Intraspecific mitochondrial DNA polymorphism within the emerging filamentous fungal pathogen *Trichoderma longibrachiatum*

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The genetic diversity of the emerging fungal pathogen *Trichoderma longibrachiatum* was examined at the level of mitochondrial DNA. The 17 investigated strains, comprising nine clinical and eight non-clinical isolates, exhibited seven and ten different mitochondrial DNA profiles by using the restriction enzymes *Bsu*RI and *Hin*6I, respectively. The sizes of mitochondrial DNAs varied from 34.9 to 39.5 kb. The discriminatory power of the method was higher than that of internal transcribed spacer sequence analysis and therefore should be more suitable for identification and epidemiological investigations. However, clinical and non-clinical isolates did not form separate clusters on the resulting dendrogram and thus there was no indication of a correlation between genetic structure and pathogenicity of the isolates.

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INTRODUCTION

An increasing number of mycoses are reported in many countries owing to a greater life expectancy associated with an increase in the quality of medical and surgical procedures, as well as the emergence of infections and diseases affecting the immune system. Additionally, more common airborne and soil-borne fungi are being added to the list of potential human pathogens. Strain typing is a necessary tool to identify the pathogenic strains, find the reservoirs, investigate the epidemiology and analyse the modes of transmission in cases of nosocomial outbreaks of fungal infections.

Trichoderma species are common, soil-borne, filamentous fungi and have long been known as non-harmful micro-organisms. They are used in biotechnology as sources of enzymes (Kubicek & Penttilä, 1998) and antibiotics (Ghisalberti & Sivasithamparan, 1991). Moreover, they are applied to agricultural crops as plant growth promoters and biofungicides (Benítez *et al.*, 2004). However, as recently emerging fungal pathogens, *Trichoderma* strains have been detected on the skin, in the lung and as causative agents of peritonitis in peritoneal dialysis patients, and have been found to be disseminated in the liver, brain, heart and stomach of immunocompromised patients (Loeppky *et al.*,

1983; Jacobs *et al.*, 1992; Seguin *et al.*, 1995; Tanis *et al.*, 1995; Munoz *et al.*, 1997; Bren, 1998; Furukawa *et al.*, 1998; Guarro *et al.*, 1999; Richter *et al.*, 1999; Rota *et al.*, 2000; Myoken *et al.*, 2002; Kredics *et al.*, 2003a). Although clinical isolates have been reported from the species *Trichoderma koningii* (Campos-Herrero *et al.*, 1996) and *Trichoderma pseudokoningii* (Gautheret *et al.*, 1995), sequence analysis of the internal transcribed spacer (ITS) region of these isolates revealed that they had been misidentified (Kuhls *et al.*, 1999; Kredics *et al.*, 2003b), and that the majority of the pathogenic *Trichoderma* isolates belong to *Trichoderma longibrachiatum*, a species closely related to – although not the anamorph of – *Hypocrea orientalis*, a member of the *Hypocrea schweinitzii* complex (Samuels *et al.*, 1998).

Potential virulence factors of *T. longibrachiatum* strains, derived from clinical or soil samples, have been examined to compare their capacity to cause human infection (Antal *et al.*, 2005), but, on the whole, there have been no significant differences in the examined features. The present study was aimed at the investigation of intraspecific variability at the DNA level on similar sets of strains by using the method of mitochondrial (mt)DNA RFLP, which has already proved to be applicable to the identification and epidemiological investigation of medically important members of the very diverse group of dematiaceous fungi (De Cock 1994; Ishizaki *et al.*, 1995; Kawasaki *et al.*, 1993, 1999a, b; Yamagishi *et al.*, 1997).

Abbreviations: ITS, internal transcribed spacer; RAPD, random amplification of polymorphic DNA.

METHODS

The isolates used in the experiments were identified as *T. longibrachiatum* by sequence analysis of the ITS region. mtDNA RFLPs of eight non-clinical and nine clinical *T. longibrachiatum* isolates were examined (Table 1). For mtDNA characterization, the fast typing method of Varga *et al.* (1993) was used. Total DNA samples were isolated from lyophilized mycelia by the method of Leach *et al.* (1986) and digested with *Bsu*RI (GG/CC) or *Hin*6I (G/CGC) restriction enzymes. DNA fragments were separated by agarose gel electrophoresis and visualized under UV light. The sizes of the mtDNA fragments were determined by using GelBase/GelBlot Pro Gel Analysis software (UltraViolet Products), using λ -pUC mix (Fermentas) as the molecular mass marker. The mtDNA profiles were converted to a similarity matrix and phylogenetic distances were calculated by using PhylTools software (Buntjer, 1997) to create a dendrogram by the unweighted pair group method with arithmetic means, using the NEIGHBOR program of the PHYLIP software package (version 3.57c; Felsenstein, 1995).

