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The authors declare that they have no conflicting interests in relation to this study.

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RESEARCH NOTE

High-affinity iron permease (*FTR1*) gene sequence-based molecular identification of clinically important Zygomycetes

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ABSTRACT

The clinical importance of zygomycosis, an emerging and frequently fatal mycotic disease, has increased during recent years. This report describes an identification method based on PCR amplification and sequencing of the high-affinity iron permease 1 gene (*FTR1*). Primers and amplification protocols were established and tested for the identification of *Rhizopus oryzae*, *Rhizopus microsporus* var. *rhizopodiformis*, *R. microsporus* var. *oligosporus*, *Rhizopus schipperae*, *Rhizopus niveus* and *Rhizopus stolonifer*. *Rhizomucor* and *Syncephalastrum* could be identified at the genus level. PCR–restriction fragment length polymorphism analysis of the amplified gene fragment using *Alu*I digestion distinguished three subgroups among the *R. oryzae* isolates.

Keywords Identification, iron permease 1 gene, Mucorales, PCR–restriction fragment length polymorphism analysis, *Rhizopus*, zygomycosis

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Zygomycetes have been reported to be agents of opportunistic mycoses that are frequently fatal [1]. The diagnosis of zygomycosis is based on the detection of hyphae in clinical specimens. Species determination is laborious and usually requires the expertise of a reference laboratory [2]; thus, clinical laboratories often identify such infections as zygomycosis only, without further species determination [3,4]. Although these mycoses are relatively rare, the associated high mortality, difficulty in diagnosis and resistance to the most widely used antifungal drugs emphasise the importance of developing new diagnostic assays [3,5]. Progress has already been made in the design of taxon-specific primer pairs based on 28S rDNA sequences [6], and Schwarz *et al.* [7] have reported an identification method based on internal spacer sequences and 5.8S rDNA regions.

The present study aimed to use a structural gene for molecular diagnostic purposes. A fragment of the high-affinity iron permease 1 gene (*FTR1*) was used to generate a sequence dataset in order to design PCR primer pairs for the rapid and accurate detection of Zygomycetes. Twenty-six strains, comprising *Rhizopus oryzae* (CBS 395.54, SZMC 8100, CBS 146.90, SZMC 0497, NRRL 2908, CBS 112.07, CBS 260.28, TJM 24B2, CBS 109.939), *Rhizopus schipperae* (CBS 138.95, UHF 3053), *Rhizopus microsporus* var. *rhizopodiformis* (CBS 220.92, CBS 102.277), *R. microsporus* var. *oligosporus* (NRRL 514), *Rhizopus niveus* (CBS 403.51), *Rhizopus stolonifer* (CBS 347.49, CBS 320.35), *Rhizomucor miehei* (CBS 360.92), *Rhizomucor pusillus* (WRLCN(M) 231), *Syncephalastrum racemosum* (SZMC 2011), *Mucor racemosus* (NRRL 3640), *Mucor circinelloides* (FRR 2109, CBS 277.49), *Mucor plumbeus* (ATCC 42423), *Mucor rouxii* (ATCC 24905) and *Backusella lamprospora* (NRRL 1422), were included in the study. As *Rhizopus* spp., particularly *R. oryzae*, are the predominant zygomycotic organisms, this study focused primarily on the members of this genus. Nine strains of *R. oryzae* were included, and the corresponding *FTR1* region of the clinical isolate 99–880 [8] was also added to the sequence analysis. Clinical isolates of *R. microsporus* var. *rhizopodiformis* and *R. schipperae*, a newly described species isolated exclusively from zygomycoses [9], were also investigated. *Rhizomucor miehei* and *Rhizomucor pusillus* were represented by isolates from human or animal mycoses. The strains of *Mucor*, *Backusella* and *Syncephalastrum* spp. were included in the study for comparison.

