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Use of Amplified Fragment Length Polymorphism To Identify 42 *Cladophialophora* Strains Related to Cerebral Phaeohyphomycosis with In Vitro Antifungal Susceptibility▽

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The amplified fragment length polymorphism technique has been applied to identify neurotropic chaetothyrialean black yeasts and relatives from clinical sources. *Cladophialophora bantiana*, *C. emmonsii*, *C. arxii*, *C. devriesii*, and *C. modesta*, previously identified on the basis of sequencing and phenotypic and physiological criteria, were confirmed by cluster analysis, demonstrating the clear separation of *C. bantiana* as a rather homogeneous group from the other species. *C. bantiana* is a neurotropic fungus causing cerebral abscesses with a mortality of up to 70%. Successful therapy consists of neurosurgical intervention and optimal antifungal therapy. Since the latter is not clearly defined in a large series, we tested the *in vitro* activities of eight antifungal drugs against clinical isolates of *C. bantiana* ($n = 37$), *C. modesta* ($n = 2$), *C. arxii* ($n = 1$), *C. emmonsii* ($n = 1$), and *C. devriesii* ($n = 1$), all of which had caused invasive infections. The resulting MIC_{90}s for all neurotropic *C. bantiana* strains were as follows, in increasing order: posaconazole, 0.125 μg/ml; itraconazole, 0.125 μg/ml; isavuconazole, 0.5 μg/ml; amphotericin B, 1 μg/ml; voriconazole, 2 μg/ml; anidulafungin, 2 μg/ml; caspofungin, 4 μg/ml; and fluconazole, 64 μg/ml. On the basis of these *in vitro* results and the findings of previous clinical and animal studies, posaconazole seems to be a good alternative to the standard treatment, amphotericin B, for *C. bantiana* cerebral infections. The new agent isavuconazole, which is also available as an intravenous preparation, has adequate activity against *C. bantiana*.

The genus *Cladophialophora* represents anamorph members of the ascomycetes in the order *Chaetothyriales* in the family *Herpotrichiellaceae* comprising the black yeasts and relatives (10). These dematiaceous fungi are normally associated with soil or vegetative matter; however, they are increasingly being seen as causative agents of mycoses in humans (27, 37, 48) and domestic (14, 23) and wild (29) animals. *Cladophialophora carrionii* is the type species and an agent of chromoblastomycosis, a cutaneous and subcutaneous disease. The genus *Cladophialophora* encompasses several other clinically significant species which are potentially able to cause severe fungal infections in otherwise immunocompetent patients. In human infections, the brain is frequently involved (27, 37, 48). Within the genus, the majority of brain abscesses with fatal outcomes are associated with *Cladophialophora bantiana* (formerly *Cladosporium bantianum*, *Cladosporium trichoides*, *Cladosporium trichoides* var. *chlamydosporum*, *Torula bantiana*, and *Xylohypha bantiana*), a neurotropic fungus, although severe phaeohyphomycotic infections are also caused by novel *Cladophialophora* species like *C. modesta* (40, 44), *C. arxii* (53, 56), *C. emmonsii* (*Xylohypha emmonsii*) (45), *C. devriesii* (22, 28, 42) *C. saturnica* (4), and *C. boppii* (34). Moreover, *Exophiala dermatitidis* and *Rhinocladiella mackenziei*, other members of the black yeast group, are also frequently isolated from cerebral infections (8, 27, 37). Central nervous system infection due to *C. bantiana* is reported worldwide, though a general preference for warmer climates with high humidity is apparent (27). Indeed, many cases are reported from India (19, 30, 33, 55), as opposed to arid climatic zones (8). The first case of *C. bantiana* (*Cladosporium trichoides*) infection was reported in 1952, when the fungus was isolated from a human brain abscess and was demonstrated to be neurotropic in laboratory animals (6). A review of 17 cases of brain abscess, published in the English language literature by the mid-1970s, reported that the majority of patients had no underlying disease (41). The most recent series of 48 patients with brain abscess due to *C. bantiana* showed that 35 patients (72%) had no risk factors and that only 13 patients (28%) survived the infection, despite combined surgical and antifungal treatment (48). The minority of immunocompromised patients are transplant recipients, intravenous drug abusers, or individuals on steroids (2, 13, 15, 24, 31, 35, 36, 51, 54, 57, 59).

