of the 2,000-bp fragment of different species of filamentous fungi and yeasts. M: Mid-range molecular mass marker (Cellco); 1) Iso1 (*R. toruloides*); 2) Iso3 (*Penicillium* spp.); 3) Iso4 (*Penicillium* spp.); 4) Iso5 (*R. mucilaginosa*); 5) CP1 (*C. albicans*); 6) Iso8 (*C. guilliermondii*); 7) Iso9 (*A. versicolor*); 8) Iso11 (*C. guilliermondii*); 9) Iso15 (*Scopulariopsis* spp.); 10) Iso16 (*Chaetomium* spp.); 11) Iso17 (*Aspergillus* spp.). M: Mid-Range Molecular Mass Marker (Cellco). Panel B: Amplification of the 2000-bp fragment of different species of filamentous fungi and yeasts. M: Mid-range molecular mass marker (Cellco); 1) Iso1(*R. toruloides*); 2) Iso2 (*R. toruloides*); 3) Iso3 (*Penicillium* spp.); 4) Iso4 (*Penicillium* spp.); 5) CP1 (*C. albicans*); 6) CP2 (*C. albicans*); 7) Iso8 (*C. guilliermondii*); 8) Iso9 (*A. versicolor*); 9) Iso11 (*C. guilliermondii*); 10) Iso12 (*Moniliella* spp.); 11) Iso13 (*Aspergillus unguis*); 12) Iso14 (*Paecilomyces lilacinus*); 13) Iso15 (*Scopulariopsis* spp.); 14) Iso16 (*Chaetomium* spp.); and 15) Iso17 (*Aspergillus* spp.).