For DNA isolation, strains were grown in yeast extract–glucose medium (yeast extract 0.5% w/v, glucose 2% w/v) with continuous shaking at 200 rpm for 3 days. Genomic DNA was isolated as described by Iturriaga *et al.* [10]. *FTR1* fragments were amplified from the DNA samples using PCR and a degenerate primer pair designated as FTR-A (5′-GGTCTAGAGARGAYATHT GGGARGG) and FTR-B (5′-GGCTCGAGCC ANCCNARDATNGCRTTRAA). Primer design

Table 1. Oligonucleotide primers and annealing temperatures used for specific amplification of *FTR1* gene fragments from the species indicated, together with the corresponding sizes of the amplification products

PCR primer pairs (5′–3′)	Zygomycetes species identified	Size of PCR product (bp)	Annealing temperature (°C)
M1 GGGYCAAAAGATYGGWTTSAA *M2* GCAAMAGACTTCCACCKCGAT	*Backusella lamprospora* *Mucor plumbeus* *Mucor rouxii* *Mucor circinelloides* *Mucor racemosus*	215	63
Rhm1 GTATCACCATGCTTCGA *Rhm2* TGATGGATCCTGACTCCT	*Rhizopus microsporus* var. *oligosporus*	438	65
Rhr1 CTAGCACTGAAAAGACTGGCT *Rhr2* GGCAGAAATGTTTAATTCAGGAT	*R. microsporus* var. *rhizopodiformis*	431	68
Rsc1 CCTTCAAAGACAAACTCCAGAAG *Rsc2* CGTTTGTGTCAACATTCA	*Rhizopus schipperae*	417	60
Rho3 GATCATGATCACTGCCAT *Rho2* GCGGTWGAGACTCTGTARCYA	*Rhizopus oryzae*	465	68
Rhs1 GTCCAACTTYAAGGAAAAGAT *FTRB* GGCTCGAGCCANCCNARDATNGCRTTRAA	*Rhizopus stolonifer*	434	49
Rhn1 CGCAAGAGCGTTCTTCTTTCA *FTRB* GGCTCGAGCCANCCNARDATNGCRTTRAA	*Rhizopus niveus*	444	60
Sr1 GAAGACACTTAGCGCACGCA *Sr2* CAGCGCAGGGCAATCATAT	*Syncephalastrum racemosum*	273	64
R1 GGAAACCGATGCYTTGCA *R2* CRTCACCRCCTTCTTCGGC	*Rhizomucor miehei* *Rhizomucor pusillus*	432/441	68

R = A, G; Y = C, T; M = A, C; K = G, T; S = C, G; W = A, T; H = A, C, T; B = C, G, T; V = A, C, G; D = A, G, T; N = A, C, G, T.

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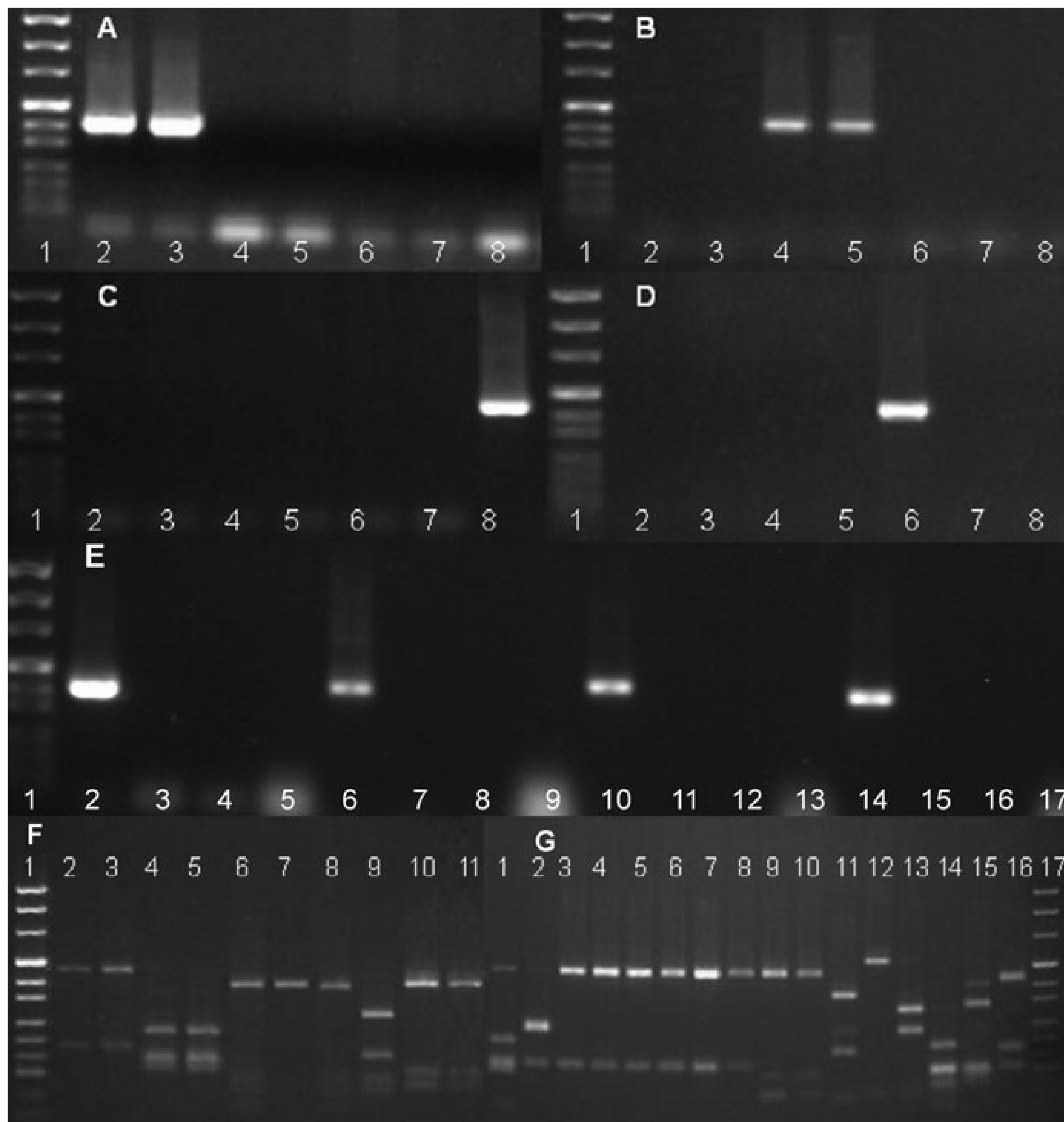