RESULTS AND DISCUSSION

Based on *Bsu*RI RFLP profiles, the saprotrophic and clinical isolates belonged to three and five different haplotypes, respectively (Fig. 1a, Table 1). Certain non-clinical isolates collected near Szeged, Hungary, exhibited the same mtDNA

Fig. 1. mtDNA types of the *T. longibrachiatum* isolates examined using the restriction enzymes *Bsu*RI (a) and *Hin*6I (b). Lanes: 1, CECT 20105; 2, CECT 2412; 3, CECT 2606; 4, UAMH 9573; 5, UAMH 9515; 6, ATCC 208859; 7, ATCC 201044; 8, IP 2110.92; 9, UAMH 7955; 10, UAMH 7956; M, λ -pUC mix molecular mass marker.

Table 1. Origin, mtDNA types and size of the *T. longibrachiatum* isolates examined

Collections: ATCC, American Type Culture Collection; UAMH, University of Alberta Microfungus Collection and Herbarium; CBS, Centraalbureau voor Schimmelcultures; CM, Centro Nacional de Microbiología, Virología y Immunología Sanitarias; IP, Institut Pasteur; SZMC, Szeged Microbiological Collection; CECT, Colección Española de Cultivos Tipo.

Isolate	Origin	mtDNA type (<i>Bsu</i> RI)	mtDNA type (<i>Hin</i> 6I)	mtDNA size (kb)
CECT 20105	Soil; Egypt	I	I	39.5
CECT 2412	Mushroom compost; UK	II	II	37.2
CECT 2606	Soil; Sierra Leone	III	III	36.4
SZMC 1012	Soil; Hungary	I	I	39.5
SZMC 1158	Soil; Hungary	I	I	39.5
SZMC 1159	Soil; Hungary	I	I	39.5
SZMC 0886	Soil; Hungary	II	II	37.2
SZMC 0887	Soil; Hungary	II	II	37.2
UAMH 9573	Peritoneal catheter; Newfoundland, Canada	IV	IV	34.9
UAMH 9515	Peritoneal fluid; Newfoundland, Canada	V	V	36.4
ATCC 208859	Human immunodeficiency virus-positive patient; Texas, USA	VI	VI	35.1
CM 382	Peritoneal fluid; Gran Canaria, Spain	VI	VI	35.1
CBS 446.95	Lung; Austria	VI	VI	35.1
ATCC 201044	Skin biopsy; Texas, USA	VII	VII	37.4
IP 2110.92	Lung, brain, heart, stomach; France	II	VIII	37.2
UAMH 7955	Sinus of a transplant patient; Pennsylvania, USA	VI	IX	35.1
UAMH 7956	Faeces, liver, lung; Iowa, USA	II	X	37.2

patterns as either the non-clinical isolate CECT 20105 from Egypt or CECT 2412 from the UK (Table 1). More interestingly, the haplotype of two clinical isolates (IP 2110.92 from France and UAMH 7956 from the USA) was identical to those of non-clinical strains (SZMC 0886 and SZMC 0887 derived from soil in Hungary and CECT 2412 originating from mushroom compost from the UK; Fig. 1a, Table 1), suggesting close phylogenetic relationships among strains. Nevertheless, using the restriction enzyme *Hin*6I, the clinical and non-clinical isolates could be separated from each other (Fig. 1b, Table 1). However, neither enzyme could distinguish among three clinical isolates of widely dispersed geographical origin (ATCC 208859 from the USA, CM 382 from Spain and CBS 446.95 from Austria). Altogether, seven haplotypes could be distinguished by both restriction enzymes, while three additional ones could be distinguished only by *Hin*6I. In addition to the diversity shown in the RFLP patterns of the examined *T. longibrachiatum* strains, the sizes of their mtDNAs were also different, varying from 34.9 to 39.5 kb (Table 1). Previously, we characterized a mtDNA of 28.5 kb in a *Trichoderma harzianum* isolate (Antal *et al.*, 2002). Meyer (1991) observed mtDNAs in the range 32.6–32.8 kb in *Trichoderma viride* isolates, while Chambergro *et al.* (2002) determined the mtDNA of *Trichoderma reesei* to be 42.1 kb. The observed size variability of mtDNAs of *T. longibrachiatum* isolates is possibly caused by loss of introns or intergenic sequences, which has been observed in several *Aspergillus* species (Hamari *et al.*, 2001, 2003).

