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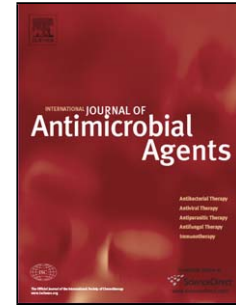
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## Genotyping and in vitro antifungal susceptibility of *Neoscytalidium dimidiatum* isolates from different origins

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## ABSTRACT

This study evaluated the genetic variability and in vitro susceptibility patterns of isolates of *Neoscytalidium dimidiatum* and *Scytalidium hyalinum* from different geographical origins. Partial sequences of four loci (the ITS region and D1/D2 domains of the 28S rRNA gene and the tubulin and chitin synthase genes) were analysed. Among a total of 1970 bp sequenced in 24 isolates, 7 polymorphic positions (0.36%) were detected, representing five different sequence types (ST1 to ST5), from which two (ST2 and ST3) were detected exclusively in isolates from plants, two (ST1 and ST5) were found only in clinical isolates and one (ST4) was observed in isolates from humans and from a mango tree. We propose subordinating *S. hyalinum* as a variety of *N. dimidiatum*. Amphotericin B was the most active drug, but low minimum inhibitory concentrations were also detected for voriconazole, terbinafine and anidulafungin.

## 1. Introduction

*Neoscytalidium dimidiatum* is a dematiaceous fungus causing diseases in numerous plant hosts [1]. In humans it produces mostly chronic superficial infections of the hands and feet, clinically resembling dermatophytosis [2]. Cases of mycetoma, subcutaneous lesions, cerebral phaeohyphomycosis and other deep-seated infections have been reported less frequently, mainly affecting immunocompromised patients [2–5]. The fungus is endemic in tropical and subtropical areas of South America, the Caribbean, Asia and Africa but has been increasingly reported from other non-endemic regions owing to immigration and travel [3].

In culture, *N. dimidiatum* shows cottony, fast-growing colonies that may appear hyaline at first but soon become dark brown to blackish. Cylindrical to ellipsoidal both hyaline and darkly pigmented arthroconidia are invariably observed. In old cultures, a pycnidial synanamorph may also be produced [1,2]. Owing to the presence of arthroconidia, earlier literature included this mould in the genus *Scytalidium* as *Scytalidium dimidiatum* [3,4]. A very similar but non-pigmented fungus, *Scytalidium hyalinum*, has also been reported from human skin and nail infections [2]. Previous molecular studies have suggested that both taxa might be conspecific [6,7]. Nevertheless, the synonymy has yet not been formally proposed. Recent phylogenetic analyses using sequences of rDNA demonstrated that the type species of the genus *Scytalidium*, *Scytalidium lignicola*, and *S. dimidiatum* grouped into different classes [1]. Therefore, the new genus *Neoscytalidium* was proposed to accommodate *S. dimidiatum*, under the name *N. dimidiatum* [1]. The list of synonyms also included

*Hendersonula toruloidea* and *Fusicoccum dimidiatum*, but *S. hyalinum* was not mentioned. *Nattrassia mangiferae*, another binomial widely used in earlier literature to refer to *N. dimidiatum* isolates, was considered a different species, which differs from the latter taxon in lacking an arthroconidial anamorph.

Genotyping of pathogenic fungi can provide useful information regarding their epidemiology, routes and sources of infection, cryptic speciation, and the possible association between particular genotypes and medically relevant traits such as resistance to antifungal drugs and virulence. In recent years, several multilocus sequence approaches have been developed for pathogenic fungi [8,9]. These methods overcome the drawbacks of low reproducibility of image-based methods such as restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP), and facilitate global sharing of genetic data via the Internet. Optimal therapy has not been developed for diseases caused by *Neoscytalidium* [2]. Newer antifungal agents and alternative therapeutic options need to be explored. This study aimed to (i) assess the genetic relationships among isolates of *N. dimidiatum* and its hyaline counterpart by analysing sequences of four nuclear loci and (ii) determine whether the sequence types (STs) identified showed different antifungal susceptibility patterns.

## 2. Materials and methods

### 2.1. Fungal isolates

Twenty-four isolates morphologically identified as *N. dimidiatum* or *S. hyalinum* were studied (Table 1). The isolates were cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) and were incubated at 25 °C for 5–6 days.