Fig. 1. Examples of PCR amplifications demonstrating the specificity of the primers for *Rhizopus schipperae*, *Rhizopus microsporus* var. *rhizopodiformis*, *Rhizopus oryzae* and *R. microsporus* var. *oligosporus* (A–E) and PCR–restriction fragment length polymorphism (RFLP) patterns of the corresponding strains following *Alu*I digestion of the *FTR1* fragments (F–G). (A–D) Amplifications with the primer pairs Rsc1–Rsc2 (A), Rhr1–Rhr2 (B), Rho1–Rho2 (C) and Rhm1–Rhm2 (D). The order of the samples is the same in (A–D): pUC mix; CBS 138.95; UHF 3053; CBS 220.92; CBS 102.277; NRRL 514; CBS 403.51; and CBS 146.90. (E) Results of amplification with primer pairs Rsc1–Rsc2 (lanes 2–5), Rhr1–Rhr2 (lanes 6–9), Rho1–Rho2 (lanes 10–13) and Rhm1–Rhm2 (lanes 14–17); lanes: 1, pUC mix; 2, CBS 138.95; 3, 7, 11 and 15, *Candida albicans* ATCC 10231; 4, 8, 12 and 16, *Saccharomyces cerevisiae* CBS 1171; 5, 9, 13 and 17, *Aspergillus fumigatus* SZMC 1389; 6, CBS 220.92; 10, CBS 146.90; and 14, NRRL 514. (F, G) PCR-RFLP patterns of the strains involved in the study. (F) Lanes 1–11: pUC mix; CBS 220.92; CBS 102.277; CBS 138.95; UHF 3053; NRRL 1422; ATCC 24905; FRR 2109; ATCC 42423; CBS 277.49; NRRL 3640. (G) Lanes 1–17: NRRL 514; CBS 395.54; SZMC 8100; CBS 146.90; SZMC 0497; NRRL 2908; CBS 112.07; CBS 260.28; TJM 24B2; CBS 109.939; CBS 403.51; CBS 347.49; CBS 320.35; CBS 360.92; WRLCN(M) 231; SZMC 2011; pUC mix. The fragment sizes of the pUC mix marker were 1118, 881, 692, 501/489, 404, 331, 242, 190, 147, 111/110 and 67 bp, respectively. Source of strains: ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures, The Netherlands; NRRL, Agricultural Research Service Culture Collection, USA; SZMC, Szeged Microbial Collection, Hungary; TJM, T. J. Michailides, University of California, USA; WRLCN, Wellcome Bacterial Collection, UK; FRR, CSIRO Food Research Culture Collection, Australia; UH, Fungal Testing Laboratory, University of Texas Health Science Center, USA.