The mode of infection is either by hematogenous spread from an unrecognized pulmonary focus, through direct extension from adjacent paranasal sinuses, or by penetrating trauma to the head. However, the majority of patients had had no recent evidence of pulmonary or sinus infections (27). *Cladophialophora* species are prone to identification problems (3, 20). Due to the high degree of phenotypic similarity between recently described new *Cladophialophora* spe-

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cies and *C. bantiana*, identification problems are imminent. For most cases published in the older literature, identification down to the species level cannot be repeated or confirmed by molecular methods due to the absence of the original isolates; hence, the etiological agent described in older publications may often have been misidentified. It is now well established that molecular identification methods, which have driven new developments in fungal taxonomy, are more reliable than classical morphological methods (3, 20). Amplified fragment length polymorphism (AFLP) is a technique based on the detection of genomic restriction fragments by PCR amplification, which can be used with the DNA of any organism (58). The purpose of this study was to study the inter- and intraspecific genomic variations of 42 *Cladophialophora* isolates stored in the CBS collection and recovered from cases with cerebral phaeohyphomycosis and other infections. Antifungal therapy is mainly based on the experience gathered from and published in isolated case reports and mostly involved amphotericin B, itraconazole, and flucytosine singly or in different combinations (48). Animal studies (1) and human experience suggested that amphotericin B has no value in treating cerebral infections, probably due to poor penetration of the central nervous system (CNS), but that triazoles might be of value (26, 38). A recent study of antifungal therapy in a murine model of disseminated infection by *C. bantiana* confirmed the poor activity of amphotericin B but found that posaconazole and flucytosine extended survival (39). This suggests that new antifungal drugs with broad-spectrum activity and suitable pharmacokinetic profiles compared with those of conventional antifungal agents might be more effective against *C. bantiana* (1, 39). Only limited data on *in vitro* antifungal activities against the neurotropic fungus *C. bantiana* are available. Therefore, with no standard therapy available, unfavorable results in animal experiments, and only a small published series of susceptibility testing with itraconazole and voriconazole (47), the second objective of this study was *in vitro* testing of this large collection of *Cladophialophora* strains for their susceptibilities to eight antifungal drugs, including the new triazole isavuconazole.

(Part of this work was presented as a poster at Trends in Medical Mycology, Athens, Greece, October 2009 [4a].)

MATERIALS AND METHODS

Fungal strains. Table 1 summarizes the data for and characterizes a total of 42 isolates of *Cladophialophora* spp. that originated from different human and veterinary clinical sources with cerebral phaeohyphomycosis or other infections. Strains were obtained from the reference collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands, and were handled under biosafety level 3 conditions. Stock cultures for transient working collections were initially grown on oatmeal agar (OA; Difco Oatmeal; Brunschwig Chemie, Amsterdam, Netherlands) at 24°C for 1 week, and the organisms were identified to the species level by sequencing of the internal transcribed spacer regions of the rDNA region and partial translation of the elongation factor 1-alpha and beta-tubulin genes (3).

DNA extraction. The fungal mycelia were grown on 2% malt extract agar plates for 2 weeks at 24°C. A sterile blade was used to scrape the mycelium from the surface of the plate. DNA was extracted using an Ultra Clean microbial DNA isolation kit (Mobio, Carlsbad, CA), according to the manufacturer's instructions. DNA extracts were stored at −20°C prior to use.

AFLP analysis. Approximately 50 ng of genomic DNA was subjected to a combined restriction ligation procedure containing 50 pmol of HpyCH4 IV adapter, 50 pmol MseI adapter, 2 U of HpyCH4 IV (New England Biolabs, Beverly, MA), 2 U of MseI (New England Biolabs), and 1 U of T4 DNA ligase (Promega, Leiden, Netherlands) in a total volume of 20 μl of 1× reaction buffer for 1 h at 20°C. Next, the mixture was diluted five times with 10 mM Tris-HCl (pH 8.3) buffer. Adapters were made by mixing equimolar amounts of complementary oligonucleotides (5′-CTCGTAGACTGCGTACC-3′ and 5′-CGGGTA CGCAGTC-3′ for HpyCH4 IV; 5′-GACGATGAGTCCTGAC-3′ and 5′-TAG TCAGGACTCAT-3′ for MseI), heating to 95°C, and subsequently cooling slowly to ambient temperature. One microliter of the diluted restriction-ligation mixture was used for amplification in a volume of 25 μl under the following conditions: 1 μM HpyCH4 IV primer with one selective residue (underlined) (5′-fluophore-GTAGACTGCGTACCCGTC-3′), 1 μM MseI primer with four selective residues (underlined) (5′-GATGAGTCCTGACTAATGAG-3′), 0.2 mM each deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase (Roche Diagnostics, Almere, Netherlands) in 1× reaction buffer containing 1.5 mM $MgCl_2$. Amplification was done as follows. After an initial denaturation step for 4 min at 94°C in the first 20 cycles, a touchdown procedure was applied: 15 s of denaturation at 94°C, 15 s of annealing at 66°C with the temperature for each successive cycle lowered by 0.5°C, and 1 min of extension at 72°C. Cycling was then continued for a further 30 cycles at an annealing temperature of 56°C. After completion of the cycles, incubation at 72°C for 10 min was performed before the reaction mixtures were cooled to room temperature. The amplicons were then combined with an ET400-R size standard (GE Healthcare, Diegem, Belgium) and analyzed on a MegaBACE 500 automated DNA platform (GE Healthcare), according to the manufacturer's instructions.

