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In Vitro Antifungal Susceptibility and Molecular Characterization of Clinical Isolates of *Fusarium verticillioides* (*F. moniliforme*) and *Fusarium thapsinum*▽

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A microdilution method was used to test 11 antifungal drugs against clinical isolates of *Fusarium thapsinum* and three different phylogenetic clades of *Fusarium verticillioides* that were characterized by sequencing a region of the β-tubulin gene. Terbinafine was the most-active drug against both species, followed by posaconazole against *F. verticillioides*.

Fusarium verticillioides (*F. moniliforme*) is one of the most-common species involved in fusariosis (7). These infections are frequently refractory to treatment because species of *Fusarium* are generally resistant to the currently available antifungal agents (1, 12). The information available on clinical infections by *F. verticillioides* is limited because in most cases of fusariosis, the identification of the causative agent is not performed, due to the difficulties in species recognition. *F. verticillioides* can be morphologically confused with other species of the *Gibberella fujikuroi* species complex (11, 14, 15). The purposes of this study have been (i) to verify molecularly the morphological identification of numerous clinical isolates of *F. verticillioides*, (ii) to determine whether they constitute a unique phylogenetic group, and in the case that different genetic groups were detected, (iii) to determine if they demonstrate various antifungal susceptibility patterns.

For these first two aims we have sequenced a region of the β-tubulin gene which has proven to be highly informative at the phylogenetic level in different molecular studies of the *G. fujikuroi* complex (13, 14, 15). In this phylogenetic study, we included a total of 46 strains, mainly from clinical sources, that have been morphologically identified as *F. verticillioides* (3). Twelve sequences retrieved from GenBank were also included, 10 of them corresponding to related species of the complex other than *F. verticillioides* and *Fusarium thapsinum* (14, 15) (Table 1). The procedures for DNA extraction and amplification and sequencing of the region analyzed have been previously described (5). With the primers used, TUB-F and T22 (2, 13), we were able to amplify and sequence a fragment of 433 bp. Surprisingly, a BLAST search demonstrated that four of the isolates did not belong to *F. verticillioides*; instead, they were identified as *F. thapsinum*. The morphological differentiation of *F. thapsinum* and *F. verticillioides* is problematic. According to Klittich et al. (8), the production of a yellow diffusible pigment on potato dextrose agar is the main phenotypic feature distinguishing the two species, but this pigment is not produced by all of the strains.

Parsimony analysis of the data set yielded 120 phylogenetic trees of 79 steps in length (Fig. 1). *F. verticillioides* and *F. thapsinum* were clearly separated from the other species; however, *F. verticillioides* showed a high molecular variability, which was reflected in the existence of three different molecular clades (I, II, and III) and nine different haplotypes. Whether these clades represented different reproductively isolated subgroups can only be determined by the analysis of additional, independent, variable sequence data sets.

We then evaluated the in vitro activity of 11 antifungal drugs against 5 isolates of *F. thapsinum* and 24 of *F. verticillioides* that were randomly selected from the different clades. The isolates were grown on potato dextrose agar plates and incubated at 25°C for 7 days. We used a microdilution reference method (10), with some modifications. The inocula were adjusted to a final concentration of 4×10^3 to 5×10^4 conidia/ml. Final drug concentrations ranged from 64 to 0.12 μg/ml for fluconazole and flucytosine, from 128 to 0.25 μg/ml for micafungin, and from 16 to 0.03 μg/ml for albaconazole, amphotericin B, itraconazole, ketoconazole, posaconazole, ravuconazole, terbinafine, and voriconazole. The MIC endpoint for amphotericin B, terbinafine, and most triazoles was defined as the lowest concentration that produced complete inhibition of growth; for fluconazole, flucytosine, ketoconazole, and micafungin, the endpoint was defined as the lowest concentration that produced 50% inhibition of growth. Testing was performed twice on two different days, and in those instances where the results did not coincide it was repeated a third time. For those strains, the MIC was considered as the mode of the three MICs.

