



Rapid screening for genotypes as possible markers of virulence in the neurotropic black yeast *Exophiala dermatitidis* using PCR-RFLP

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ABSTRACT

A simple method for fungal genotype screening was developed for the black yeast *Exophiala dermatitidis* based on RFLP of ribosomal ITS regions currently used as potential virulence markers. In a study set of 502 strains of the species, two main genotypes were recognized. Only 0.97% of lanes were difficult to interpret as they did not clearly present one of the expected genotypes. Twenty strains were deviating and proved to be *E. spinifera* after sequencing. Eight common, related species (based on SSU data) with clinical significance yielded different patterns with *TaqI* digestion, and thus the method is also usable for routine diagnostics.

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1. Introduction

Nearly all filamentous fungal agents of human disease are environmental, i.e. infections are caused by species that have their natural habitat outside the host. In view of understanding routes of transmission and evaluating eventual prophylactic measures, insight into the natural occurrence of infectious agents is essential. Revealing the natural ecological niche of an opportunist with a worldwide distribution requires an extended sampling effort, because the operational criteria for the determination of a niche are of a statistical nature, as follows (a) the presence of the fungus at a significantly higher frequency than outside the niche, and (b) occurrence of active assimilation in that habitat, leading to relatively rapid growth when appropriate conditions are provided. For environmental screening, high-throughput methods are compulsory.

Exophiala dermatitidis has been recognized as one of the most common black yeasts in clinical routine and is involved in a multitude of human diseases. Most significant is its occasional appearance as an etiologic agent of fatal infections of the central nervous system in otherwise healthy individuals in East Asia (Hiruma et al., 1993; Matsumoto et al., 1993; Chang et al., 2000). Outside Asia nosocomial pseudoepidemics have been reported in elderly patients after intravenous administration of contaminated medication (Woollons et al., 1996; Engemann et al., 2002). The species relatively frequently

resides in warmer, somewhat osmotic body parts, such as inner ears (Kerkmann et al., 1999) and bronchiectases (Mukaino et al., 2006) and it regularly colonizes mucous lungs of patients with cystic fibrosis (Haase et al., 1991; Horré et al., 2003) and the intestinal tract of individuals without immune disorder (de Hoog et al., 2005). The possibility that this colonization eventually leads to hematogenous dissemination and subsequent neurotropic encephalitis cannot be excluded. Such infections may be rare, but, given their high morbidity and mortality, understanding of the source and transmission route of the infection is of prime importance.

A practical problem with typing of black yeasts is that they have very few stable morphological or physiological parameters for recognition. Differences between species may be small, and phenotypic plasticity within species may be very high. Most molecular parameters still have not been validated. In a multilocus study, Zeng et al. (2007a,b) recently proved that the ribosomal Internal Transcribed Spacer (ITS) region is a reliable marker for distinction of black yeast entities. For this reason we focused on the ITS region for the development of rapid methodologies to enhance ecologic studies of this group of opportunistic organisms.

Investigations on environmental occurrence of *E. dermatitidis* have been performed by Matos et al. (2002) and Sudhadham et al. (2008). The species was found to be remarkably abundant in several types of human-made environments. In Asia, *E. dermatitidis* has been found in bathing facilities (Nishimura and Miyaji, 1987), while Matos et al. (2002) reported that it is very common in public Turkish steam bath facilities in Europe. Judging from its very high prevalence these authors regarded the steam bath as a novel niche of *E. dermatitidis*.

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Table 1

Strains listed according to the site locations from Thailand.

Source	Province	Strains isolated	Gen A	Gen B	Gen C	Deviating result
Bathing facilities	ChiengRei	61	8	53		2
Mango surface	Bangkok	5	–	5		–
Pineapple surface	Chonburi	24	3	1		20
Railway station	Srakraew	31	31	–		–
Railway station	Prachinburi	113	108	5		–
Railway station	Nakornsawan	207	176	29	2	2
Railway station	Pitsanulok	61	59	–	1	1

Increased exposure of humans to the fungus might lead to higher frequencies of infection. Sudhadham et al. (2008) found an entirely different human-made niche in the tropics in creosote-treated wood, such as railway ties. Given its asymptomatic occurrence in the human intestinal tract (de Hoog et al., 2005), growth on tropical railway ties may therefore be due to fecal dissemination, combined with tropical outside temperatures. In summary, *E. dermatitidis* has a worldwide distribution in humans and in human-made environments, but its natural ecological niche is likely to be located in the tropics.

