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Development of a new MLST scheme for differentiation of *Fusarium solani* Species Complex (FSSC) isolates

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ABSTRACT

Fungi belonging to the *Fusarium solani* Species Complex (FSSC) are well known plant pathogens. In addition to being the causative agent of some superficial infections, FSSC has recently emerged as a group of common opportunistic moulds, mainly in patients with haematological malignancies. Molecular typing methods are essential in order to better understand the epidemiology of such opportunistic agents with the final goal of preventing contamination. A three-locus typing scheme has thus been developed for FSSC; based on polymorphisms in the domains of the ITS, EF-1 alpha, and RPB2 genes. This method is now considered to be a useful reference for phylogenetic and taxonomic studies. In other significant clinical fungi (e.g., *Candida* sp., *Cryptococcus neoformans*, and *Aspergillus fumigatus*), genes coding for metabolic enzymes have been widely used and proven to be very informative for diagnosis and epidemiology. The contribution of these genes has never been evaluated for *Fusarium* sp. and more specifically for *F. solani* Species Complex.
Here, we have evaluated the contribution of 25 genes for diagnosis and epidemiological purposes. We then report a new five-locus MLST scheme useful for diagnosis and typing of clinical FSSC isolates. The method has been validated on 51 epidemiologically unrelated strains of FSSC and presents a high power of discrimination calculated at 0.991.

1. Introduction

Members of the *Fusarium solani* Species Complex (FSSC) are common saprophytes, frequently isolated from environmental sources such as soil, air and plants. These fungi are also well known as plant, animal and human pathogens and account for approximatively two-thirds of all human and animal fusariosis worldwide (De Hoog et al., 2004; Dignani and Anaissie, 2004). During the last decade, *F. solani* has been increasingly reported in humans, causing localised cutaneous infections in immunocompetent hosts (e.g., onychomycosis, keratitis or endophtalmitis), usually associated with trauma (Chang et al., 2006). In immunocompromised patients, superficial or subcutaneous lesions are susceptible to disseminate and induce serious invasive mycoses with a high mortality rate, ranging from 50% to 80% (Nucci and Anaissie, 2007). Presently, *F. solani* has emerged as the second most common opportunistic pathogenic mould (after *Aspergillus*), mainly in patients with haematological malignancies, in recipients of solid organ and allogenic bone marrow or stem cell transplants (Boutati and Anaissie, 1997; Nucci and Anaissie, 2007). More specifically, neutropenia, lymphopenia, graft-versus host disease, corticosteroid therapy or any other immunosuppressive treatments represent high risk factors for disseminated fusariosis (Guarro and Gene, 1995; Nucci and Anaissie, 2007).

Due to their increasing clinical relevance, several molecular phylogenetic studies have been performed involving sequence typing, restriction fragment length polymorphism and microsatellite analysis (Dyavaiah et al., 2007; Godoy et al., 2004; Mehl and Epstein, 2007; Zhang et al., 2006). Multilocus sequence typing (MLST) is a highly accurate method used to distinguish between isolates of microbial species. MLST was first developed to facilitate studies of epidemiology and population structure in several bacterial populations (Maiden et al., 1998). This method compares nucleotide polymorphisms within five to seven gene regions, traditionally housekeeping genes. The different polymorphisms giving rise to allelic variants are recorded and the resulting combinations correspond to the strain sequence types (ST). Because MLST data can be easily accessible at a global scale through dedicated websites, it has the advantage of allowing multiple users to compare their results. MLST has already been used to investigate

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populations of human pathogenic fungi, including *Candida albicans* (Bougnoux et al., 2002), *Candida glabrata* (Dodgson et al., 2003), *Candida tropicalis* (Tavanti et al., 2005), *Candida krusei* (Jacobsen et al., 2007), *Cryptococcus neoformans* var. *gattii* (Feng et al., 2008 ; Meyer et al., 2009), *C. neoformans* var. *grubii* (Litvintseva et al., 2006 ; Meyer et al., 2009) and *Aspergillus fumigatus* (Bain et al., 2007).