### 2.2. DNA extraction, amplification and sequencing

DNA extraction was performed directly from fungal colonies following the FastDNA<sup>®</sup> Kit protocol (BIO 101 Inc., Vista, CA), with the homogenisation step repeated five times. Portions of the following loci were chosen for genotyping: the internal transcribed spacer (ITS) region; the D1/D2 domains of the 28S rRNA gene; the  $\beta$ -tubulin gene (TUB); and the chitin synthase gene (CHS). Amplification of the ITS region and TUB were performed as described by Gilgado et al. [8]. D1/D2 and CHS were amplified according to Cano et al. [10] and Marimon et al. [9], respectively. Polymerase chain reaction (PCR) products were purified using a GFX<sup>™</sup> PCR DNA Kit (Pharmacia Biotech, Cerdanyola, Spain) and were stored at –20 °C until sequencing. PCR products were sequenced using the same primers used for amplification and following the *Taq* DyeDeoxy<sup>™</sup> Terminator Cycle Sequencing Kit protocol (Applied Biosystems, Gouda, The Netherlands). DNA sequencing reaction mixtures were analysed on an Applied Biosystems 310 DNA Sequencer. Consensus sequences were obtained with the AutoAssembler program (Applied Biosystems).



### 2.3. Nucleotide sequence data analysis

Multiple sequence alignments for each locus were obtained using ClustalX version 1.81 software for Windows. Substitutions as well as insertions and/or deletions (indels) were identified using the program DnaSP v. 4.10. STs were defined for the unique nucleotide sequences obtained for the four loci analysed.

### 2.4. Antifungal susceptibility testing

The in vitro activities of the following seven antifungal drugs, obtained as pure powders, were evaluated: amphotericin B (AMB) (USP, Rockville, MD); voriconazole (VCZ) (Pfizer Inc., New York, NY); posaconazole (PCZ) (Schering-Plough, Kenilworth, NJ); ravuconazole (Bristol-Myers Squibb Company, New Brunswick, NJ); terbinafine (TRB) (Novartis, Basel, Switzerland); anidulafungin (ANI) (Pfizer Inc.); and micafungin (MFG) (Astellas Pharma Inc., Tokyo, Japan). All the antifungal agents were diluted in dimethyl sulphoxide (Panreac Química S.A., Barcelona, Spain), except for MFG that was diluted in sterile distilled water. Microplates were prepared following the standard protocol of the Clinical and Laboratory Standards Institute [11]. Final drug concentrations were 128–0.25 µg/mL for MFG, 32–0.06 µg/mL for ANI and 16–0.03 µg/mL for all other drugs. The microdilution reference method [11] was used, with some modification, by flooding the surface of the agar plate with sterile saline, scraping the sporulating mycelium with a culture loop and drawing up the resultant suspension with a sterile Pasteur pipette. The suspensions were then filtered once through sterile gauze to remove hyphae. The inocula were adjusted to a final concentration of  $4 \times 10^3$  to  $5 \times 10^4$  conidia/mL with a haemocytometer and verified by quantitative colony counts on PDA plates.

*Paecilomyces variotii* ATCC 36257 and *Candida parapsilosis* ATCC 22019 were included in each batch of tests as quality control strains. The microplates were incubated at 35 °C and read after 48 h. The minimum inhibitory concentration (MIC) endpoint for the triazoles, AMB and TRB was defined as the lowest concentration that produced complete inhibition of growth, and for MFG and ANI as the lowest concentration that produced 50% growth inhibition. All the tests were repeated and showed the same tendency (data not shown). However, in the few cases that did not coincide, the test was repeated and a modal MIC of the three values was considered.

### 3. Results

#### 3.1. Nucleotide sequence variability

With the primers used, it was possible to amplify and sequence 284, 444, 434 and 807–808 bp of the CHS, TUB, D1/D2 and ITS loci, respectively. Among a total of 1970 aligned characters of these loci, only 7 polymorphic positions (0.36%) were detected (Table 2), 4 of them belonging to the ITS locus, which exhibited a T→C transition, a G→A transition and one A→T transversion as well as a single-site indel. The CHS locus had two variable positions, consisting of a synonymous T→C transition in the third position of a glycine codon and a synonymous T→A transversion observed in the third position of another glycine codon. The only polymorphism detected in TUB was a synonymous C→T transition in the third position of a serine codon.

Combined analysis of the polymorphic loci allowed five sequence types (ST1 to ST5) to be defined (Table 2). ST1 was detected in a clinical isolate from Brazil. An Egyptian isolate from *Prunus* sp. showed ST2. Two isolates from *Juglans regia* and *Citrus aurantium*, from the USA and with unknown origin, respectively, had ST3. An isolate from Mali, obtained from *Mangifera indica*, had ST4, which was also detected in 17 clinical isolates from Brazil and the UK. The two hyaline isolates tested had ST5.