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was based on an analysis of *R. oryzae* and *Candida albicans FTR1* gene sequences obtained from the EMBL database (accession numbers: AY344587 and AF195775, respectively). Each 25-μL PCR mixture contained 1.25 U of *Pfu* polymerase (Fermentas, St Leon-Rot, Germany), 2.5 μL of 10× reaction buffer, 2.5 mM $MgSO_4$, 400 μM each dATP, dCTP, dGTP and dTTP (Fermentas), 2 μM primers and 20 ng of genomic DNA. Amplification comprised 3 min at 94°C, followed by five cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 2 min, with a slow ramp time between the annealing and the extension segments (0.12°C/s instead of the 2°C/s used between each of the other steps), followed by 30 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 2 min, followed by 72°C for 10 min. The sizes of the amplification products varied between 585 and 740 bp.

Nucleotide sequences of the amplified *FTR1* fragments were aligned and compared to find motifs applicable for oligonucleotide design. Primers useful for specific identification of the involved *Rhizopus* species were identified (Table 1). Primer pairs Sr1–Sr2 and R1–R2 were able to detect strains belonging to the genera *Syncephalastrum* and *Rhizomucor*, respectively. However, motifs useful for differentiating *Rhizomucor miehei* and *Rhizomucor pusillus* were not found in the *FTR1* sequences of these two species; thus, the primer pair R1–R2 identifies *Rhizomucor* to the genus level only. Similarly, the M1 and M2 primers differentiated all members of the *Mucor–Backusella* group from other strains. For *R. stolonifer* and *R. niveus*, the degenerate FTR-B primer was used as part of a pair with a species-specific primer. Some primers (e.g., M1, M2, Rho2, Rhs1, R1 and R2) also contained degenerate positions to ensure amplification from every strain of the corresponding species. For species identification, the PCR mixtures had the same composition as described above, except for the primers. Amplification comprised 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, annealing (see temperatures in Table 1) for 30 s and 72°C for 1 min, with a final extension at 75°C for 10 min. The annealing temperatures were optimised for each primer pair separately.

Fig. 1A–D shows examples of amplification patterns obtained with the various primer pairs. DNA extracts from strains of *Saccharomyces cerevisiae*, *C. albicans* and *Aspergillus fumigatus* were also used to test the specificity of the PCR (Fig. 1E). The primer pairs used in this study were not able to amplify any products from these strains, thereby demonstrating the specificity of the method.

All of the species examined were differentiated by PCR–restriction fragment length polymorphism analysis. Amplification products obtained with the FTR-A/FTR-B primers and then digested with *Alu*I yielded fragment patterns characteristic of the species studied (Fig. 1F–G). The PCR used the same conditions and settings as described above for the degenerate primers. The method distinguished three subgroups within *R. oryzae* according to the sequence variations detected in the *FTR1* fragments of the nine strains investigated. Diagnosis of zygomycosis at an early stage is essential for a successful patient outcome. Use of species- and strain-specific PCR-based analysis could reveal important data concerning the epidemiology of zygomycosis.

EMBL accession numbers for the *FTR1* sequences determined in this study are as follows: *R. oryzae*: AM286222, AM286221, AM286201, AM286214, AM286223, AM286202, AM286200, AM286199, AM286198; *R. schipperae*: AM286216, AM286217; *R. microsporus* var. *rhizopodiformis*: AM286218, AM286219; *R. microsporus* var. *oligosporus*: AM286220; *R. niveus*: AM286205; *R. stolonifer*: AM286204, AM286206; *Rhizomucor miehei*: AM286225; *Rhizomucor pusillus*: AM286224; *S. racemosum*: AM286213; *M. circinelloides*: AM286210, AM286207; *M. racemosus*: AM286208; *M. plumbeus*: AM286209; *M. rouxii*: AM286211; and *B. lamprospora*: AM286212. Clustal W alignment of the *FTR1* sequences and the corresponding identity matrix are available at http://www.sci.u-szeged.hu/microbiology/alignmentFTR1.doc.

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