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Species diversity of *Aspergillus* section *Versicolores* in clinical samples and antifungal susceptibility

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ABSTRACT

Aspergillus section *Versicolores* includes species of clinical relevance and many others that have been poorly studied but are occasionally found in clinical samples. The aim of this study was to investigate, using a multilocus phylogenetic approach, the spectrum of species of the section *Versicolores* and to determine their *in vitro* antifungal susceptibility. The study was based on a set of 77 clinical isolates from different USA medical centres, which had been previously identified as belonging to this section. The genetic markers used were internal transcribed spacer (ITS), β-tubulin (*BenA*), calmodulin (*CaM*), and RNA polymerase II second largest subunit (*RPB2*), and the drugs tested, following the CLSI guidelines, were amphotericin B (AMB), itraconazole, posaconazole, voriconazole, anidulafungin, caspofungin, micafungin, terbinafine (TBF), and flucytosine (5FC). The most frequent species were *Aspergillus sydowii* (26 %), *Aspergillus creber* (22 %), and *Aspergillus amoenus* (18.2 %), followed by *Aspergillus protuberus* (13 %), *Aspergillus jensenii* (10.4 %), and *Aspergillus tabacinus* (5.2 %); while *Aspergillus cvjetkovicii*, *Aspergillus fructus*, *Aspergillus puulaauensis*, and *Aspergillus versicolor* were represented by only one isolate each (1.3 %). This is the first time that *A. jensenii* and *A. puulaauensis* have been reported from clinical samples. Considering the high number of isolates identified as belonging to this fungal group in this study, its clinical relevance should not be overlooked. *Aspergillus versicolor*, traditionally considered one of the most common species in this section in a clinical setting, was only rarely recovered in our study. The *in vitro* antifungal results showed that echinocandins and TBF were the most potent drugs, the azoles showed variable results, AMB was poorly active, and 5FC was the less active.

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Introduction

Aspergillus is one of the most ubiquitous genera of ascomycetes. It includes many species of biotechnological and industrial relevance (Houbraken *et al.* 2014). Some of them, particularly *Aspergillus fumigatus*, are involved in allergic diseases and severe infections in both animals and humans (de Hoog *et al.* 2011). Therefore, the correct identification of the fungal isolates is crucial for a better knowledge of the actual prevalence of the different species in their habitats and substrates. Traditionally, *Aspergillus* identification is based on macro- and micromorphological characteristics, and the species organized in groups or sections (Raper & Fennell 1976; Gams *et al.* 1985). Recent molecular studies have demonstrated that most of the *Aspergillus* sections are in fact monophyletic groups of closely related species. However, the boundaries of some sections still remain unclear (Houbraken & Samson 2011; Houbraken *et al.* 2014; Samson *et al.* 2014; Hubka *et al.* 2015). The section *Versicolores* is a clear example. It includes a group of relevant species but with a taxonomy not yet resolved. Some authors consider the delimitation of the members of this section from those of the section *Nidulantes* to be unresolved (Peterson 2008; Buzina 2013; Houbraken *et al.* 2014; Negri *et al.* 2014), while others treat *Versicolores* and *Nidulantes* as different sections (Jurjevic *et al.* 2012; Samson *et al.* 2014; Visagie *et al.* 2014; Hubka *et al.* 2015). Despite their being closely related and being two monophyletic clades with low statistical support, both sections show some phenotypic characteristics that allow their distinction. Specifically, the *Versicolores* species are characterized by conidiophores with subglobose to pyriform vesicles, biseriate conidial heads, usually radiated, with greenish rough-walled usually globose to subglobose conidia (Raper & Fennel 1976; Klich 1993; Jurjevic *et al.* 2012). However, they are particularly difficult to distinguish among species because even though their cultural morphology is considerably different, their microscopic structures are very similar (Klich 1993; Jurjevic *et al.* 2012). The taxonomy of *Versicolores* has been investigated molecularly in recent years and 20 species have so far been accepted (Jurjevic *et al.* 2012; Samson *et al.* 2014; Visagie *et al.* 2014), *Aspergillus versicolor* and *Aspergillus sydowii* being the most well-known and studied species. The interest of the species of this section lies in their common occurrence in indoor environments (Zahradnik *et al.* 2013; Sharpe *et al.* 2015), the ability to produce sterigmatocystin, a carcinogenic, and mutagenic precursor to aflatoxin B_1, and in their different biotechnological applications (Schmitt *et al.* 2002; Batista *et al.* 2003; Jurjevic *et al.* 2013; Dou *et al.* 2014; Li *et al.* 2015). Moreover, they have been reported as human and animal opportunistic pathogens (de Hoog *et al.* 2011; Buzina 2013) able to cause a variety of infections, including onychomycosis (Torres-Rodrígues *et al.* 1998; Takahata *et al.* 2008), endophthalmitis (Perri *et al.* 2005), ear infection (Rotoli *et al.* 2001), invasive pulmonary infections (Charles *et al.* 2011), aspergilloma (Kane *et al.* 2014), homograft valve infection (Huh *et al.* 2013), endodontic infection (Gomes *et al.* 2015), and vaginitis (Borsa *et al.* 2015); as well as infections in animals, such as dogs (Zhang *et al.* 2012) and horses (Ludwig *et al.* 2005; Lee *et al.* 2012). However, the spectrum of species of the section *Versicolores* in the clinical setting, considering modern taxonomic criteria proposed for *Aspergillus* (Jurjevic *et al.* 2012; Samson *et al.* 2014; Visagie *et al.* 2014), has not been fully explored. Additionally, the antifungal susceptibility of these species is practically unknown because it has only occasionally been reported (Torres-Rodríguez *et al.* 1998; Chavez *et al.* 2010; Negri *et al.* 2014). The aim of this study, therefore, was to investigate, using a multilocus sequence analysis, the diversity of species of *Aspergillus* section *Versicolores* in clinical samples in the USA and to determine their *in vitro* susceptibility to the currently available antifungal drugs.