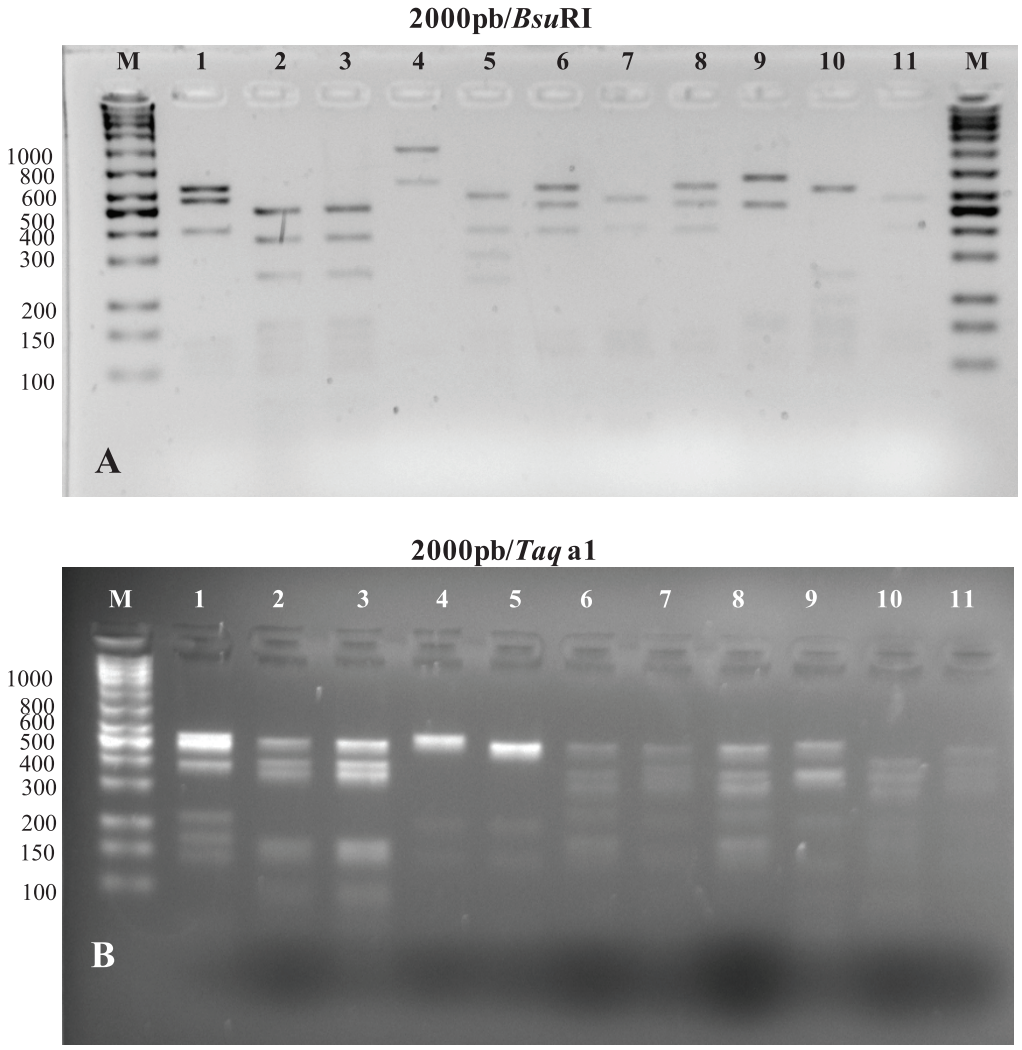

Figure 5. Restriction profile of 2,000-bp PCR products digested with *Bsu*RI and *Taq*$^{\alpha}$1 enzymes. Panel A: Restriction profile of PCR products digested with *Bsu*RI enzyme. M: Mid-range molecular mass marker (Cellco); 1) Iso1 (*R. toruloides*); 2) Iso3 (*Penicillium* spp.); 3) Iso4 (*Penicillium* spp.); 4) Iso5 (*R. mucilaginosa*); 5) CP1 (*C. albicans*); 6) Iso8 (*C. guilliermondii*); 7) Iso9 (*A. versicolor*); 8) Iso11 (*C. guilliermondii*); 9) Iso15(*Scopulariopsis* spp.);10) Iso16 (*Chaetomium* spp.); 11) Iso17 (*Aspergillus* spp.). Panel B: Restriction profile of digestion with *Taq*$^{\alpha}$1 enzyme. M: Mid-range molecular mass marker (Cellco); 1) Iso1 (*R. toruloides*); 2) Iso3 (*Penicillium* spp.); 3) Iso4 (*Penicillium* spp.); 4) Iso5 (*R. mucilaginosa*); 5) CP1 (*C. albicans*); 6) Iso8 (*C. guilliermondii*); 7) Iso9 (*A. versicolor*); 8) Iso11 (*C. guilliermondii*); 9) Iso15 (*Scopulariopsis* spp.);10) Iso16 (*Chaetomium* spp.); 11) Iso17 (*Aspergillus* spp.).

They consider the ITS region as an appropriate fungal barcode. The sequences of the ITS1 and ITS2 regions and 5.8S and 28S rDNA genes were used by Benedetti *et al*. (2016) to correctly identify the yeast species.

The restriction profile analysis of the 2,000-bp fragment digested with *Bsu*RI enzyme generated eight different profiles for 13 microorganisms tested. The isolates such as Iso8, Iso9, Iso18, and Iso19, corresponding to *C. guilliermondii* species, presented the same restriction profile (120, 140, 400, 500, and 610 bp). No difference was found in the restriction profiles of the two *Penicillium* spp. samples related to 250-, 390-, and 500-bp bands (Fig. 5, panel A; Table 2). It was also possible to verify that the band profiles of the *A. versicolor* isolate were identical to the profile presented by *Aspergillus* spp., suggesting that they are the same microorganism. The restriction profile analysis of the 2,000-bp fragment digested with *Taq*$^{\alpha}$1 enzyme generated nine different profiles for 11 microorganisms tested. The isolates such as Iso8, Iso9, Iso18, and Iso19, corresponding to *C. guilliermondii* species, presented the same restriction profile (140, 200, 300, 400, and 500 bp). The two isolates identified as *Penicillium* spp. also presented the same restriction profile (90, 140, 150, 350, 400, and 500 bp) sharing the 250, 390, and 500 bp bands (Fig. 5, panel B; Table 2).