In the framework of environmental studies (Sudhadham et al., 2008) *E. dermatitidis* was isolated selectively by pre-incubation in Raulin's solution and incubation at 40 °C on agar with meso-erythritol as sole source of carbon. The target species is then recognized by the presence of multiple, short annellated zones, absence of nitrite assimilation and growth at 42 °C. Several molecular genotypes were detected, which had uneven prevalence in the compared habitats. In order to process large numbers of samples at high throughput in view of further ecological studies, the present article aims to develop a rapid black yeast screening method based on PCR-Restriction Length Polymorphism (RFLP) of the ITS region.

2. Material and methods

2.1. Fungal strains

Strains studied with RFLP are 502 isolates acquired after selective isolation described by Sudhadham et al. (2008), supplemented with 125 references, mainly clinical strains taken from the CBS culture collection—including the ex-type strain of the species, CBS 207.35 to establish the degree of prevalence and stability of genotypes in this dataset. The environmental samples had been added in 5.5 ml Raulin's solution. Tubes were incubated on a near-horizontal shaker at 10 rpm at 25 °C for 2–3 days. Samples were vortexed for 10 s and 0.5 ml was

spread onto Erythritol Chloramphenicol Agar (ECA) and Sabouraud's Glucose Agar (SGA) plates and dispersed with a Drigalski spatula. Plates were sealed with Parafilm and incubated for 30 days at 40 °C. Small black colonies were purified by 0.1% Tween80 on Potato Dextrose Agar (PDA). Stock cultures were maintained on Malt Extract Agar (MEA) and PDA agar slants at 4 °C. For DNA extraction, fungi were subcultured on MEA at 24 °C for 3–5 days.

2.2. DNA extraction and sequencing

Voucher strains for verification, and strains producing deviating RFLP profiles were sequenced as follows. About 1 cm² of mycelium was transferred to a 2 ml Eppendorf tube containing 300 mg glass beads (Sigma G9143) and 400 µl TE buffer pH 9.0 (100 mM Tris, 40 mM Na-EDTA). Samples were homogenized for 1 min using MoBio vortex (Bohemia, New York, U.S.A.) adding 120 µl 10% SDS. Samples were incubated in a water bath at 55 °C for 30 min with the addition of 10 µl proteinase K, mixed well, and vortexed for 3 min in 120 µl 5 M NaCl and 65 µl of 10% CTAB (Cetyltrimethylammoniumbromide) was added. After incubation at 55 °C for 60 min and 3 min MoBio vortexing, 700 µl SEVAG was added, mixed carefully by hand and spun for 5 min at 4 °C at 14,000 rpm (20,400 r.c.f). After transferring the aqueous supernatant to a new Eppendorf tube (~600 µl), 225 µl 5 M NH₄-acetate was added and mixed gently. Samples were kept on ice for at least 30 min, spun again, and subsequently the supernatant (~800 µl) was transferred to a clean, sterile Eppendorf tube. 510 µl isopropanol was added to the supernatant, mixed, incubated at –20 °C for 30–60 min and spun down for 5 min. The supernatant was decanted and pellets were washed with 100 µl with ice cold 70% ethanol. Pellets were dried at room temperature and resuspended in 100 µl TE-buffer pH 9.0 (100 mM Tris, 40 mM Na-EDTA), incubated at 37 °C for 30 min and refrigerated. For molecular identification the sequences were adjusted using the program SeqMan II of Lasergene software (DNASTar, Wisconsin, U.S.A.) and aligned iteratively using Ward's averaging in the BioNumerics package v. 4.5 (Applied Maths, Kortrijk, Belgium).

2.3. Selection of genotype-specific restriction enzymes

Two main genotypes (A and B) of *E. dermatitidis* are known to differ by three substitutions at positions 162, 184 and 196 in ITS1 (Sudhadham et al., 2008). Characteristic sites were selected using a database on black yeasts and relatives for research purposes maintained at CBS, Utrecht, The Netherlands, containing about 7000 ITS sequences of members of *Dothideales* and *Chaetothyriales*. Nearest