Data analysis. Data were inspected visually and were also imported into BioNumerics (version 5.1) software (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed by the unweighted-pair group method using average linkages (UPGMA) clustering using the Pearson correlation coefficient. The analysis was restricted to DNA fragments in the range from 80 to 250 bp.

In vitro antifungal susceptibility testing. Amphotericin B (AMB; Bristol-Myers-Squib, Woerden, Netherlands), fluconazole (FLU; Pfizer Central Research Sandwich, United Kingdom), itraconazole (ITR; Janssen Research Foundation, Beerse, Belgium), voriconazole (VOR; Pfizer), posaconazole (POS; Schering-Plough, Kenilworth, NJ), isavuconazole (ISA; Basilea Pharmaceuticals, Switzerland), caspofungin (CAS; Merck Sharp & Dohme BV, Haarlem, Netherlands), and anidulafungin (ANI; Pfizer) were obtained from the manufacturers as pure powders. As described in the Clinical and Laboratory Standards Institute (CLSI) M38-A2 guidelines for *in vitro* susceptibility studies, additive drug dilutions were prepared at 100 times the final concentrations in different solutions (9). The drugs were diluted in standard RPMI 1640 medium (Sigma Chemical) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) with L-glutamine without bicarbonate to yield the following concentrations: amphotericin B, itraconazole, voriconazole, posaconazole, and caspofungin, 0.016 to 16 μg/ml; fluconazole, 0.063 to 64 μg/ml; and isavuconazole and anidulafungin, 0.008 to 8 μg/ml. The plates were stored at −70°C until they were used. Broth microdilution was performed as described by the CLSI, in accordance with the guidelines in document M38-A2 (9). Briefly, all clinical isolates were grown on potato dextrose agar plates (PDA; Difco) at 35°C for up to 7 days for sporulation. Inoculum suspensions were prepared under biosafety laboratory level 3 regulations by slightly scraping the surface of mature colonies with a loop in sterile saline solution with Tween 40 (0.05%). After the heavy particles were allowed to settle, the homogeneous conidial suspensions were transferred to sterile tubes and adjusted spectrophotometrically at a 530-nm wavelength to optical densities (ODs) that ranged from 0.17 to 0.15 (68 to 71% transmission). The inoculum suspensions, including mostly nongerminated conidia, were diluted 1:50 in RPMI 1640 medium. The final concentration of the stock inoculum suspensions of the tested isolates ranged from 0.5×10^4 to 3.1×10^4 CFU/ml, as determined by the use of quantitative colony counts to determine the viable numbers of CFU per milliliter. After inoculation, the microdilution plates were incubated at 35°C and examined visually and spectrophotometrically at 420 nm after 72 h of incubation. The MIC endpoints were defined with the aid of a reading mirror as the lowest concentration of drug that prevents any recognizable growth (100% inhibition) for amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole. For fluconazole, a prominent reduction of growth (≥50%) compared to the growth of the drug-free control was used. The minimum effective concentration (MEC) was defined microscopically as the lowest concentration of drug that leads to the growth of small, rounded, compact hyphal forms rather than the long, unbranched hyphal clusters that were seen in the growth control (9). *Paecilomyces variotii* (ATCC 22319), *Candida parapsilosis* (ATCC 22019), and *Candida krusei* (ATCC 6258) were used as quality control organisms. Values for MIC_{50} and MIC_{90} were obtained by ordering the MIC data for each antifungal in ascending arrays and selecting the median and 90th quartile of the MIC distribution, respectively. Geometric mean MICs were computed using the Microsoft Office Excel 2003 SP3 program, for which purpose values less than *x* were set equal to 0.5*x*.