The susceptibility results are shown in Table 2. For *F. verticillioides*, terbinafine was the most-active drug, followed by posaconazole, ravuconazole, voriconazole, amphotericin B, ketoconazole, albaconazole, and itraconazole in decreasing order of potency. Among these, itraconazole has practically no ac-

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TABLE 1. Isolates included in the study and their origin[a]

Species	Isolate no.	Isolate source	GenBank TUB
F. verticillioides	CBS 576.78 (T)	Clinical source, USSR	AM933108
F. verticillioides	CBS 102699	Clinical source, Germany	AM933097
F. verticillioides	CBS 108922	Clinical source, Germany	AM933102
F. verticillioides	CBS 115135	Clinical source, Sweden	AM933089
F. verticillioides	FMR 7236	Clinical source, Spain	AM933098
F. verticillioides	FMR 8585	Clinical source, Spain	AM933094
F. verticillioides	FMR 8694	Clinical source, Spain	AM933111
F. verticillioides	UTHSC R-1027	Clinical source, United States	AM933092
F. verticillioides	UTHSC R-1213	Clinical source, United States	AM933112
F. verticillioides	UTHSC R-1214	Clinical source, United States	AM933115
F. verticillioides	UTHSC 90-715	Clinical source, United States	AM933118
F. verticillioides	UTHSC 93-459	Clinical source, United States	AM933116
F. verticillioides	UTHSC 94-106	Clinical source, United States	AM933105
F. verticillioides	UTHSC 95-2483	Clinical source, United States	AM933099
F. verticillioides	UTHSC 96-7	Clinical source, United States	AM933110
F. verticillioides	UTHSC 96-449	Clinical source, United States	AM933101
F. verticillioides	UTHSC 96-2334	Clinical source, United States	AM933113
F. verticillioides	UTHSC 99-1013	Clinical source, United States	AM932522
F. verticillioides	UTHSC 99-1936	Clinical source, United States	AM933109
F. verticillioides	UTHSC 00-1810	Clinical source, United States	AM933119
F. verticillioides	UTHSC 02-185	Clinical source, United States	AM933122
F. verticillioides	UTHSC 03-72	Clinical source, United States	AM933114
F. verticillioides	UTHSC 03-504	Clinical source, United States	AM933100
F. verticillioides	UTHSC 03-1454	Clinical source, United States	AM933103
F. verticillioides	UTHSC 03-1455	Clinical source, United States	AM933104
F. verticillioides	UTHSC 03-2552	Clinical source, United States	AM933106
F. verticillioides	UTHSC 04-506	Clinical source, United States	AM933130
F. verticillioides	UTHSC 04-695	Clinical source, United States	AM933132
F. verticillioides	UTHSC 05-430	Clinical source, United States	AM933131
F. verticillioides	UTHSC 05-431	Clinical source, United States	AM932521
F. verticillioides	UTHSC 05-1039	Clinical source, United States	AM933090
F. verticillioides	UTHSC 05-3141	Clinical source, United States	AM933091
F. verticillioides	UTHSC 06-134	Clinical source, United States	AM933121
F. verticillioides	UTHSC 06-1103	Clinical source, United States	AM933128
F. verticillioides	UTHSC 06-1639	Clinical source, United States	AM933129
F. verticillioides	UTHSC 06-3023	Clinical source, United States	AM933120
F. verticillioides	CBS 139.40	*Phyllocactus hybridus*, Italy	AM933107
F. verticillioides	FMR 9323	Corn, Spain	AM933117
F. verticillioides	FMR 9324	Pig feed, Spain	AM933093
F. verticillioides	FMR 9325	Horse feed, Spain	AM933096
F. verticillioides	FMR 8976	Unknown	AM933095
F. verticillioides			U34413[b]
F. thapsinum[c]	CBS 539.79	Clinical source, Italy	AM933124
F. thapsinum[c]	UTHSC 98-1202	Clinical source, United States	AM933126
F. thapsinum[c]	UTHSC 03-2158	Clinical source, United States	AM933125
F. thapsinum[c]	UTHSC 03-3093	Clinical source, United States	AM933123
F. thapsinum	CBS 733.97	*Sorghum bicolor*, South Africa	AM933127
F. thapsinum			U34418[b]
F. denticulatum			U61550[b]
F. fujikuroi			U34415[b]
F. lactis			U61551[b]
F. napiforme			U34428[b]
F. nygamai			U34426[b]
F. pseudoanthophilum			U61553[b]
F. pseudocircinatum			U34427[b]
F. ramigenum			U61554[b]
F. sacchari			U34414[b]
F. subglutinans			U34417[b]