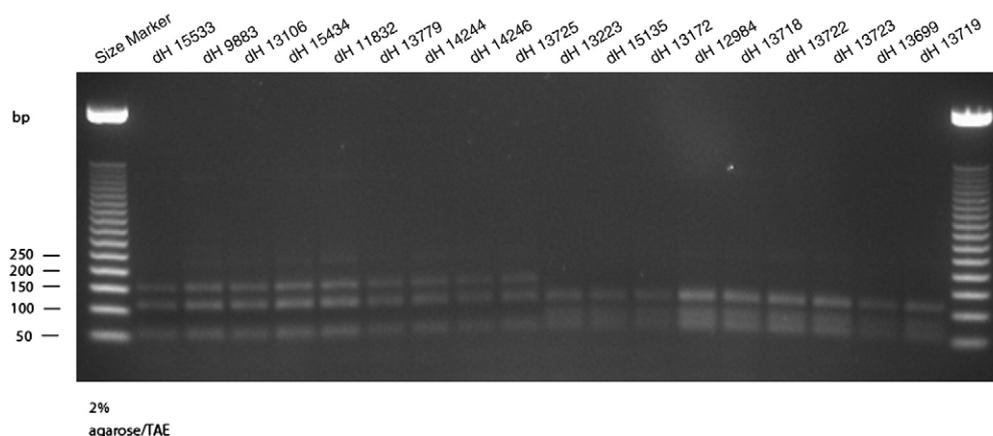


Fig. 1. Overview of *TaqI* restriction sites of common, clinically relevant *Exophiala* species. *E. bergeri* CBS 353.52, *E. spinifera* CBS 899.68, *E. xenobiotica* CBS 118157, *E. jeanselmei* CBS 507.90, *E. oligosperma* CBS 725.88, *E. phaeomuriformis* dH 13442, *E. attenuata* IFM 46115, *E. dermatitidis* (A) CBS 207.35, *E. dermatitidis* (B) CBS 971.87. Numbers below the bars depict *TaqI* restriction sites, numbers at the far right indicate the total length of ITS1–5.8S–ITS2 region in nucleotides.

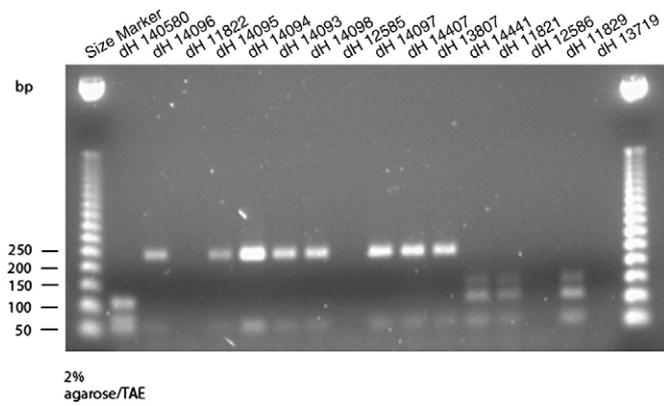


Fig. 2. RFLP patterns showing the differences between *Exophiala dermatitidis* genotypes A and B.

neighbours were found by local Blast searches. Genotype-specific restriction sites were selected with MapDraw with a cut-off at 100 bp.

2.4. Restriction Fragment Length Polymorphism (RFLP)

PCR of the rDNA internal transcribed spacer region was performed with primers ITS1 and ITS4 (TCC GTA GGT GAA CCT GCGG resp. TCC TCC GCT TAT TGA TAT GC) in reaction mixtures of 25 μ l containing 24 μ l of Gotaq® GreenMasterMix (Promega, Leiden, The Netherlands) and 1 μ l of extracted DNA. PCR conditions were as follows: denaturation 30 s at 94 °C, annealing 30 s at 52 °C, extension 1 min at 72 °C, with a final extension 7 min at 72 °C. Amplicons were verified by electrophoresis in a 1.2% agarose gel in TAE buffer with ethidium bromide staining. Digestion was performed using 8 μ l of amplicons and 10 U of *TaqI* endonuclease (recognition site at 5'-TCGA-3'; New England Biolabs, Leiden, The Netherlands) in a mastermix of 10 μ l H₂O, 2 μ l of endonuclease buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT at pH 7.9) and 0.25 μ l endonuclease (*TaqI*), with incubation at 65 °C for 3 h. The fragments were separated using 2% submarine agarose gels (Invitrogen, Breda, The Netherlands) containing ~25 μ g ethidium bromide/ml in 50 \times TAE buffer (40 mM Tris, pH 8.0, 20 mM acetic acid, 1 mM EDTA) at 4–6 V/cm using 50 bp DNA Step Ladder (Promega, Leiden, The Netherlands). Gels were photographed with BioRad Photograph and digitized by Quality one program v. 4.5.2.

3. Results

A multialignment of 125 strains of *E. dermatitidis* of a worldwide selection of clinical as well as environmental strains was used for the

analysis. A total of 103 haplotypes were found, of which 96 were rare (46 mutations, 50 indels) and were excluded from the analysis. Of the phylogenetically informative mutations, 8 were positioned in ITS1 (202 bp), 3 in ITS2 (209 bp), while the 5.8S gene (150 bp) was invariable (Table 1). The number of base changes per genotype varied from 1 to 3 of the retained polymorphisms. Only one bipartition of the dataset was known to be reflected in another, independent gene; this polymorphism has been indicated with genotypes A and B (Sudhadham et al., 2008). A genotype C, characterized by a significant deletion of 26 bases in ITS1 (genotype III of Uijthof et al., 1998), generated a profile that deviated from genotype A by a single band shift (data not shown).