### 3.2. A variety status for *Scytalidium hyalinum*

In this study, sequences of D1/D2 were identical in all isolates, and nucleotide sequence analysis of ITS or CHS separately could not distinguish hyaline from pigmented isolates in all cases. However, a point mutation in the TUB locus clearly distinguished them (Table 2). Based on the high level of genetic similarity observed between hyaline and dematiaceous isolates, we agree with other authors' views that the fungus previously known as *S. hyalinum* should be classified as a variety of *N. dimidiatum*, for which the following new combination is proposed:

*Neoscytalidium dimidiatum* var. *hyalinum* (C.K. Camp. & J.L. Mulder) Madrid, Cano, Stchigel & Guarro, *comb. nov.*

≡ *Scytalidium hyalinum* C.K. Camp. & J.L. Mulder, *Sabouraudia* 15: 163, 1977 (basionym).

### 3.3. Antifungal susceptibility testing in vitro

Results of antifungal susceptibility are shown in Table 3. AMB was the most active drug, showing an MIC range of 0.125–0.50  $\mu\text{g/mL}$  [geometric mean (GM) MIC 0.32  $\mu\text{g/mL}$ ]. VCZ was more active than PCZ against all isolates tested [range 0.06–4  $\mu\text{g/mL}$  (GM 1.37  $\mu\text{g/mL}$ ) vs. range 0.06–32  $\mu\text{g/mL}$  (GM 6.15  $\mu\text{g/mL}$ ), respectively]. For PCZ, most strains showed MICs  $>4$   $\mu\text{g/mL}$ . In the present work, TRB was the second most active drug, with an MIC range of 0.125–2  $\mu\text{g/mL}$  and a GM MIC of 0.70  $\mu\text{g/mL}$ . MFG was inactive against all STs with the exception of ST5. ANI showed an MIC range of 0.25–4  $\mu\text{g/mL}$  with a GM MIC of 1.05  $\mu\text{g/mL}$ .

## 4. Discussion

With the aim of clarifying the taxonomy and genetic relationships between *N. dimidiatum* and *S. hyalinum*, Roeijmans et al. [6] studied their interspecific and intraspecific variability using rDNA RFLP. Although differences in amplicon lengths were detected in the 18S and 28S rRNA genes among isolates of *N. dimidiatum*, the RFLP profiles did not distinguish this taxon clearly from *S. hyalinum*. These results agree with the high nucleotide sequence conservation observed in this study in regions of rDNA and, in addition to the low variability detected in CHS and TUB, provide strong molecular support to consider *S. hyalinum* as a mere variety of *N. dimidiatum*.

Interestingly, whilst several cases of opportunistic deep-seated infections have been caused by pigmented *N. dimidiatum* isolates, the hyaline variety is

extremely uncommon in this kind of infection and is involved almost exclusively in skin and nail disease [2,12]. This suggests that melanin-deficient isolates might be less virulent than the pigmented ones, highlighting the importance of their correct identification in clinical laboratories. Diagnostic procedures by conventional methods may take several days because *N. dimidiatum* may not appear clearly as dematiaceous elements in tissue, and cultures often need some days of incubation to develop their typical dark colour [2]. Machouart et al. [7] analysed partial sequences of the 18S rRNA gene of pigmented and hyaline isolates of *N. dimidiatum*. In that study, a single nucleotide polymorphism was detected, with A in most pigmented isolates and G in all hyaline isolates. Intron inserts were observed only in pigmented isolates. However, some pigmented isolates had G in the polymorphic position and lacked intron insertions and thus a reliable identification could not be achieved in all cases by sequence analysis of this locus. The low genetic variability observed by these authors within coding regions agrees with data obtained in the other loci analysed in this study. However, in the highly conserved TUB locus we observed a point mutation that allowed us to distinguish hyaline from pigmented isolates, and discrimination between them could also be achieved if the ITS and CHS loci were analysed together (Table 2). Additional isolates should be tested to determine whether these polymorphisms have a potential diagnostic use.