Recently, a three-locus typing scheme has also been developed for the *F. solani* Species Complex that is based on polymorphisms in portions of the internal transcribed spacer region and domains D1 plus D2 of the nuclear large-subunit rRNA, the translation elongation factor 1 alpha gene (EF-1alpha), and the second largest subunit of the RNA polymerase II gene (RPB2). This multilocus method is particularly useful for phylogenetic and taxonomic studies and has led to a new nomenclature of FSSC containing three main clades. Twenty phylogenetically distinct species have thus been found in the medical FSSC clade 3 (Zhang et al., 2006; O'Donnell et al., 2008). By using this typing method, authors theorise that species-level studies using additional phylogenetically informative data may lead to the discovery of unsuspected cryptic species (O'Donnell et al., 2008). In other clinical fungi, MLST schemes have been developed using metabolic genes, for example glyceraldehyde 3P deshydrogenase (Feng et al., 2008) or isocitrate lyase (Tavanti et al., 2005). Although widely used for fungal MLST, these metabolic genes have never been evaluated for the epidemiology of *F. solani*.

In the present study, we aim to evaluate the contribution of 25 genes to specifically differentiate clinical FSSC isolates. A new five-locus MLST scheme has thus been described for the typing of these fungi. Tested over a panel of 51 epidemiologically unrelated strains, the method was shown to be reproducible, generates a stable profile and presents a power of discrimination calculated at 0.991. These characteristics made this method suitable for tracing strains in a hospital context.

2. Materials and methods

2.1. Isolates

Fifty-one epidemiologically unrelated isolates of *F. solani* Species Complex were used in this study. These strains originated from clinical, plant and environmental sources and were obtained from our own collection or provided by collaborators. All strains were deposited at the CBS-KNAW Fungal Biodiversity Centre (http://www.cbs.knaw.nl). The corresponding collection numbers and the characteristics of all strains are listed in Table 1.

Strains were stored at −80 °C in water/glycerol (1/1) and then streaked onto Malt Agar and grown at 30 °C until conidia formed.

2.2. Genetic markers

Based on previous MLST studies, we initially selected the sequences of 25 genes used for *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *A. fumigatus* and *C. neoformans*, for which homologues were available in GenBank for several species of *Fusarium* (http://www.ncbi.nlm.nih.gov/sites/entrez) (Bain et al., 2007; Bougnoux et al., 2002; Dodgson et al., 2003; Jacobsen et al., 2007; Litvintseva et al., 2006; Tavanti et al., 2005). These genes code for enzymes involved in metabolic functions, such as isocitrate lyase (ICL), an enzyme unique to the glyoxylate cycle, which catalyses the cleavage of isocitrate to glyoxylate and succinate (Table 1). For each gene, sequences were then aligned using the Alibee program available on the GeneBee website (http://www.genebee.msu.su/services/malign_reduced.html). Pairs of primers were designed in conserved regions of these genes in order to amplify 500- to 700-bp fragments. Both strands of the successfully amplified genes were then sequenced in order to finally reduce the set to gene fragments, which, in combination, yielded the largest number of different strain types. Primers and corresponding PCR product sizes are described in Table 2.

2.3. DNA extraction

DNA was extracted from cultures by using the High Pure PCR Template Preparation kit (Roche Diagnostics, Meylan, France). The standard protocol was slightly modified by a short pre-treatment: an aliquot of mycelium was suspended in 200 µl of tissue lysis buffer and incubated for 30 min at 37 °C in the presence of lyticase (10 U/µl, final concentration). DNA was then extracted by following the manufacturer's instructions.

2.4. Amplification and nucleotide sequence determination

PCR assays were carried out in 50 µl reaction volumes containing 2 or 5 µl of DNA extract, 0.5 µl of each primer (40 µM), 0.4 µl of Fast Start Taq DNA Polymerase (5 U/µl), 5 µl of 10× buffer-$MgCl_2$ and 5 µl of a deoxynucleoside triphosphate mix (2 mM). PCRs were run in a iCycler IQ Thermal cycler (Biorad, France) under the following conditions: an initial 3-min denaturation step at 94 °C, followed by 30 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min, with a final extension step of 5 min at 72 °C. Five microliters of PCR product were electrophoresed in a 2% agarose gel in the presence of ethidium bromide and visualized under UV light. PCR products were purified using the QIAquick PCR purification kit (Qiagen, France) and sequenced on both strands using the amplification primers. Sequencing reactions were performed by using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, France) on an ABI PRISM Genetic Analyzer (PE Applied Biosystems).

2.5. Sequence data analysis

The one-letter code for nucleotides from the International Union of Pure and Applied Chemistry (IUPAC) nomenclature was used for data analyses. Forward and reverse DNA sequence chromatograms were analysed with the BioEdit software and nucleotide polymorphic sites were then identified within multiple alignments. Numbers were assigned to unique allelic variants for each haploid locus. These numbers were then combined to yield a sequence type. Sequence type identifiers were compiled for all isolates in the Table 1.