TABLE 1. Isolation data for the tested *Cladophialophora* strains[a]

Strain	CBS strain no.	Other strain reference(s)	Source	Geography
Cladophialophora bantiana	CBS 101251	ATCC 58037, CDC B-3112	Human, brain abscess	USA
Cladophialophora bantiana	CBS 100428	ATCC 22649	Human, brain abscess	USA
Cladophialophora bantiana	CBS 119547	dH 16810	Human, brain abscess	USA, North Carolina
Cladophialophora bantiana	CBS 564.82	ATCC 46715, dH 16022	Human, brain abscess	USA, Maryland
Cladophialophora bantiana	CBS 110013	dH 12086	Human, brain abscess	USA, Kentucky
Cladophialophora bantiana	CBS 194.54	dH 15506	Human origin, unknown source	USA, Pennsylvania
Cladophialophora bantiana	CBS 100432	ATCC 58035, CDC B-1551	Human, brain abscess	USA, Georgia
Cladophialophora bantiana	CBS 173.52	ATCC 10958 CDC B-1940 (T)	Human, brain abscess	USA
Cladophialophora bantiana	CBS 101252	ATCC 58040, CDC B-3466	Human, brain abscess	USA, Washington, DC
Cladophialophora bantiana	CBS 110009	dH 12082	Human origin, brain abscess	USA, North Carolina
Cladophialophora bantiana	CBS 110011	dH 12084	Human origin, unknown source	USA, West Virginia
Cladophialophora bantiana	CBS 110008	dH 12081	Human origin, unknown source	USA, Missouri
Cladophialophora bantiana	CBS 110010	dH 12083	Human origin, unknown source	USA, Virginia
Cladophialophora bantiana	CBS 110007	dH 12080	Human, brain abscess	USA
Cladophialophora bantiana	CBS 644.96	IFM 41438	Human origin, unknown source	Japan
Cladophialophora bantiana	CBS 101253	IFM 41439, dH 15162	Human origin, unknown source	Japan
Cladophialophora bantiana	CBS 642.96	IFM 41436, dH 16119	Human origin, unknown source	Japan
Cladophialophora bantiana	CBS 641.96	IFM 41434, dH 16116	Human origin, unknown source	Japan
Cladophialophora bantiana	CBS 643.96	IFM 41437, dH 16121	Human origin, unknown source	Japan
Cladophialophora bantiana	CBS 646.96	IFM 4820, DCU 651	Human origin, unknown source	Japan
Cladophialophora bantiana	CBS 101158	ATCC 44223, CDC B-3426	Human, brain abscess	Japan
Cladophialophora bantiana	CBS 100431	ATCC 44217	Human, subcutaneous phaeohyphomycosis	India
Cladophialophora bantiana	CBS 155.53		Human, brain abscess	Belgium
Cladophialophora bantiana	CBS 102586	dH 11331	Human, brain abscess	Brazil
Cladophialophora bantiana	CBS 119719	dH 14515	Human, subcutaneous phaeohyphomycosis	Thailand
Cladophialophora bantiana	CBS 100429	ATCC 24928, dH 10739	Human, brain abscess	Unknown
Cladophialophora bantiana	CBS 123392	dH 16363	Human, eumycetoma	Mexico
Cladophialophora bantiana	CBS 984.96	SAIMR J-1872	Human, brain abscess	South Africa
Cladophialophora bantiana	CBS 649.96	SAIMR W262	Human, subcutaneous phaeohyphomycosis	South Africa
Cladophialophora bantiana	CBS 981.96	UAMH 6501, dH 16330	Human, brain abscess	Canada, Edmonton
Cladophialophora bantiana	CBS 120376	dH 16362	Human, subcutaneous phaeohyphomycosis leg	Sweden
Cladophialophora bantiana	CBS 100436	ATCC 58039, CDC B-1897	Cat, brain abscess	USA, California
Cladophialophora bantiana	CBS 648.96	UAMH 3830	Dog, liver abscess	Canada, Edmonton, Alberta
Cladophialophora bantiana	CBS 118738	CNRMA 2004/222	Dog, thorax tumefaction	France, Montpellier
Cladophialophora bantiana	CBS 444.96		Dog, disseminated infection	South Africa
Cladophialophora bantiana	CBS 328.65	CDC B-3394, NCMH 1168	Dog, liver abscess	Netherlands Antilles
Cladophialophora bantiana	CBS 647.96	CDC 3432, IFM 4821	Mouse, experimentally infected by sawdust	USA
Cladophialophora modesta	CBS 985.96	UAMH 4004, dH 16331 (T)	Human, brain abscess	USA, North Carolina
Cladophialophora emmonsii	CBS 979.96	CDC B-3875, NCMH 2247 (T)	Human, subcutaneous phaeohyphomycosis	USA, Virginia
Cladophialophora emmonsii	CBS 102594	CDC B-5420, dH 11918	Human, subcutaneous phaeohyphomycosis, hand	USA, Virginia
Cladophialophora arxii	CBS 102461	CDC B-5887, dH 11524	Human, brain abscess	USA, Florida
Cladophialophora devriesii	CBS 147.84	ATCC 56280, dH 15405 (T)	Human, disseminated infection	Grand Cayman Islands