89 of the sequences analyzed had concomitant mutations at ITS1 positions 162, 184, 196 (genotype A), while the 32 counterparts were classified as genotype B. A single enzyme (*TaqI*) proved to have a restriction site in the polymorphic area of ITS1 (Fig. 1), and thus generated different profiles of genotypes A and B after electrophoresis of digestion products (Fig. 2). All ex-type strains of clinically relevant *Exophiala* species were verified with *TaqI*. The profiles generated with the restriction enzyme proved to generate diagnostic profiles for each species (Fig. 1), with the exception of *Exophiala phaeomuriformis* which was indistinguishable from *E. dermatitidis* genotype A.

Subsequently a total of 502 environmental strains isolated with an *E. dermatitidis*-specific protocol were analyzed by *TaqI* RFLP. Of these, 82 strains were verified by ITS-sequencing and proven to belong to the expected genotypes. Twenty deviating samples (Table 1) were sequenced and found to be *Exophiala spinifera*. Examples of deviating bands are shown in Fig. 3 (lanes 17, 18, 21). Of the remaining strains, 385 were interpreted to be genotype A, 93 genotype B, and 3 genotype C.

The entire procedure of genotype recognition from ITS-PCR till gel photography takes about 9.5 h.

4. Discussion

In the human-dominated environment, *E. dermatitidis* is one of the most common black yeasts worldwide, but it is very rarely isolated from natural habitats. The species lists among the microbes potentially able to cause disease in humans and having a natural reservoir in association with wild animals in the tropical rain forest (Sudhadham et al., 2008). Judging from the low actual infection rate, humans are unlikely to be a primary host of the fungus, and its pathology is therefore judged to be of opportunistic nature. The introduction of animal-associated fungi into human environments including hospitals may pose a great health risk, particularly in the growing population of immunocompromised patients suffering from AIDS, leukemia, or who underwent organ transplant or major surgery. Man-made environments, which have no counterpart in nature, may create opportunities for positive selection of these fungi and thus bring about an evolution which in nature would not have taken place.

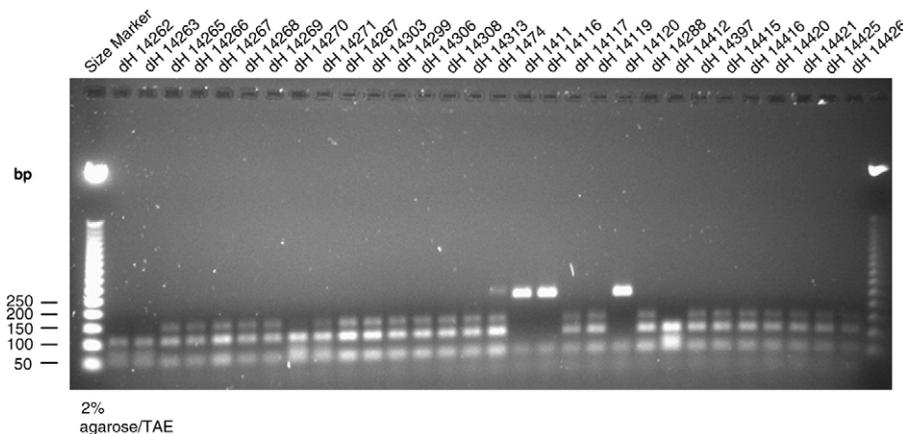


Fig. 3. Banding patterns of *Exophiala dermatitidis* genotypes A and B, and deviating profiles (lanes 17, 18, and 21) of *E. spinifera*.

In their natural reservoir they are likely to be relatively harmless: asymptomatic intestinal passage in frugivorous birds and bats is hypothesized (Sudhadham et al., 2008). Asymptomatic carriage has also been found in humans and was estimated to occur in 3% of the healthy population (de Hoog et al., 2005).