Combined analysis of polymorphic loci allowed five different STs to be detected that were associated with characteristics such as pigmentation, source and country of origin of the isolates. Interestingly, three different STs were observed among four *N. dimidiatum* isolates from different plant species, suggesting that

different strains could be associated with different plants in nature. However, this apparent association might have been influenced by the different geographical origins of the isolates. Additional isolates from different countries and different plant hosts should be studied to corroborate or reject this hypothesis. Interestingly, ST4, the most common ST in clinical isolates, was also detected in an isolate from a mango tree, *M. indica*. This tree has been proposed as the possible source of infection in a case of mycetoma caused by *N. dimidiatum* in a Venezuelan agricultural field worker with diabetes [4] and should be considered as a potential reservoir of pathogenic strains of the fungus. It is noteworthy that ST4 was observed in 16 of 19 Brazilian clinical isolates studied as well as in a clinical isolate from the UK, representing 75% of the total number of isolates studied. The overrepresentation of this multilocus genotype and the apparent inability of the fungus to produce ascospores in culture and in natural substrata suggest a clonal mode of propagation. However, as the number of isolates studied is low, more isolates should be tested before drawing conclusions on the type of reproduction of *N. dimidiatum*.

The low MICs of AMB are consistent with other in vitro data reported by Guarro et al. [13] and Lacroix and Feuilhade de Chauvin [14] and agree with the favourable clinical outcomes observed in infections by *N. dimidiatum* in an immunosuppressed patient treated with AMB [5]. The in vitro activity of VCZ also agrees with previous reports of successful treatment of subcutaneous and sinus infection with this drug [3,12]. The high in vitro activity detected for TRB agrees with a previous study [14]. The lack of activity of MFG against most isolates tested was to some extent expected owing to the high MICs reported

for this drug against filamentous fungi, with the exception of *Aspergillus* spp. and some infrequent opportunistic moulds [15]. Similar results were expected for ANI, another echinocandin, but surprisingly it showed low total GM MICs against the isolates tested. There are no previous in vitro data or reports of infections caused by these fungi and treated with this compound with which to compare the results. In summary, these data indicate that VCZ and ANI, an echinocandin, could be possible candidates for the treatment of systemic *Neoscytalidium* infections, however further studies with animal models and clinical trials are needed to determine their clinical value.

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**Table 1**

Isolates included in this study with their origin and EMBL accession numbers

Isolate	Source	Origin	EMBL accession no.			
			ITS region	D1/D2	CHS	TUB
CBS 204.33	Branch of <i>Prunus</i> sp.	Egypt	FM211429	FM211331	FM211195	FM211164
CBS 251.49	<i>Juglans regia</i>	USA	FM211430	FM211333	FM211197	FM211166
CBS 380.36	<i>Citrus aurantium</i>	Unknown	FM211431	FM211332	FM211196	FM211165
CBS 499.66	<i>Mangifera indica</i>	Mali	FM211432	FM211334	FM211198	FM211167
CBS 661.77	Human	UK	FM211433	FM211335	FM211199	FM211168
FMR 8342 <sup>a</sup>	Human	Brazil	FM211427	FM211329	FM211193	FM211162
FMR 8343 <sup>a</sup>	Human	Brazil	FM211428	FM211330	FM211194	FM211163
FMR 9345	Onychomycosis, toe nail	Brazil	FM211434	FM211336	FM211200	FM211169
FMR 9347	Onychomycosis, toe nail	Brazil	FM211435	FM211337	FM211201	FM211170
FMR 9348	Human	Brazil	FM211436	FM211338	FM211202	FM211171
FMR 9349	Onychomycosis, toe nail	Brazil	FM211437	FM211339	FM211203	FM211172
FMR 9351	Onychomycosis, toe nail	Brazil	FM211438	FM211340	FM211204	FM211173
FMR 9353	Dermatomycosis, skin of the sole	Brazil	FM211439	FM211341	FM211205	FM211174
FMR 9354	Onychomycosis, toe nail	Brazil	FM211440	FM211342	FM211206	FM211175
FMR 9358	Human	Brazil	FM211441	FM211343	FM211207	FM211176

FMR 9359	Onychomycosis, toe nail	Brazil	FM211442	FM211344	FM211208	FM211177
FMR 9360	Interdigital lesion of foot	Brazil	FM211443	FM211345	FM211209	FM211178
FMR 9361	Dermatomycosis, skin of the sole	Brazil	FM211444	FM211346	FM211210	FM211179
FMR 9366	Interdigital lesion of foot	Brazil	FM211445	FM211347	FM211211	FM211180
FMR 9375	Onychomycosis, toe nail	Brazil	FM211446	FM211348	FM211212	FM211181
FMR 9376	Onychomycosis, toe nail	Brazil	FM211447	FM211349	FM211213	FM211182
FMR 9378	Onychomycosis, toe nail	Brazil	FM211448	FM211350	FM211214	FM211183
FMR 9379	Onychomycosis, toe nail	Brazil	FM211449	FM211351	FM211215	FM211184
FMR 9383	Onychomycosis, toe nail	Brazil	FM211450	FM211352	FM211216	FM211185

ITS, internal transcribed spacer; D1/D2, D1/D2 domains of the 28S rRNA gene; CHS, partial chitin synthase gene; TUB, partial tubulin gene; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; FMR, Facultat de Medicina i Ciències de la Salut, Reus, Spain.

