International Journal of Food Microbiology 234 (2016) 24–35

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

A polyphasic approach for characterization of a collection of cereal isolates of the *Fusarium incarnatum-equiseti* species complex

Alessandra Villani [a], Antonio Moretti [a], Sarah De Saeger [b], Zheng Han [b], Jose Diana Di Mavungu [b], Célia M.G. Soares [c], Robert H. Proctor [d], Armando Venâncio [c], Nelson Lima [c], Gaetano Stea [a], Costantino Paciolla [e], Antonio F. Logrieco [a], Antonia Susca [a,*]

[a] Institute of Sciences of Food Production, National Research Council, Via Amendola, 122/O, 70126 Bari, Italy
[b] Laboratory of Food Analysis, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium
[c] CEB-Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710, –057 Braga, Portugal
[d] National Center for Agricultural Utilization Research, U.S. Department of Agriculture, 1815 N. University St., Peoria, IL 61604, USA
[e] Department of Biology, University of Bari, Via E. Orabona 4, 70125, Bari, Italy

ARTICLE INFO

Article history:
Received 22 January 2016
Received in revised form 18 April 2016
Accepted 20 June 2016
Available online 22 June 2016

Keywords:
FIESC
Secondary metabolites
Phylogeny
TRI genes

ABSTRACT

DNA-based phylogenetic analyses have resolved the fungal genus *Fusarium* into multiple species complexes. The *F. incarnatum-equiseti* species complex (FIESC) includes fusaria associated with several diseases of agriculturally important crops, including cereals. Although members of FIESC are considered to be only moderately aggressive, they are able to produce a diversity of mycotoxins, including trichothecenes, which can accumulate to harmful levels in cereals. High levels of cryptic speciation have been detected within the FIESC. As a result, it is often necessary to use approaches other than morphological characterization to distinguish species. In the current study, we used a polyphasic approach to characterize a collection of 69 FIESC isolates recovered from cereals in Europe, Turkey, and North America. In a species phylogeny inferred from nucleotide sequences from four housekeeping genes, 65 of the isolates were resolved within the *Equiseti* clade of the FIESC, and four isolates were resolved within the *Incarnatum* clade. Seven isolates were resolved as a genealogically exclusive lineage, designated here as FIESC 31. Phylogenies based on nucleotide sequences of trichothecene biosynthetic genes and MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry) were largely concordant with phylogeny inferred from the housekeeping gene. Finally, Liquid Chromatography (Time-Of-Flight) Mass Spectrometry [LC-(TOF-)MS(/MS)] revealed variability in mycotoxin production profiles among the different phylogenetic species investigated in this study.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Cereals contribute to approximately 30% of the human diet in industrialized countries (FAOStat, 2013) as well as roughly 50% of animal feed in Europe (European Commission, Agricultural and Rural Development, Short-term outlook , 2015). Therefore, problems related to cereal crops can be of economic and public health concern. Several pathogenic fungi threaten yield and quality of cereals worldwide due to their ability to cause disease and produce mycotoxins, secondary metabolites that are toxic to humans and other animals (Rocha et al., 2005; Ma et al., 2013; Moretti et al., 2013). The genus *Fusarium* includes some of the cereal-infecting fungi that are of greatest concern to agricultural production and food/feed safety worldwide (Desjardins, 2006). Collectively, *Fusarium* species cause multiple diseases that reduce yield and quality, and they produce several mycotoxins, including fumonisins, trichothecenes and zearalenone. Trichothecenes (TRI) are among the most economically significant mycotoxins worldwide, and are of particular concern on barley, maize and wheat (Maier et al., 2006; McCormick, 2013). These terpene-derived mycotoxins are strong inhibitors of eukaryotic protein biosynthesis, which in turn can cause a wide range of toxic effects, including vomiting, hemorrhaging and immunosuppression (Desjardins, 2006). Over 200 trichothecene analogs have been described (Grovey, 2007), and can be subdivided into four classes (A, B, C, and D) based on variation in chemical structure. Only type A and type B trichothecenes are produced by *Fusarium* (Desjardins, 2006). Type B trichothecenes are distinguished by the presence of a keto group at the carbon eight position (C8), and include deoxynivalenol (DON), its acetylated derivatives 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), as well as nivalenol (NIV) and its acetylated derivative 4-acetylnivalenol/fusarenone X (4-ANIV/FUS-X). The health risks for humans and other animals associated with exposure