[a] TUB, β-tubulin gene; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; FMR, Facultat de Medicina i Ciències de la Salut, Reus, Spain; UTHSC, University of Texas Health Science Center at San Antonio, San Antonio, TX; (T), type strain.
[b] Sequences retrieved from GenBank.
[c] Isolates morphologically identified as *F. verticillioides*.

tivity. For *F. thapsinum*, terbinafine was the most-active drug. Voriconazole and amphotericin B followed terbinafine with equivalent potencies. The rest of the tested drugs were not active against this species. In general, the differences among the MICs of the molecular clades, determined by using the Mann-Whitney U test ($P < 0.05$), were not statistically significant, with the exception of those for ketoconazole and ravuconazole, which showed less activity against the isolates of

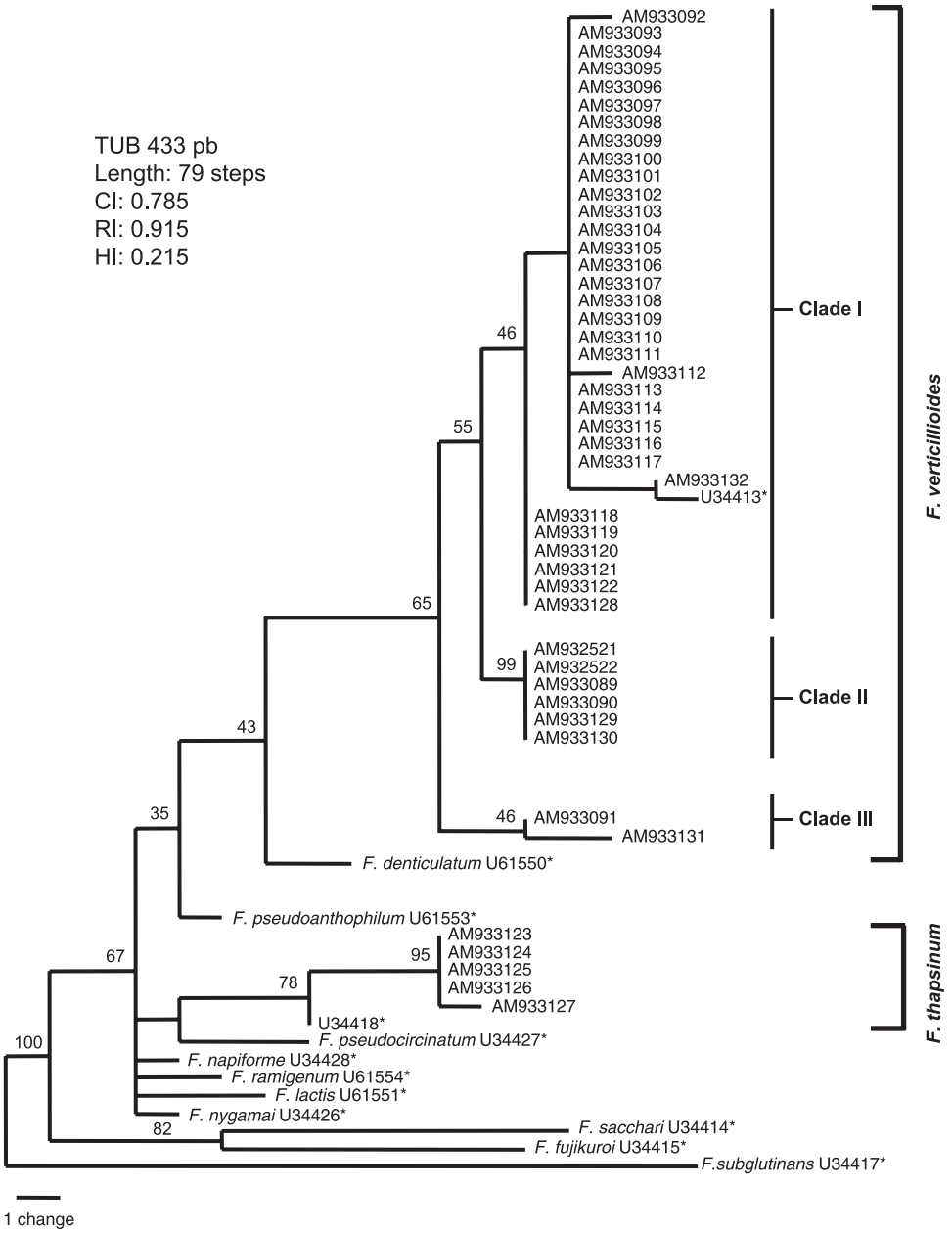

FIG. 1. One of the 120 most-parsimonious trees obtained from heuristic searches based on β-tubulin gene (TUB) sequences. Bootstrap support values are indicated at the nodes. CI, consistency index; RI, retention index; HI, homoplasy index. Asterisks indicate accession numbers of sequences retrieved from GenBank.

clades II and III than those of clade I. Although amphotericin B and voriconazole are the recommended drugs for treating fusariosis (4) and reasonable levels of clinical success (45.5%) have been attained with voriconazole (18), here both drugs showed more-limited activity than that of terbinafine for *F. thapsinum* and of terbinafine and posaconazole for *F. verticillioides*. Unlike *F. verticillioides*, posaconazole was not active against *F. thapsinum*. Fluconazole, flucytosine, and micafungin demonstrated no activity against any of the isolates tested, as had already been demonstrated (6, 19, 21). In a previous in vitro study, terbinafine combined with different azoles, such as albaconazole, ravuconazole, and voriconazole, showed synergistic activity against the three isolates of *F. verticillioides* that were tested (17). No data exists on the clinical use of terbinafine to treat infections by *F. verticillioides*. In some clinical trials, successful outcomes have been reported in patients with fusariosis treated with posaconazole, but the species involved in such cases were not determined (20).