[a] Abbreviations: ATCC, American Type Culture Collection, Manassas, VA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; dH, G. S. de Hoog working collection, Utrecht, Netherlands; CDC, Centers for Disease Control and Prevention, Atlanta, GA; UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Canada; IFM, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan; SAIMR, South African Institute for Medical Research, Johannesburg, South Africa; NCMH, North Carolina Memorial Hospital, Chapel Hill, NC; DCU, Department of Dermatology, School of Medicine, Chiba, Japan; CNRMA, Collection of the National Reference Center for Mycoses and Antifungals at the Pasteur Institute of Paris; (T), ex-type culture.

RESULTS

Figure 1 depicts a dendrogram of the AFLP analysis with the type strains of *C. bantiana*, *C. emmonsii*, *C. devriesi*, *C. modesta*, and *C. arxii* demonstrating that *C. bantiana* is phylogenetically distinct from the other *Cladophialophora* spp. The AFLP patterns of *C. bantiana* strains from different geographical regions (Table 1), such as the United States, Japan, India, Belgium, France, South Africa, Mexico, and Brazil, clustered together. The structuring of this data set suggests that *C. bantiana* populations dispersed quickly across the world. There was one main cluster of *C. bantiana*, including the type strain (CBS 173.52), with similarities of more than >75%. The *C. emmonsii* strains segregate in one cluster, including the reference strain (CBS 979.96) and one clinical taxon originating from a subcutaneous lesion, which had >50% similarity to each other. The remaining taxa from clinical sources were represented by single strains, and the AFLP pattern shows that they are completely distinct from the *C. bantiana* and *C. emmonsii* clade (<20% similarity).

Table 2 summarizes the results of the geometric mean MICs, MIC ranges, and MIC_{50} and MIC_{90} distributions of eight antifungal agents for 37 *C. bantiana* isolates. Five other clinical