* Corresponding author.
E-mail address: antonella.susca@ispa.cnr.it (A. Susca).

http://dx.doi.org/10.1016/j.ijfoodmicro.2016.06.023
0168-1605/© 2016 Elsevier B.V. All rights reserved.

to trichothecenes are widely recognized, and levels for DON in grains and cereal-based products are regulated by the European Commission (EC No. 1881/2006). Type A trichothecenes have hydrogen (4.15-diacetoxyscirpenol), hydroxyl (neosolaniol), or ester moiety (T-2 toxin) at C-8, and they are generally more toxic to animals than type B trichothecenes. Some type A compounds, such as 4.15-diacetoxyscirpenol (DAS) and T-2 toxin, are also included on the National Select Agent list in the US (http://www.selectagents.gov).

Within the genus *Fusarium*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. sporotrichioides*, and members of *F. incarnatum-equiseti* species complex (FIESC) are reported to produce trichothecenes (Desjardins, 2006). Although members of the FIESC are rarely associated with major disease epidemics, they have been identified as co-occurring with plant and human infections. Members of the FIESC are able to produce not only trichothecenes (both type A and type B), but also a number of other secondary metabolites, such as butenolide, beauvericin, equisetin, fusarochromanone and zearalenone (Desjardins, 2006). Recently, O'Donnell et al. (2009, 2010, 2012) demonstrated by multilocus GCPSR (Genealogical Concordance Phylogenetic Species Recognition) analysis that the FIESC comprises a large number of cryptic species (i.e., that cannot be distinguished by traditional morphology-based identification methods). Evolutionary analysis through Multi-Locus Sequence Typing (MLST), based on four genes sequences (O'Donnell et al., 2009, 2012), has facilitated recognition of 30 phylogenetic species (FIESC 1 through FIESC 30) within the FIESC. Some of these species contain multiple haplotypes which are designated by addition of a lowercase letter to the phylogenetic species designation (e.g., FIESC 5-a, FIESC 5-b, FIESC 5-c). FIESC species were resolved into two major clades: *Equiseti* and *Incarnatum*. The *Equiseti* clade was represented by 15 phylogenetic species, only three of which were linked to published species: *F. equiseti* (FIESC 14), *F. lacertarum* (FIESC 4), and *F. scirpi* (FIESC 9) (O'Donnell et al., 2009). The *Incarnatum* clade also included 15 phylogenetic species. *Fusarium equiseti* (Corda) Saccardo (1886) is a soil-inhabiting saprophyte able of colonizing roots of agriculturally important crops including cereals. The fungus has also been associated with mycotic infection of humans and other animals (O'Donnell et al., 2009, 2012; Short et al., 2011; van Diepeningen et al., 2015). *Fusarium scirpi* (Lambotte and Fautrey, 1894) is morphologically similar to *F. equiseti*, and the two species are frequently confused. However, *F. scirpi* tends to occur in arid and semi-arid regions, particularly in soil (Leslie and Summerell, 2006).

DNA-based phylogenetic analyses have provided significant insight into genetic diversity and phylogenetic relationships among members of this complex (O'Donnell et al., 2009, 2012). However, plant pathogenicity, plant disease epidemiology, and mycotoxin production profiles of members of the FIESC are poorly understood. Mycotoxin production profiles have been used in polyphasic approaches to understand diversity and species boundaries within *Fusarium* (Moretti et al., 1996; Watanabe et al., 2013). Over the past two decades, genes responsible for biosynthesis of the *Fusarium* mycotoxin, such as trichothecenes and fumonisins (Brown et al., 2001; Proctor et al., 2003) have been identified. This has provided a tool to predict mycotoxin production potential of field isolates (Jurado et al., 2005, 2006; Ward et al., 2002). In addition, the proteomic approach of Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) has emerged as a powerful tool for fast and reliable identification of species within several genera of fungi, including *Fusarium* (Marinach-Patrice et al., 2009; Rodrigues et al., 2011; Ranque et al., 2014; Silva et al., 2015).