2.6. Discriminatory power

The discriminatory power was measured with Simpson's index of diversity, calculated according to Hunter and Gaston's modification (Hunter and Gaston, 1988). This index is based on the probability that two unrelated strains sampled from a test population will be placed into different typing groups. The Simpson's index of diversity must be associated with confidence intervals (Simpson, 1949). The index should be at least 0.95 in order to consider a typing system to have an effective discrimination power (Struelens 1996).

2.7. Stability and reproducibility of the MLST method

The stability of the MLST method was evaluated by sequencing the amplicons obtained using the DNA from the last subculture of the strain *F. solani* CBS 124896 after 10 transfers.

To evaluate the intra-laboratory reproducibility, the DNAs from three different isolates were extracted from three independent cultures and subsequently submitted to the MLST scheme.

Table 1
Characteristics and genotypes of the 51 isolates of *Fusarium solani* species complex tested in the study.

CBS number	Site of isolation	Geographic origin	Allelic variants					Sequence type (ST)
			ACC	ICL	GDP	MDP	SOD	
124633	Nail	France (Nancy)	1	1	1	1	1	1
124894	Skin	France (Paris)	1	2	1	1	2	2
124632	Skin	France (Paris)	2	3	2	2	3	3
124901	Skin	France (Paris)	1	4	1	1	1	4
124631	Nail	France (Paris)	3	5	3	3	4	5
124630	Skin	France (Paris)	2	3	2	2	3	3
124629	Nail	France (Paris)	3	5	3	3	3	6
124895	Skin	France (Paris)	4	6	4	4	3	7
124896	Blood	Belgium	5	7	5	5	5	8
124628	Nail	France (Paris)	3	5	3	3	5	9
124623	Environment	France (Paris)	3	5	3	3	3	6
124889	Nail	France (Paris)	1	8	1	1	1	10
124627	/	/	6	9	2	6	3	11
124890	Nail	France (Nancy)	7	10	6	4	3	12
124897	Skin	Gabon	1	1	7	1	1	13
124891	Skin	Gabon	8	11	8	1	1	14
124898	Skin	Gabon	1	2	1	7	2	15
124892	Nail	Gabon	9	12	9	8	3	16
124899	Nail	Gabon	1	8	1	1	1	10
124893	Nail	France (Limoges)	4	10	6	4	3	17
124900	Foot	France (Limoges)	4	10	6	4	3	17
124626	/	/	3	5	10	3	1	18
125013	/	France (Lille)	5	7	11	9	5	19
124625	Cornea	France (Lille)	3	5	3	3	6	20
124624	Nail	France (Nancy)	3	5	3	3	3	6
102824	Plated litter fragment	Colombia	10	13	3	10	7	21
241.93	Mycetoma	Suriname	11	14	12	11	8	22
115695	/	/	12	5	3	12	9	23
208.29	*Hyacinthus orientalis*	Germany	10	13	3	10	7	21
181.29	Potato	Germany	5	7	13	5	5	24
119996	Carrot	Netherlands	4	10	4	4	3	25
119223	Carrot	Spain	13	15	14	13	10	26
118931	Tomato	UK	14	16	2	14	11	27
117481	*Liriodendron tulipfera*	USA	15	17	15	15	9	28
115659	*Potato cultivar Maritta*	Germany	16	18	14	16	10	29
165.87	Potato	Denmark	4	10	6	4	3	17
318.73	*Trichosanthes dioica*	India	17	19	16	17	3	30
222.49	*Euphorbia fulgens*	Netherlands	18	16	2	7	11	31
231.31	*Quercus garyana*	USA	5	7	5	5	5	8
121450	Declined grape vine	Syria	6	20	17	6	12	32
115660	Potato	Egypt	19	21	14	18	10	33
115658	Potato	Israel	13	21	14	18	3	34
115045	*Calamus Palmae*	Hong Kong	20	5	3	19	13	35
166.87	Soil under chestnut tree	USA	21	10	4	4	3	36
109028	Subcutaneous nodule	Switzerland	22	22	18	20	4	37
108942	Big toe	Netherlands	4	10	4	4	3	25
490.63	Man	Japan	1	8	1	1	2	38
224.34	Toe nail	Cuba	3	5	3	3	4	5
112100	Human vocal prosthesis	Belgium	4	6	4	21	3	39
117608	Arm lesion human dermis	Turkey	23	23	19	22	14	40
116746	Hairy head	Ghana	6	24	2	22	14	41

/:unavailable data.