Digestion of the 1,000-bp fragments with *Bsu*RI enzyme generated nine different profiles for 15 microorganisms tested. *C. guilliermondii* species (Iso8 and Iso11) presented the same band profile (150, 350, and 500 bp). The homogeneous profiles were also observed among *Penicillium* spp., *Rhodosporidium* spp., and *C. albicans* species (Fig. 6, panel A; Table 3). Digestion of the 1,000 bp fragment with *Taq*$^{\alpha}$1 resulted in eleven profiles for 15 microorganisms tested. Homogeneous profiles were also

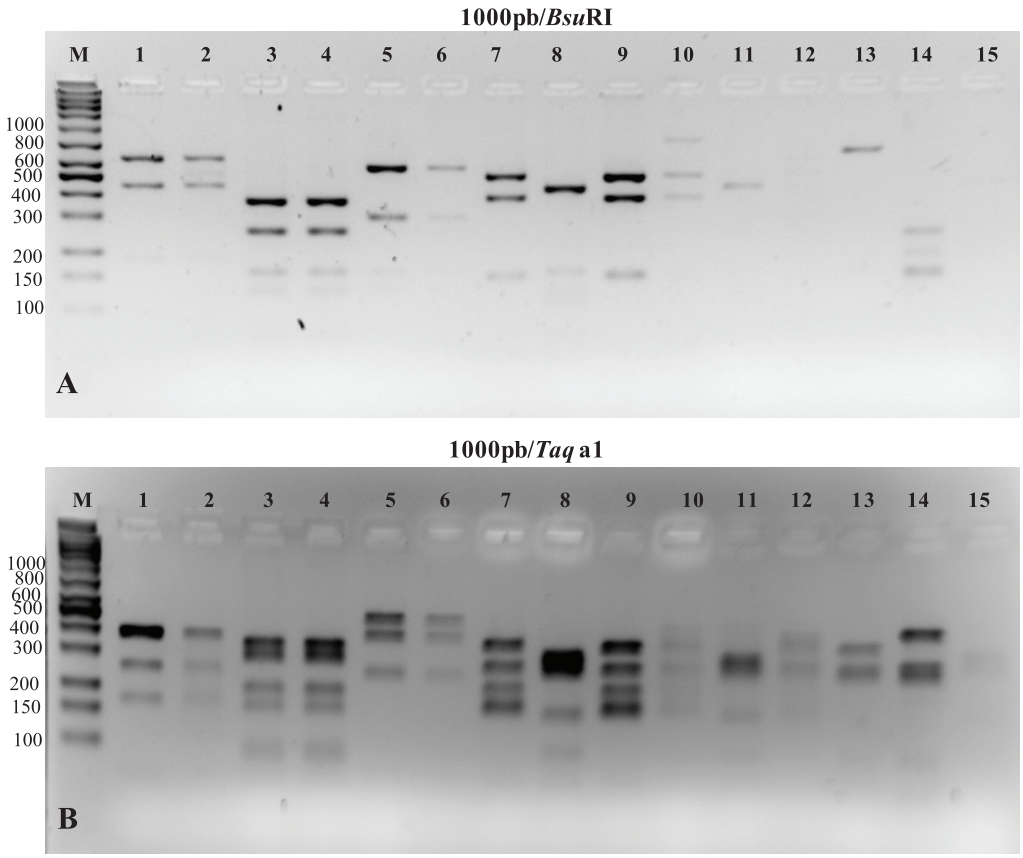

Figure 6. Restriction profile of 1000 bp PCR products digested with *Bsu*RI and *Taq*$^{\alpha}$1 enzymes. Panel A: Restriction profile of PCR products digested with *Bsu*RI enzyme. M: Mid-Range Molecular Mass Marker (Cellco); 1) Iso1 (*R. toruloides*); 2) Iso2 (*R. toruloides*); 3) Iso3 (*Penicillium* spp.); 4) Iso4 (*Penicillium* spp.); 5) CP1 (*C. albicans*); 6) CP2 (*C. albicans*); 7) Iso8 (*C. guilliermondii*); 8) Iso9 (*A. versicolor*); 9) Iso11 (*C. guilliermondii*); 10) Iso12 (*Moniliella* spp.*);* 11) Iso13 (*A. unguis*); 12) Iso14 (*P. lilacinus)*; 13) Iso15 (*Scopulariopsis* spp.); 14) Iso16 (*Chaetomium* spp.); and 15) Iso17 *(Aspergillus* spp.). Panel B: Restriction profile of digestion with *Taq*$^{\alpha}$1 enzyme. M: Mid-Range Molecular Mass Marker (Cellco); 1) Iso1 (*R. toruloides*); 2) Iso2 (*R. toruloides*); 3) Iso3 (*Penicillium* spp.); 4) Iso4 (*Penicillium* spp.); 5) CP1 (*C. albicans*); 6) CP2 (*C. albicans*); 7) Iso8 (*C. guilliermondii*); 8) Iso9 (*A. versicolor*); 9) Iso11 (*C. guilliermondii*); 10) Iso12 (*Moniliella* spp.*);* 11) Iso13 (*A. unguis*); 12) Iso14 (*P. lilacinus)*; 13) Iso15 (*Scopulariopsis* spp.); 14) Iso16 (*Chaetomium* spp.); and 15) Iso17 *(Aspergillus* spp.).

Table 3. Size in base pairs obtained by PCR-RFLP (1000-bp fragment digested with *Bsu*RI and *Taq*$^{\alpha}$1).

Microorganism	Designation	*Bsu*RI profile (bp)	*Taq*$^{\alpha}$1 profile (bp)
A. versicolor	Iso9	160, 450	40, 150, 250, 290
***Aspergillus* spp.**	Iso17	-	250
***Aspergillus* fungi**	Iso13	400	140, 300
C. albicans	CP1	300, 550	230,400, 500
C. albicans	CP2	300, 550	230,400, 500
C. guilliermondii	Iso8	150, 350, 500	150, 200, 250, 290
C. guilliermondii	Iso11	150, 350, 500	150, 200, 250, 290