Thus, the objective of this study was to characterize a collection of 69 fusaria from cereals that exhibited FIESC-like morphology. This was accomplished using DNA-based phylogenetic, MALDI-TOF and mass spectrometry-based mycotoxin analyses. The phylogenetic analyses indicate that all of the isolates are members of FIESC, and resolved most of them in four previously described phylogenetic species. However, seven isolates were resolved as a novel species lineage designated FIESC 31. The results also indicate that phylogenetic species can exhibit distinct MALDI-TOF MS spectra. Finally, isolates within and among phylogenetic species can vary in their ability to produce trichothecene mycotoxins. These findings provide important insight into the diversity of FIESC isolates that occur on cereals.

2. Materials and methods

2.1. Fungal strain collection

Sixty-nine isolates morphologically defined as *F. equiseti* and other FIESC isolated from cereals, mainly maize, oat and wheat, were included in the present study (Table 1). Strains were selected from the Agri-Food

Table 1
List of isolates analyzed in this study. *: Isolates selected for MALDI-TOF MS analysis §: Isolates selected for LC MS/MS analysis.

ITEM*	Host/Substrate	origin	year
190	barley	Italy	1978
10774	maize		2008
11037	maize	Italy	2008
11080	maize	Italy	2010
13569*§-13575*§-13576–13577	maize	Netherlands	2010
13580*§-13585–13601*-13607			
11285–11294*§-11296*§-11297*§	oat	Canada	2006
11276*§-11320*§-11322*§-11323	oat	Canada	2008
11345*§-11348–11354*§-11359*§			
11363*§-11392–11393-11400*§			
11401*§-11403*§-11404*§-11407*§			
11410–11414*§-11419			
11417–11418*§-11420*§-11426*§-11427	oat	Canada	2009
3673–3682	rice	Italy	1999
6808	rice	Italy	2004
10906	soil	Italy	
192	wheat	Italy	1979
6461–6464	wheat	Italy	2003
11189	wheat	Italy	2008
10387–10389-10391*§-10392*§	wheat	Italy	2009
10393–10395–10416-10433			
10440–10445-11126			
13005	wheat	Mexico	
10669–10671–10673-10674-10675-10786-10787-10788-10789	wheat	Spain	2009

* = ITEM, Agri-Food Toxigenic Fungi Culture Collection, http://www.ispa.cnr.it/Collection

Table 2
List of *Fusarium incarnatum-equiseti* species complex reference strains. *: strains selected for MALDI-TOF MS analysis §: strains selected for LC MS/MS analysis.

species	ITEM	source
Fusarium equiseti (FIESC 5)	7633*§	KSU 11460
Fusarium equiseti (FIESC 5)	15511*	KSU 20979
Fusarium sp. (FIESC 25)	6748*§	FRC R-4237
Fusarium sp. (FIESC 16)	7547*§	NRRL 20425
Fusarium scirpi (FIESC 9)	7621*§	KSU 11409
Fusarium scirpi (FIESC 9)	15556*	KSU 20985
Fusarium scirpi (FIESC 9)		FRC R-06979
Fusarium sp. (FIESC 12)		NRRL 13405
Fusarium camptoceras		NRRL 13381
Fusarium sp. (FIESC 15)		NRRL 31160
Fusarium sp. (FIESC23)	7155	
Fusarium longipes		NRRL 20695

Toxigenic Fungi Culture Collection (ITEM, Institute of Sciences of Food Production, Bari, Italy; http://www.ispa.cnr.it/Collection). They were isolated over a span of 30 years and from a wide range of geographical areas (Canada, Italy, Mexico, The Netherlands, Spain, and Turkey). Eleven other strains used as references and/or outgroups were received from the following culture collections: Fusarium Research Center (FRC) at Pennsylvania State University, State College, Pennsylvania; the ARS Culture Collection (NRRL) at the US Department of Agriculture, National Center for Agricultural Utilization Research in Peoria, Illinois); and the collection at Kansas State University, Manhattan, Kansas (Table 2).