Based on the RFLP profiles and sizes of mtDNA, four groups could be identified. One group included the clinical isolates CM 382, ATCC 208859, CBS 446.95, UAMH 9515 and UAMH 7955 with mtDNAs in the range of 35.1–36.4 kb (Figs 1 and 2, Table 1). Isolates CECT 2412, SZMC 0886, SZMC 0887, IP 2110.92 and UAMH 7956 belonged to the second group with mtDNAs of 37.2 kb, while the third group comprised isolates ATCC 201044, SZMC 1012, SZMC 1158, SZMC 1159 and CECT 20105 with mtDNAs of between 37.4 and 39.5 kb. Another group consisted of isolates CECT 2606 and UAMH 9573 with mtDNAs of 36.4 and 34.9 kb, respectively. These two isolates were also positioned on a separate branch based on phylogenetic analysis of sequences of the ITS region (Kredics *et al.*, 2003b). In the case of the other isolates, the nuclear rDNA sequence data did not correlate with mtDNA-based clustering. Such a phenomenon has been observed elsewhere within other fungal taxa (Geiser *et al.*, 1996), possibly due to differences in inheritance and mutation rates of the nuclear and mitochondrial genomes.

Although five clinical isolates formed a well-defined group, other non-clinical and clinical isolates did not form separate clusters on the dendrogram constructed from mtDNA RFLP profiles (Fig. 2). Based on the observation that clinical isolates could not be distinguished from non-clinical ones, the question of whether every environmental isolate has the capacity to cause infection remains unanswered. Similar observations have been made for the widespread

Fig. 2. Dendrogram of the isolates based on their mtDNA profiles, constructed using the unweighted pair group method with arithmetic means. The scale bar represents genetic distance.

opportunistic fungal pathogen *Aspergillus fumigatus* (Latgé, 2001; Varga & Tóth, 2003).

DNA-based molecular methods have become routinely used tools in clinical microbiological laboratories for species identification and epidemiological investigations of emerging filamentous fungal pathogens (Nagy *et al.*, 2004). Random amplification of polymorphic DNA (RAPD), using both fingerprinting and random primers, has been used successfully to analyse isolates of the section *Longibrachiatum* (Kuhls *et al.*, 1999). However, RAPD has been suggested to be prone to errors due to the low annealing temperatures (Rieseberg, 1996). ITS sequence analysis is a reliable method for phylogenetic analysis and species identification within the section *Longibrachiatum* of the genus *Trichoderma*. In recent years, efforts involving this molecular method have been made for the identification and taxonomic investigation of different *Trichoderma* species, including members of the section *Longibrachiatum* (Kuhls *et al.*, 1997, 1999; Kindermann *et al.*, 1998; Kredics *et al.*, 2003b; Gherbawy *et al.*, 2004; Druzhinina & Kubicek, 2005). However, as the variability of ITS sequences is low within the species *T. longibrachiatum*, this method is not appropriate for intraspecific population studies and strain typing. Analysis of mtDNA RFLP seems to be a more suitable method for the determination of intraspecific relationships, as the evolutionary rates of mtDNA are higher than those of nuclear DNA, which results in higher intraspecific variability.

In conclusion, the mtDNA RFLP method seems to be more reliable than the frequently used RAPD technique, and has a much higher discriminatory power on the same set of strains than ITS sequence analysis (Kredics *et al.*, 2003b).

Therefore, this technique seems to be useful for epidemiological studies to clarify the relationships between clinical *T. longibrachiatum* isolates, and for the investigation of the occurrence of potential emerging pathogenic strains in the environment. Further studies are in progress to characterize the mtDNAs of *T. longibrachiatum* strains in more detail.

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