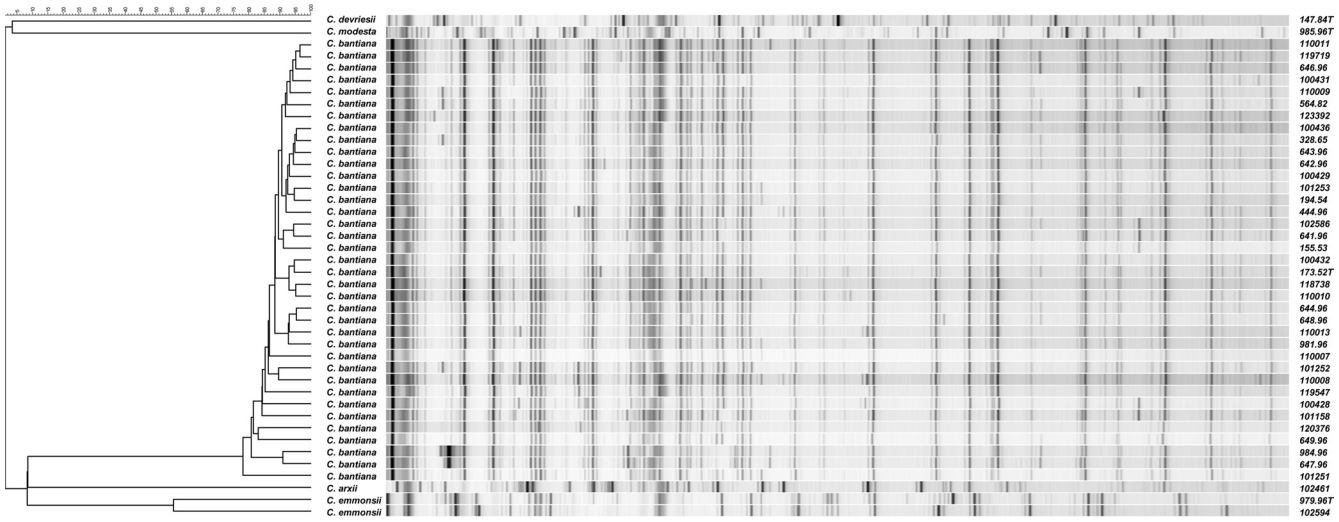

FIG. 1. Dendrogram of AFLP analysis of 42 *Cladophialophora* isolates (numbers to the right of the figure are CBS strain designations; the scale bar on the left indicates the percent similarity).

Cladophialophora species have individual MIC results similar to the *C. bantiana* MIC_{90} data. For each antifungal, the MIC_{50} and geometric mean MIC values differed by <1 $\log_2$ dilution step, indicating that in all cases the MIC_{50}s obtained by inspection reasonably reflect the central tendency of the antifungal susceptibility of the population. Overall, all of the isolates showed a uniform pattern of low MICs for itraconazole, posaconazole, and isavuconazole. The widest ranges and the highest MICs were seen for fluconazole (range, 16 to 64 μg/ml). Amphotericin B MICs ranged from 0.125 to 2 μg/ml; and itraconazole, posaconazole, and isavuconazole had MIC ranges of <0.016 to 0.25 μg/ml, <0.016 to 0.25 μg/ml, and 0.008 to 1 μg/ml, respectively. There were no differences in the activities of itraconazole and posaconazole; and they were generally more active than amphotericin B, fluconazole, voriconazole, and isavuconazole. *C. modesta*, *C. arxii*, *C. emmonsii* (*Xylohypha emmonsii*), and *C. devriesii* were also inhibited by low concentrations of azoles, similar to *C. bantiana*. Isavuconazole exhibited potent activity against *C. bantiana* and the other neurotropic strains with a MIC_{90} of 0.5 μg/ml. The voriconazole MIC_{90} (2 μg/ml) was 2-$\log_2$-dilution steps less active than isavuconazole (0.5 μg/ml), which in turn was 2-$\log_2$-dilution steps less active than itraconazole and posaconazole (MIC_{90}, 0.125 μg/ml). The only available isolate of *C. modesta* had a high MIC (2 μg/ml) for voriconazole and isavuconazole, similar to the MIC_{90} of voriconazole for *C. bantiana*. Of the two echinocandins, anidulafungin had the best activity, with the geometric mean MEC being 5 $\log_2$ dilution steps more active than caspofungin, although the MEC_{90} of 2 μg/ml would not qualify anidulafungin as an agent which can be used as monotherapy.

DISCUSSION

Primary cerebral phaeohyphomycosis in humans without obvious predisposing factors is rare, but it is increasingly recognized as an infectious disease associated with high mortality and a poor prognosis (13, 26, 37, 48). The most common agent of cerebral phaeohyphomycosis that belongs to the order of *Chaetothyriales* is *C. bantiana*, which has been reported in both healthy and immunocompromised hosts. In addition, other species causing similar clinical presentations have been de-

TABLE 2. Geometric mean MICs, MIC ranges, MIC_{50}s, and MIC_{90}s of eight antifungal drugs against 42 strains of *Cladophialophora*

Drug	MIC (μg/ml)								
	Cladophialophora bantiana (*n* = 37)				*C. modesta* (*n* = 1)	*C. arxii* (*n* = 1)	*C. devriesii* (*n* = 1)	*C. emmonsii* (*n* = 2)	
	Range	Geometric mean	50%	90%				Strain 1	Strain 2
Amphotericin B	0.125–2	0.7	1	1	1	1	2	0.5	1
Fluconazole	16–64	35.14	32	64	32	8	16	32	32
Itraconazole	<0.016–0.25	0.064	0.063	0.125	0.5	0.016	0.031	0.125	0.125
Voriconazole	0.125–4	0.769	1	2	2	0.125	0.25	2	0.5
Posaconazole	<0.016–0.25	0.044	0.031	0.125	0.25	0.016	0.031	0.063	0.063
Isavuconazole	0.008–1	0.259	0.25	0.5	2	0.063	0.031	1	1
Caspofungin[a]	1–8	2.551	2	4	4	2	2	2	4
Anidulafungin[a]	0.016–4	0.073	0.063	2	0.5	1	1	1	1