2.8. Nucleotide sequence accession numbers

The DNA sequence data obtained in this study have been deposited in Genbank and the accession numbers are listed in supplemental material (Table A).

3. Results

3.1. Selection of genes for F. solani MLST

Amplification of each of the 25 initially selected genes was attempted on 5 isolates. Among them, eight were successfully amplified for *F. solani* strains and then sequenced (Table 2). Five show high variability whereas three were not very variable. Based on this highest inter-strain discrimination, we selected the five most variable loci to build our MLST scheme: FsACC, FsICL, FsGPD, FsMPD and FsSOD that encode respectively for acetylCoenzyme A carboxylase, isocitrate lyase, glyceraldehyde 3P deshydrogenase, mannitol 1P deshydrogenase and superoxyde dismutase. For each locus, start and stop nucleotidic positions used for mutiple alignment are given in Table 3.

FsSOD showed the highest typing efficiency, distinguishing 14 genotypes for 13 polymorphisms observed (Table 3). FsICL was the least efficient marker with 24 genotypes differentiated for 67 polymorphisms observed.

3.2. Criteria of validation of MLST

Sequences obtained from primary cultures and the 10th sub-cultures were identical and validated the stability of the method.

The reproducibility, which was tested by using three separate isolates, was also confirmed by identical sequences obtained from the corresponding DNA independently extracted.

Table 2
Oligonucleotide primers of the 8 successfully amplified genes. The first 5 genes were selected for the *Fusarium solani* MLST because of their higher variability.

Genes*	Coded proteins	Size	Forward primer	Reverse primer
FsACC	AcetylCoenzyme A carboxylase	683	FsACCF 5′-CTCGTGAGATCATGATCCAGT-3′	FsACCR 5′-GTTGATAACAGCGGAGAGCT-3′
FsICL	Isocitrate lyase	626	FsICLF 5′-GGAGGTTGAGGCTGTCAAG-3′	FsICLR 5′-GCTTGGTGAGCTTCATGACA-3′
FsGPD	Glyceraldehyde 3P deshydrogenase	633	FsGPDF 5′-CATGTACGTCGTCGGTGTCA-3′	FsGPDR 5′-CGCTTACTTGGAGGCATCG-3′
FsMPD	Mannitol 1P deshydrogenase	688	FsMPDF 5′-CGTCGAGAACACCATCACAAA-3′	FsMPDR 5′-ATGGGGGTTGCCAATTCGCT-3′
FsSOD	Superoxyde dismutase	479	FsSODF 5′-TGGGACATCACCGGTAACGA-3′	FsSODR 3′-CAGTCTTGAGAGACTCCTCG-3′
FsTOP1	Topoisomerase I	482	FsTOP1F 5′-AGGAGCACATGACGACCAAG-3′	FsTOP1R 5′-GATCCTGATCAGCCATGATC-3′
FsUGP1	UTP glucose1P uridylyltransferase	582	FsUGP1F 5′-CAGATGCGAAATGCTCTGAC-3′	FsUGP1R 5′-AGGATATCGACGTTGTGGC-3′
FsHMG	HydroxyMethlylGlutarylCoA reductase	569	FsHMGF 5′-GGCAAGATTCCTGGTTACGC-3′	FsHMGR 5′-TTCATACCCATAGCGTCACC-3′

*Amplifications failed for 17 genes; ATP dependant permease (ADP1), Annexin (ANX); Beta 1.3 glucanosyltransferase (BGT1), Catalase (CAT1); Imidazole glycerol P dehydratase (HIS3); Laccase (LAC1); Lipase (LIP); Mating type protein (MAT1-2); Myristoyl CoA transferase (NMT1); Phospholipase (PLB1); 26S proteasome regulatory subunit (RPN2); Alanyl RNA synthetase (SYA1c); Orotidine 5′P decarboxylase (URA3); Urease (URE1); D xylose reductase I ou II (XYR1); Zinc transporter (ZRF2); Putative G6PD (ZWF1alpha).

3.3. Discriminatory power

Simpson's formula applied to the dataset obtained from the 51 typed isolates infers a high discriminatory power of the MLST scheme of 0.991 with confidence intervals $CI_{95\%}$: [0.984; 0.997]. Fig. 1 shows the relation between the variation of diversity index and the number of genes used in the MLST scheme. According to our results, the discriminatory power increased when more genes were included in the scheme (Fig. 1).

Forty one different genotyping profiles have been determined among the 51 selected strains (Table 1).