2.2. DNA extraction

Fungal isolates were grown in Wickerham medium (40 g of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract per liter) on a rotary shaker at 120 rpm for 48 h in the dark. Fresh mycelia were collected by vacuum filtration through No. 4 Whatman filter paper (Whatman Biosystems Ltd., Maidstone, UK), then frozen and lyophilized. Total genomic DNA was extracted using the "Wizard® Magnetic DNA Purification System for Food" kit (Promega, USA) according to the manufacturer's protocol, using 10 mg of dried mycelium.

2.3. Sequence analysis

Phylogenetic relationships of isolates were investigated by amplifying and sequencing the housekeeping genes calmodulin (*CAL1*), the second largest subunit of RNA polymerase II (*RPB2*), translation elongation factor (*TEF1*), and β-tubulin (*TUB2*), as well as the trichothecene biosynthetic genes *TRI1*, *TRI4*, *TRI5*, *TRI8* and *TRI11*. The sequence for Gene F was also included in this analysis. Gene F is predicted to encode a Zn(II)2Cys6 transcription factor and is located within the *TRI* cluster in members of the FIESC, but not in cluster of other trichothecene-producing fusaria (Proctor et al., 2009). Primers used to amplify and sequence *TRI8*, *TRI11*, and Gene F were designed during the course of this study based on previously described sequences for these genes (Proctor et al., 2009), using the following PCR program: denaturation at 96 °C for 2 min; 35 cycles of the denaturation at 94 °C for 30 s, annealing at 55 °C for 30s, extension at 68 °C for 75 s; and final extension at 68 °C for 10 min. All other primers have been described previously (Table 3). Before sequencing, PCR products were purified with the enzymatic mixture EXO/FastAP (Exonuclease I, FastAP thermosensitive alkaline phosphatase, Thermo Scientific, Lithuania, Europe). Sequence reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit for both strands, after which they were purified by gel filtration through Sephadex G-50 (5%) (Amersham Pharmacia Biotech, Piscataway, NJ, USA), before they were analyzed on the 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequences were determined by Sequencing Analysis 5.2 software (Applied Biosystems) and aligned by the Clustal W algorithm (Thompson et al., 1994) with MEGA 5.2 software (Tamura et al., 2011). Representative sequences of the four loci were submitted to GenBank; accession numbers are LN901584-LN901599 for *CAL1*, LN901600-LN901619 for *RPB2*, LN901566-LN901583 for *TEF1*, and LN901620-LN901632 for *TUB2* (Table 4).

2.4. Phylogenetic analyses

Phylogenetic relationships were inferred with MEGA 5.2 software. Bootstrap analysis (Felsenstein, 1985) was conducted to determine the confidence for the internal nodes using a heuristic search with 1000 replicates. The combined data set consisting of partial sequences of the *CAL1*, *RPB2*, *TEF1*, and *TUB2* genes (2454 bp) and of the six *TRI* genes (4542 bp) were analyzed using Maximum Parsimony. Representative sequences for *TRI* genes have been submitted to Genbank as accession numbers from LN995562-LN995612. Some phylogenetic analyses also included *CAL1*, *RPB2*, *TEF1* and *TUB2* sequences from previously defined FIESC phylogenetic species. The latter sequences were obtained from GenBank.

Table 3
Sequence of oligonucleotide primers used in this study.

Target gene	Primers	5′ > 3′ nucleotide sequence	Ta (°C)	exp. size	references
Gene F	3891	GCTGTCAYAGYCAGAAGYTACGATG	54	1200	This study
	3894	AGAYATGBAGGACARGGCTTAGGGT			
TRI1	1285	GCGTCTCAGCTTCATCAAGGCAKCKAMTGAWTCG	54	1200	Proctor et al. (2009)
	1292	CTTGACTTSMTTGGCKGCAAAGAARCGACCA			
TRI4	2576	CCAATCAGYCAYGCTRTTGGGATACTG	55	1800	Proctor et al. (2009)
	2578	ACCCGGATTTCRCCAACATGCT			
TRI5	1558	GGCATGGTCGTGTACTCTTGGGTCAAGGT	55	1300	Proctor et al. (2009)
	1559	GCCTGMYCAWAGAAYTTGCRGAACTT			
TRI8	3904	GACCAGNAYCACSGYCAACAGTTCAG	55	1200	This study
	3906	GAACAGCCRCTCCRWAACTATTGTC			
TRI11	3895	TWCCCCACAAGRAACAYCTYGARCT	54	1300	This study
	3897	TCCCASACTGTYCTSGCMAGCATCAT			
CAL1	CL1	GARTWCAAGGAGGCCTTCTC	55	650	O'Donnell et al. (2000)
	CL2	TTTTGCATCATGAGTTGGAC			
RPB2	5F	GAYGAYMGVGATCAYTT	57	1000	Liu et al. (1999)
	7cR	CCCATRGCTTGYTTRCCCAT			
TEF1	EF1	ATGGGTAAGGARGACAAGAC	54	700	O'Donnell et al. (1998)
	EF2	GGARGTACCAGTSATCATGTT			
TUB2	Bt2a	GGTAACCAAATCGGTGCT	60	400	Glass and Donaldson (1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTYTGGC			