Materials and methods

Fungal isolates

A total of 77 isolates of *Aspergillus* section *Versicolores* were investigated (Table 1), 69 from human origin, six from animal specimens and two from an environmental source. These isolates were received at the Fungus Testing Laboratory of the University of Texas Health Science Center (USA) from other centres in the country to identify them and/or to determine their antifungal susceptibility. Most of the isolates had been provisionally morphologically identified as *Aspergillus versicolor* ($n = 74$) and three as *Aspergillus* spp.

Morphological characterization

The fungal isolates were characterized morphologically following the criteria recommended by Samson *et al.* (2014). Briefly, the macromorphology of the colonies and the growth rates were determined on Czapek Yeast Autolysate Agar (CYA, Becton, Dickinson and Company®, Sparks, MD, USA) and Malt Extract Agar (MEA, Pronadisa®, Madrid, Spain) after 7 d of incubation at 25 °C and 37 °C. The microscopic structures were examined and measured on MEA cultures after 10–14 d of incubation at 25 °C, in wet mounts with 60 % lactic acid. Photographs were taken with a Zeiss Axio Imager M1 light microscope (Zeiss, Oberkochen, Germany) with a mounted DeltaPix Infinity X digital camera using Nomarski differential interference contrast and phase contrast optics.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from MEA cultures after 7 d of incubation at 25 °C, using the FastDNA® Kit and the FastPrep® Instrument (MP Biomedicals, Irvine, CA, USA), according to the manufacturer's specifications. Four genetic markers were amplified, i.e. the internal transcribed spacer (ITS) region of the rDNA, which comprises ITS1, the 5.8S gene, and ITS2, and fragments of β-tubulin (*BenA*), calmodulin (*CaM*), and RNA polymerase II second largest subunit (*RPB2*) genes (Peterson 2008; Samson *et al.* 2014). The primers used were ITS5 and ITS4 for the ITS region (White *et al.* 1990), Bt2a and Bt2b for the *BenA* gene (Glass & Donaldson 1995), Cmd5 and Cmd6 for *CaM* gene (Hong *et al.* 2005), and 5F and 7CR