[a] Data for caspofungin and anidulafungin represent MECs (μg/ml).

scribed. Patients with bone marrow and solid organ transplantation and patients who are on steroid therapy are particularly affected (2, 13, 15, 24, 31, 35, 36, 51, 54, 57). *Cladophialophora bantiana*, a neurotropic fungus, has rarely been isolated from sources other than clinical samples. *C. bantiana* is distributed worldwide, but infections with this organism are especially encountered in subtropical and humid climate areas (8). Although *C. bantiana* has been recovered from the environment (12) and clinical infections are linked to traumatic inoculation (46), the environmental niche of *C. bantiana* is largely unknown; and only one such strain of *C. bantiana* (CBS 647.96), recovered from sawdust, was available in this study. An unambiguous connection between a clinical and an environmental strain still has to be proven.

Correct identification by mycological procedures remains difficult, due to the high degree of phenotypic similarity between these groups of fungi. For most cases published in the older literature, the etiological agent may often have been misidentified; however, reidentification down to the species level cannot be performed by molecular methods due to the absence of the original isolates. Recently, molecular data have shown that *Cladophialophora* contains a number of hitherto unknown species closely related but significantly different from *C. bantiana* (3). A study of the variability and molecular determination of the neurotropic species *C. bantiana* and sequencing data for the internal transcribed sequence indicated a low degree of variability (20). Interestingly, *C. bantiana* consistently contains an invariable intron of 558 bp at position 1768 in the small-subunit rDNA gene, while it was absent and present in all *C. emmonsii* isolates and *C. psammophila*, respectively, which are closely related to *C. bantiana* (3). Remarkably, this intron might be involved in evolutionary events, because it is not found outside the genetically homogeneous species *C. bantiana*. Other neurotropic species of *Cladophialophora* closely related to *C. bantiana* are involved in brain infections. A fatal cerebral infection with *Cladophialophora modesta* was reported in a 25-year-old black male after possible traumatic inoculation (40), and *Cladophialophora arxii* was found as the cause of cerebral phaeohyphomycosis in a 30-year-old African-American female who underwent cardiac transplantation for postpartum cardiomyopathy (44). In contrast, *Cladophialophora devriesii* was involved in a case of disseminated disease without involvement of the central nervous system (42), which years before presented as subcutaneous mycosis (22). *C.* (*Xylohypha*) *emmonsii* was the cause of a subcutaneous mycosis (45), and *C. boppii* was responsible for a pulmonary infection in a lung transplant recipient (34).

AFLP is one of a series of techniques for phylogenetic studies, plant and animal genetic mapping, and genotyping and is well suited for distinguishing closely related organisms at the species to strain level (32). The AFLP method relies on selective amplification of restriction fragments from a digest of genomic DNA and has many advantages compared to other marker technologies, including randomly amplified polymorphic DNA, restriction fragment length polymorphism, and microsatellites. AFLP not only has higher reproducibility, resolution, and sensitivity at the whole-genome level than other random amplification techniques but it also has the ability to amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification (58). Bakkeren et al. (5) have shown that the phylogenetic trees obtained from AFLP analysis are quite similar to those obtained by the use of ITS sequences in *Ustilago* species and, in addition, permit distinction of closely related isolates that cannot be resolved by ITS sequence comparison.