***Chaetomium* spp.**	Iso16	150,200, 250	200, 250, 400
***Moniliella* spp.**	Iso12	350, 450,800	150, 250, 290
P. lilacinus	Iso14	-	140, 250, 290
***Penicillium* spp.**	Iso3	160, 250, 350	40, 140, 200, 290, 350
***Penicillium* spp.**	Iso4	160, 250, 350	40, 140, 200, 290, 350
Rhodosporidium toluroides	Iso1	450, 700	180, 250, 400
R. toluroides	Iso2	450, 700	180, 250, 400
***Scopulariopsis* spp.**	Iso15	710	200, 300

Iso = isolate; CP = standard strain; NT = not tested. Fragments shared among strains of the same species with the same profile are underlined.

observed among *C. guilliermondii*, *Penicillium* spp.*, R. toruloides,* and *C. albicans* species (Fig. 6, panel B; Table 3). Thus, it can be concluded that there was no significant difference in the tests whether performed with 1,000- or 2,000-bp fragments.

Kordalewska *et al*. (2018) proved the efficiency of the PCR-RFLP method for advanced species-level identification of the filamentous fungus *Scopulariopsis*. This same method was used by Robledo-Leal *et al*. (2018) to identify approximately 60 isolates of *Candida* yeast.

CONCLUSION

The MALDI-TOF MS method identified all samples of filamentous fungi and yeasts to the species or genus level. The PCR-RFLP method of the ITS regions, performed with fragments of two sizes and two different restriction enzymes, was demonstrated to be capable of the interspecific analysis of the yeast and fungi samples analyzed in this study.

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AUTHORS' CONTRIBUTIONS

Luciana Nobre Leite, Mauro aparecido de Sousa Xavier, and Alessandra Rejane Ericsson de Oliveira Xavier designed the study. Luciana Nobre Leite, Josiane dos Santos, Léia Cardoso, Frederico Santos Barbosa, Rosimar Fonseca dos Santos, and Soraya Aparecida Maia Dias performed the experiments. Luciana Nobre Leite, Felipe José Nobre Lelis, Mauro aparecido de Sousa Xavier, and Alessandra Rejane Ericsson de Oliveira Xavier performed data analysis. Alessandra Rejane Ericsson de Oliveira Xavier and Luciana Nobre Leite wrote the manuscript. All authors read, edited, and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to the publication of this paper.

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