4. Discussion

Typing methods contribute greatly to the understanding of the epidemiology of infections and the evolution of pathogens. They are therefore important for monitoring infectious disease outbreaks. Two are predominant because of their high level of standardisation: microsatellite and MLST. Among them, MLST has previously been shown to be highly resolutive for epidemiological and population structure analysis of several fungi (Meyer et al., 2009). This technique is based on the identification of nucleotide polymorphisms in several gene regions present in all isolates within a given species. This reproducible method makes the results easy to include in online databases and to analyse with the appropriate software. This should allow longitudinal comparisons of large numbers of isolates and rapid exchanges between different laboratories. In the present study, we evaluated the utility of 25 genes that have previously been used in other fungal MLST schemes for epidemiological purposes. We finally selected five genes for the development of a new MLST scheme for the differentiation of isolates belonging to the *F. solani* Species Complex.

The MLST scheme has been validated according to previous published guidelines on the evaluation of molecular typing methods (Van Belkum et al., 2007). Our method is characterized by a discriminatory power of 0.991 with $CI_{95\%}$: [0.984; 0.997] and is thus a reliable tool for the typing of FSSC strains. According to Simpson's index calculation illustrated in Fig. 1, our method would have a sufficient discriminatory power (0.95) by using only two genes. Nevertheless, with five genes, the discriminatory power is higher, and this allows the discrimination of isolates that would otherwise have the same sequence type. We anticipate that typing and comparing a higher number of isolates using the five-locus MLST scheme will also lead to identifying additional genotypes of FSSC strains.

Our method, based on the analysis of 1616 nucleotides, is stable, reproducible and able to distinguish between closely related strains and reliable for the typing of FSSC strains. Moreover, the small size of the fragments makes the technique particularly cost-efficient and suitable for non-specialised institutions with simple sequencing facilities, compared to other methods. This new alternative scheme could then be used in cases of outbreaks to highlight the epidemiological relationships between environmental isolates and clinical strains. In our study, some isolates shared the same genotype, as for example, CBS 124630 and CBS 124632 that have been isolated from different patients in the same hospital, suggesting a possible common origin related to a nosocomial infection.

The five-locus scheme differentiates 41 genotypes from a sampling of 51 unrelated strains of *F. solani* Species Complex. This high genetic diversity found here within FSSC is consistent with the literature data,

Table 3
Characteristics of the fragments of the 5 loci studied.

Locus	Sequence start (5′)	Sequence end (3′)	Size (bp)	No. of variable nucleotidic sites	No of genotypes	No of genotypes/ polymorphism
FsACC	GTTGAC	GTACAT	371	54	23	0.43
FsICL	GCTCAG	GCATCC	369	67	24	0.36
FsGPD	AACGTC	GTGACA	355	31	19	0.61
FsMPD	CACGTT	CCTACC	334	47	22	0.47
FsSOD	CAACGG	GTAAGA	187	13	14	1.08

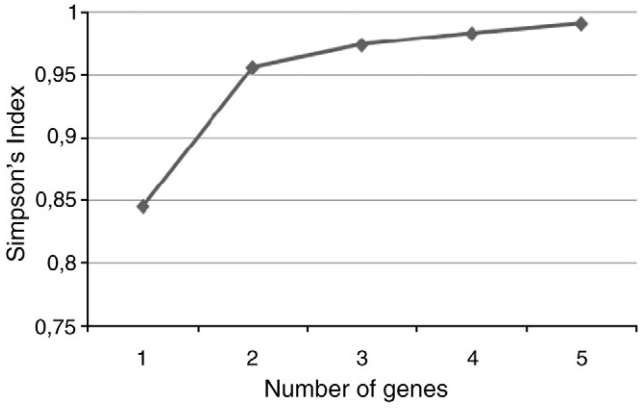

Fig. 1. Relation between the Simpson's index of diversity and the number of genes used to differentiate *F. solani* isolates.

which suggest that the *F. solani* Species Complex includes up to 45 morphologically cryptic phylogenetic species (O'Donnell et al., 2008).

The current reference typing method used for *F. solani* Species Complex based on the combined analysis of polymorphisms found within EF1α, ITS and RPB2 is well adapted for phylogenetic species recognition (O'Donnell et al., 2008). However, our new MLST scheme might bring more power for the discrimination of isolates within phylogenetic species. A comparative study is currently evaluated in order to compare the discriminatory power of the current three-locus scheme with our new typing method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mimet.2010.07.008.

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