<sup>a</sup> Isolates of *Neoscytalidium dimidiatum* var. *hyalinum*.

**Table 2**

Distribution of nucleotide polymorphisms in three nuclear loci of *Neoscytalidium dimidiatum* and *N. dimidiatum* var. *hyalinum*

Isolate	Polymorphic sites at each locus <sup>a</sup>							ST
	ITS region				CHS		TUB	
	165	488	524	581	84	220	348	
FMR 9345	T	G	A	A	T	T	C	1
CBS 204.33	C	A	T	–	C	.	.	2
CBS 251.49	.	A	.	–	.	A	.	3
CBS 380.36	.	A	.	–	.	A	.	3
CBS 499.66	.	.	.	–	.	.	.	4
CBS 661.77	.	.	.	–	.	.	.	4
FMR 9347	.	.	.	–	.	.	.	4
FMR 9348	.	.	.	–	.	.	.	4
FMR 9349	.	.	.	–	.	.	.	4
FMR 9351	.	.	.	–	.	.	.	4
FMR 9353	.	.	.	–	.	.	.	4
FMR 9354	.	.	.	–	.	.	.	4
FMR 9358	.	.	.	–	.	.	.	4
FMR 9359	.	.	.	–	.	.	.	4
FMR 9360	.	.	.	–	.	.	.	4
FMR 9361	.	.	.	–	.	.	.	4
FMR 9366	.	.	.	–	.	.	.	4
FMR 9375	.	.	.	–	.	.	.	4
FMR 9376	.	.	.	–	.	.	.	4
FMR 9378	.	.	.	–	.	.	.	4
FMR 9379	.	.	.	–	.	.	.	4
FMR 9383	.	.	.	–	.	.	.	4
FMR 8342	.	A	.	–	.	.	T	5
FMR 8343	.	A	.	–	.	.	T	5

ST, sequence type; ITS, internal transcribed spacer; CHS, partial chitin

synthase gene; TUB, partial  $\beta$ -tubulin gene; FMR, Facultat de Medicina i

Ciències de la Salut, Reus, Spain; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

<sup>a</sup> The sequences of isolate FMR 9345 (GenBank accession nos. FM211434, FM211200 and FM211169, respectively) were used as master sequences.

Nucleotides identical to the corresponding nucleotides in the master sequences are shown as dots; a dash denotes a gap.

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**Table 3**

Activities of conventional and new antifungal drugs against clinical and environmental isolates of *Neoscytalidium dimidiatum* and *N. dimidiatum* var. *hyalinum*

ST (no. of isolates tested)	MIC range (geometric mean) ( $\mu\text{g/mL}$ )						
	AMB	PCZ	VCZ	RCZ	MFG	ANI	TRB
ST1 (1)	0.25/–	4/–	4/–	32/–	8/–	2/–	0.125/–
ST2 (1)	0.25/–	0.25/–	0.25/–	0.25/–	2/–	1/–	0.5/–
ST3 (2)	0.125– 0.5/0.25	0.06– 0.25/0.12	0.06– 0.125/0.08	0.125– 0.125/0.125	2–4/2.82	0.25–1/0.5	0.125– 0.5/0.25
ST4 (18)	0.125– 0.5/0.34	1–32/12.7	0.5–4/2.07	8–32/21.7	4–32/8.31	0.25– 4/1.16	0.25–2/0.82
ST5 (2)	0.25–0.5/0.35	2–4/2.82	0.5–1/0.70	8–16/11.31	0.25– 0.5/0.35	0.25– 2/0.70	1–2/1.41
Total (24)	0.125– 0.5/0.32	0.06–32/6.15	0.06–4/1.37	0.125–32/11.31	0.25– 32/5.49	0.25– 4/1.05	0.125–2/0.70

ST, sequence type; MIC, minimum inhibitory concentration; AMB, amphotericin B; PCZ, posaconazole; VCZ, voriconazole; RCZ, ravuconazole; MFG, micafungin; ANI, anidulafungin; TRB, terbinafine.