These results are very encouraging because, unlike other pathogenic species of *Fusarium* (1), at least two drugs, posaconazole and terbinafine, seem to exert some activity against *F. verticillioides*. This fact, together with the results shown in animal studies, where *F. verticillioides* was less virulent than *Fusarium solani* (9), would suggest a better prognosis

TABLE 2. Activities of conventional and new antifungal drugs against clinical isolates of *F. verticillioides* and *F. thapsinum*[a]

Species and clade[b] (no. of isolates tested)	MIC [μg/ml; range (GM)]							
	ABC	AMB	ITC	KTC	PSC	RVC	TBF	VRC
F. verticillioides								
Clade I (16)	2–4 (3.03)	2–4 (2.41)	2–>16 (12.70)	1–4 (2.00)	0.5–1 (0.79)	1–4 (1.45)	0.125–1 (0.21)	2 (2.00)
Clade II (6)	2–4 (3.56)	2–4 (2.24)	>16 (>16)	4–>16 (8)	0.5–1 (0.89)	1–4 (2.24)	0.125–1 (0.31)	2–4 (2.83)
Clade III (2)	4–8 (5.66)	2 (2)	>16 (>16)	4–16 (8)	1 (1)	4 (4)	0.125–0.5 (0.25)	2 (2)
Total (24)	2–8 (3.34)	2–4 (2.33)	2–>16 (17.51)	1–>16 (3.24)	0.5–1 (0.83)	1–4 (1.77)	0.125–1 (0.24)	2–4 (2.19)
F. thapsinum (5)	16–>16 (18.38)	2–4 (2.64)	>16 (>16)	>16 (>16)	>16 (>16)	8–>16 (18.38)	0.25–0.5 (0.44)	2–4 (2.64)

[a] GM, geometric mean; ABC, albaconazole; AMB, amphotericin B; ITC, itraconazole; KTC, ketoconazole; PSC, posaconazole; RVC, ravuconazole; TBF, terbinafine; VRC, voriconazole.
[b] See Fig. 1.

for those infections caused by *F. verticillioides* than for those caused by *F. solani*.

This is the first in vitro study of the antifungal susceptibility of *F. thapsinum*. Although *F. thapsinum* is an important plant pathogen, several human infections have also been attributed to this species (16, 22). This study emphasizes the usefulness of molecular methods for the correct identification of species difficult to distinguish morphologically and has demonstrated important differences in the antifungal susceptibility patterns of *F. verticillioides* and *F. thapsinum*.

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