Our AFLP results are in line with previous sequencing data and also show obvious differences among clinically important *Cladophialophora* species as agents of cerebral infection. On the basis of the AFLP patterns, we did not see any misidentification for those taxa. All strains in this study were originally identified as *C. bantiana*, *C. emmonsii*, *C. arxii*, *C. devriesii*, and *C. modesta* on the basis of sequencing and phenotypic and physiological criteria, which is in the line with the results of cluster analysis demonstrating a clear separation of *C. bantiana* as a rather homogeneous group from *C. modesta*, *C. emmonsii*, *C. arxii*, and *C. devriesii*. Although dematiaceous fungi as agents of central nervous system infections are generally susceptible to most antifungal agents *in vitro*, treatment is difficult because frequent relapses and failures are observed (48). Antifungal therapy is based on the experience from single patient case reports or small series which mostly involved amphotericin B, itraconazole, and flucytosine. In animal models, amphotericin B prolonged the survival of mice infected with *C. bantiana*, but the infection did not completely disappear (1). Although the *in vitro* activity of amphotericin B against *C. bantiana* has been demonstrated in this and previous studies, the drug was ineffective in many cases of cerebral phaeohyphomycosis with or without flucytosine (15, 19, 24, 25, 40, 48, 49, 52). *In vitro* results indicate that the amphotericin B MICs for most nondermatophyte opportunistic filamentous fungal isolates clustered between 0.5 and 2 μg/ml. Very few data concerning the correlation between the MIC and the outcome of treatment with amphotericin B are available for dematiaceous fungi. Generally, filamentous fungi are not susceptible to fluconazole and most MICs were >16 μg/ml. Two case reports described the improvement of *C. bantiana* brain abscesses after treatment with fluconazole for up to 6 weeks and surgical excision (11, 55). Surgical intervention was probably the cause of the improvement. Fortunately, the antifungal armamentarium has been extended with new triazoles, potent agents that are active against drug-resistant strains and that have less toxicity. There are only limited data in the literature regarding the susceptibility of *C. bantiana* isolates to antifungals (47). Less successful outcomes of treatment with itraconazole (44) and voriconazole (17, 24, 49) for cerebral abscesses due to *C. bantiana* were observed, although itraconazole had low MICs (MIC_{90}, 0.125 μg/ml) in this study. The explanation might be the less optimal penetration into the CNS. Whereas voriconazole has a good penetration into the CNS, the MICs of *C. bantiana* were in the higher ranges (MIC_{90}, 2 μg/ml). Some authors report successful treatment of *C. bantiana* brain abscesses with voriconazole (38), while others reported clinical failure (17, 24, 49). Although it is an excellent drug for the treatment of cerebral aspergillosis, voriconazole might not be the first choice for the treatment of *C. bantiana* infections. In contrast to another study with only seven strains of *C. bantiana* (47), which gave a MIC range of 0.12 to 1 μg/ml of voriconazole, we found a large range of activity (MICs, 0.125 to 4 μg/ml). Therefore, *in vitro* susceptibility testing may be warranted before voriconazole is used to treat a CNS infection due

to *C. bantiana*. Posaconazole and itraconazole (MIC_{90}, 0.125 μg/ml) demonstrated the best *in vitro* activities, followed by isavuconazole (MIC_{90}, 0.5 μg/ml). Some clinical experience suggests that therapy with itraconazole was successfully used in treating a *C. bantiana* cerebral infection (25, 35), eumycetoma (7), and *C. arxii* osteomyelitis (53). Treatment with posaconazole is supported by our *in vitro* results, other *in vitro* data from small series ($n = 5$, range 0.06 to 0.5 μg/ml) (16), data for a clinical case (18), and data from murine infection models in which posaconazole prolonged the survival of mice and reduced the level of brain fungal burden compared to that achieved with itraconazole and amphotericin B (1, 39). Moreover, the apparently good penetration into the CNS (50) supports the use of posaconazole for this difficult-to-treat infection. Most melanized fungi appear to be resistant to echinocandins, probably due to the reduced presence of β-glucan in the cell walls (16, 43). We found that caspofungin had poor activity but that anidulafungin had activity against *C. bantiana* showing a geometric mean MIC of only 0.073 μg/ml, although several isolates had high MECs (MEC_{90}, 2 μg/ml). Another echinocandin, micafungin, was not active in animal studies when it was used as monotherapy but seemed to be promising in combination with posaconazole and flucytosine (39). The investigational agent isavuconazole shows broad-spectrum activity against many opportunistic and true pathogenic fungi (21). Here, we show that isavuconazole is also active against *C. bantiana*, but its true value needs to be confirmed in animal models.

In conclusion, itraconazole, posaconazole, and isavuconazole demonstrated *in vitro* activity against neurotropic isolates of *C. bantiana*. Some positive and negative correlating clinical experiences are available for itraconazole, voriconazole, and posaconazole; but the most important intervention for cerebral phaeohyphomycosis caused by *C. bantiana* probably remains complete neurosurgical excision of the abscesses.

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