Table 4
List of GenBank accession numbers provided for *CAL1*, *RPB2*, *TEF1*, and *TUB2*.

ITEM	*CAL1*	*TEF1*	*RPB2*	*TUB2*
6464	LN901584		LN901600	
6748	LN901598	LN901582	LN901618	LN901631
6808	LN901591	LN901575		
7155	LN901597	LN901581	LN901617	LN901630
7547	LN901596	LN901580	LN901616	LN901629
7621	LN901599	LN901583	LN901619	LN901632
7633	LN901595	LN901579	LN901615	LN901628
10387	LN901585			
10392	LN901592	LN901576	LN901610	LN901625
10393	LN901586	LN901566	LN901601	
10395	LN901593	LN901577		
10416		LN901567		
10433			LN901602	
10445		LN901568	LN901603	
10674		LN901569		
10675	LN901590	LN901573	LN901607	LN901623
10787				LN901620
10789	LN901587		LN901604	LN901621
11294		LN901571		
11296	LN901589	LN901572	LN901606	LN901622
11320			LN901608	
11363		LN901574	LN901609	
11392			LN901605	
11401	LN901594	LN901578	LN901611	LN901626
13005	LN901588	LN901570		
13569			LN901612	
13580			LN901613	LN901627
13585				LN901624
13601			LN901614	

2.5. MALDI-TOF MS analysis

A subset of 25 of the 69 isolates was selected for this investigation (Table 1) based on a phylogenetic assessment. Six strains, ITEM 7633 and 15511 (*F. equiseti*), ITEM 7621 and 15556 (*F. scirpi*), ITEM 7547 (in other papers named as *F. incarnatum*, *O'Donnell et al., 2007*) and 6748 (in other papers named as *F. semitectum*, *Susca et al., 2015*) were used as species-reference strains. Seven-day-old spores of each isolate suspended in 0.5 mL of 0.2% agar were used for a single point inoculum which were incubated at 25 °C on 6-cm diameter Petri dishes with malt extract agar (20 g of malt, 20 g of glucose, 1 g peptone, 20 g of agar). Approximately 1 μg of mycelium and spores (from colonies not older than 72 h) of each fungus was directly transferred from the culture plate to the MALDI flex target plate. Matrix solution (0.5 μL of 75 mg/mL 2.5-dihydroxybenzoic acid in ethanol/water/acetonitrile [1:1:1]) was immediately added and mixed gently.

Escherichia coli strain DH5α was used as a control for in loco protein extraction and MALDI-TOF MS calibration. Cells were grown and maintained on Luria-Bertani (LB) agar medium (LB: 10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl, 15 g bacto-agar per liter). *E. coli* DH5α incubation was standardized at 20 h and grown aerobically at 37 °C. All cultures were checked for purity prior to use and were subcultured at least once prior to analysis. Approximately 1 μg of cells (about 10^7 cells per sample) was directly transferred from a single colony and spotted onto the 48 well MALDI flex target plate. The matrix solution was added and samples were air dried at room temperature. Each sample was spotted in duplicate to test reproducibility.