In the framework of a large ecological screening study focused on revealing the natural habitat of *E. dermatitidis*, more than 500 strains were isolated from tropical outdoor environments (Sudhadham et al., 2008). Strains were obtained using a selective protocol with incubation at 40 °C (Padhye et al., 1984) but with a pre-incubation at low pH, leading to the isolation of only a limited number of black yeast species belonging to the genus *Exophiala*. In addition to *E. dermatitidis*, only *E. phaeomuriformis* and *E. spinifera* are known to grow at 37 °C or above. In this study we present a PCR-restriction enzyme for recognition of *E. dermatitidis* on the basis of the rDNA ITS region. Digestion with *TaqI* yielded two restriction profiles which were unique when compared to prevalent, clinically significant *Exophiala* species (Zeng et al., 2007b). Patterns obtained with *Exophiala attenuata*, *E. bergeri*, *E. jeanselmei*, *E. oligosperma*, *E. spinifera* and *E. xenobiotica* were found to be consistently different (Fig. 1). These species were not isolated in the present study due to the selective protocol used, with the exception of *E. spinifera*, which is also able to grow at 37 °C. The 20 strains of this species consistently isolated from pineapples were easily recognizable by their deviating RFLP pattern. After nested PCR and verification by sequencing they proved to be genotypes A and B, which was later confirmed by repeated RFLP. Thus, in total the method yields only 0.97% of ambiguous identifications. The PCR-RFLP technique presented in this study provides an easy way to recognize *E. dermatitidis* amongst other black yeasts and allows discrimination among genotypes A, B and C.

Detection of *E. spinifera* despite the rigorous isolation protocol is explained by its thermotolerance, being able to grow well at 37 °C (Vitale and de Hoog, 2002; de Hoog et al., 2003). This species is one of the major human pathogenic black yeasts in addition to *E. dermatitidis*, having been reported from mutilating and fatal phaeoophomycosis since 1968 (Nielsen and Conant, 1968; Kettlewell et al., 1989; Padhye et al., 1984; Rajendran et al., 2003). The natural niche of this species has not been found so far, but its association with sugary tropical fruit is notable: Zeng et al. (2007) reported the species from fruity juice, while all our strains were isolated from the surface of pineapples in a tropical climate. Our isolation and identification protocol thus appears to be very useful for the detection of the two most pathogenic *Exophiala* species.

Due to its simplicity and low costs, several RFLP systems have been proposed for typing of pathogenic fungi for questions at various levels of diversity, ranging from routine identification to epidemiology. The method can be adjusted to a precise detection of minimal sequence differences and is suitable for characterization of large numbers of samples (Raimondi et al., 2007). Husband et al. (2002) used this technique to investigate the diversity and distribution of more than 1300 clones of mycorrhizal fungi. Dickie et al. (2004) used RFLP in a field study to identify ectomycorrhizal fungi associated with *Quercus* hosts. In plant pathogenicity, Krokene (2004) applied the endonuclease *HaeIII* to digest β -tubulin genes in the identification of three different species of *Seiridium* species causing cypress canker. Particularly in fungal groups where classical identification requires a highly skilled expert, as well as with difficulties arising when the fungus fails to show appropriate characters used for identification (Balajee et al., 2007). Such systems are already in place for other fungi as a screening and diagnostic tool (Okhravi et al., 1998; Mirhendi et al., 2001; Machouart-Dubach et al., 2001; Monod et al., 2006). Among these, the medically significant filamentous fungi are particularly required. Latouche et al. (2003) used PCR-RFLP on phospholipase B gene (PLB1) to differentiate between *C. neoformans* vars. *grubii*, *neoformans*, and *gattii*. Eight molecular types were revealed. Similar studies were done by Meyer et al. (2003) and MacDougall et al. (2007) with *URA5* to investigate the epidemiology of clinical and environmental isolates.

Also RFLP of the rDNA IGS region proved to be useful for monitoring intraspecific dispersal and evolution, shown with *Pseudallescheria* species Zeng et al. (2007a). The result showed that IGS detected a level of diversity between EF1- α groups and individual strains. Using ITS, Ahmed et al. (1999) studied 25 clinical isolates of *Madurella mycetomatis*. In black yeasts, it may provide a relatively rapid procedure for identification of clinical *Exophiala* species for the routine laboratory. In *Fonsecaea* the ITS region was shown to be diagnostic for species or major subspecific entities. Attili et al. (1998) and Caligiore et al. (1999) showed that ITS-RFLP helped to characterize black yeast-like fungi causing chromoblastomycosis. Kawasaki et al. (2005) digested ITS with a limited panel of enzymes to develop an identification system for *Exophiala* strains; strict correlation was found with mtDNA-RFLP, underlining the stability of the results.

Multilocus studies of Zeng and de Hoog (2007) and de Hoog et al. (2006) proved that ITS is the optimal gene for species recognition in black yeasts. We here showed that ITS-RFLP can be tailored for high-throughput detection of species and major genotypes in this problematic group of fungi.

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