During the analyses all solutions were freshly prepared and kept at 5 °C. The analyses were performed on a Shimadzu Axima LNR system (Kratos Analytical, Manchester, U.K.) equipped with a nitrogen laser (337 nm), where the laser intensity was set just above the threshold for ion production. Ions of 12 well-defined ribosomal proteins of intact *E. coli* DH5α cells (4365.4, 5096.8, 5381.4, 6241.4, 6255.4, 6316.2, 6411.6, 6856.1, 7158.8, 7274.5, 7872.1, 9742 and 12,227.3 Da) were used as external calibrants. The mass spectra based on the mass range from 2 to 20 kDa were recorded using the linear mode with a delay of 104 ns and an acceleration voltage of +20 kV. The final spectra were generated by summing 20 laser shots accumulated per profile and 50 profiles produced per sample, which led to 1000 laser shots per summed spectrum.

Resulting ion peak lists were exported to the Spectral Archive And Microbial Identification System (SARAMIS™, AnagnosTec GmbH, Potsdam, Germany) software package which enabled final microbial identification. In SARAMIS™, peak lists of individual samples were compared to a database generating a ranked list of matching spectra. For each sample analyzed, only those data with match ≥99% spectral similarity were used. This software uses a point system based on ion peak lists with mass signals weighted according to their specificity. The similarity between individual spectra is expressed as the relative or absolute number of matching mass signals after subjecting the data to a single link agglomerative clustering algorithm. Microbial identifications by the SARAMIS™ package are based on the presence or absence of each ion peak in the spectra. A dendrogram of spectral proximity between isolates was created with this software (Santos et al., 2015).

2.6. LC-(TOF-)MS(/MS) analysis

Based on the phylogenetic assessment, a subset of 28 isolates (Tables 1 and 2) was selected for multitoxin analysis through a combination of untargeted LC-TOF-MS(/MS) for screening and targeted LC-MS/MS for confirmation.

2.7. Untargeted analysis of mycotoxins by LC-TOF-MS(/MS)

LC-Q-TOF (SCIEX, Framingham, MA, USA) was used for screening metabolites produced by the isolates. Chromatographic separation was achieved on a Zorbax Eclipse Plus C18 column (100 mm × 2.1 mm, 1.8 μm) (Agilent Technologies, Santa Clara, CA, US) at 40 °C, with a flow rate of 0.4 mL/min. The mobile phase consisted of (A) water/methanol (*v*/v, 5/95) containing 0.1% formic acid and 10 mM ammonium formate and (B) water/methanol (*v*/v, 95/5) containing 0.1% formic acid and 10 mM ammonium formate. A linear gradient elution program was applied as follows: 0 min 0% B, 0.5 min 0% B, 7 min 99% B, 9 min 99% B, 10 min 0% B and hold on for a further 4 min for re-equilibration, giving a total run time of 14 min. The injection volume was 5 μL. Triple TOF 4600 was operated in positive electrospray ionization mode (ESI+) for MS analysis with scan range of 50–1600 (*m*/*z*). The parameters were as follows. Source type: DuoSpray ion source, source temperature: 500 °C, injection manifold: bypass. The major primary ions (with responses >10.000) were further investigated by MS^2 for product ions with following parameters: collision energy (35 eV), cycle number (1637), period cycle time (470 m9 s), pulser frequency (12.576 kHz), accumulation time (50 ms). Calculation of the accurate mass of the analytes and their product ions were performed by PeakView software (version 1.2, SCIEX, Framingham, MA, USA). The accurate mass of the analyte was calculated by the precursor ions of $[M+H]^+$ and by $[M+Na]^+$, $[M\text{-}OH]^+$, $[M+NH4]^+$, and the precursor ions of their isotopical patterns with peak spacing tolerance of 5.0 ppm. The compounds were identified by comparing them with the library established in our lab.

2.8. Targeted LC-MS/MS confirmatory analysis

Reference DON, 15-ADON, 3-ADON, chaetoglobosin A (CHA), diacetoxyscirpenol (DAS), DON-3-glucoside (DON-3G), fusarenone X (FUS-X), mycophenolic acid (MPA), neosolaniol (NEO), and nivalenol (NIV) standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-high performance liquid chromatography (UHPLC) analysis was performed with a Waters Acquity UHPLC system (Waters, Zellik, Belgium). Analytes were separated on an Acquity CSH Fluoro-Phenyl column (150 mm*2.1 mm, 1.7 μm) (Waters, Zellik, Belgium) maintained at 40 °C, with a mobile phase flow rate of 0.40 mL/min. The

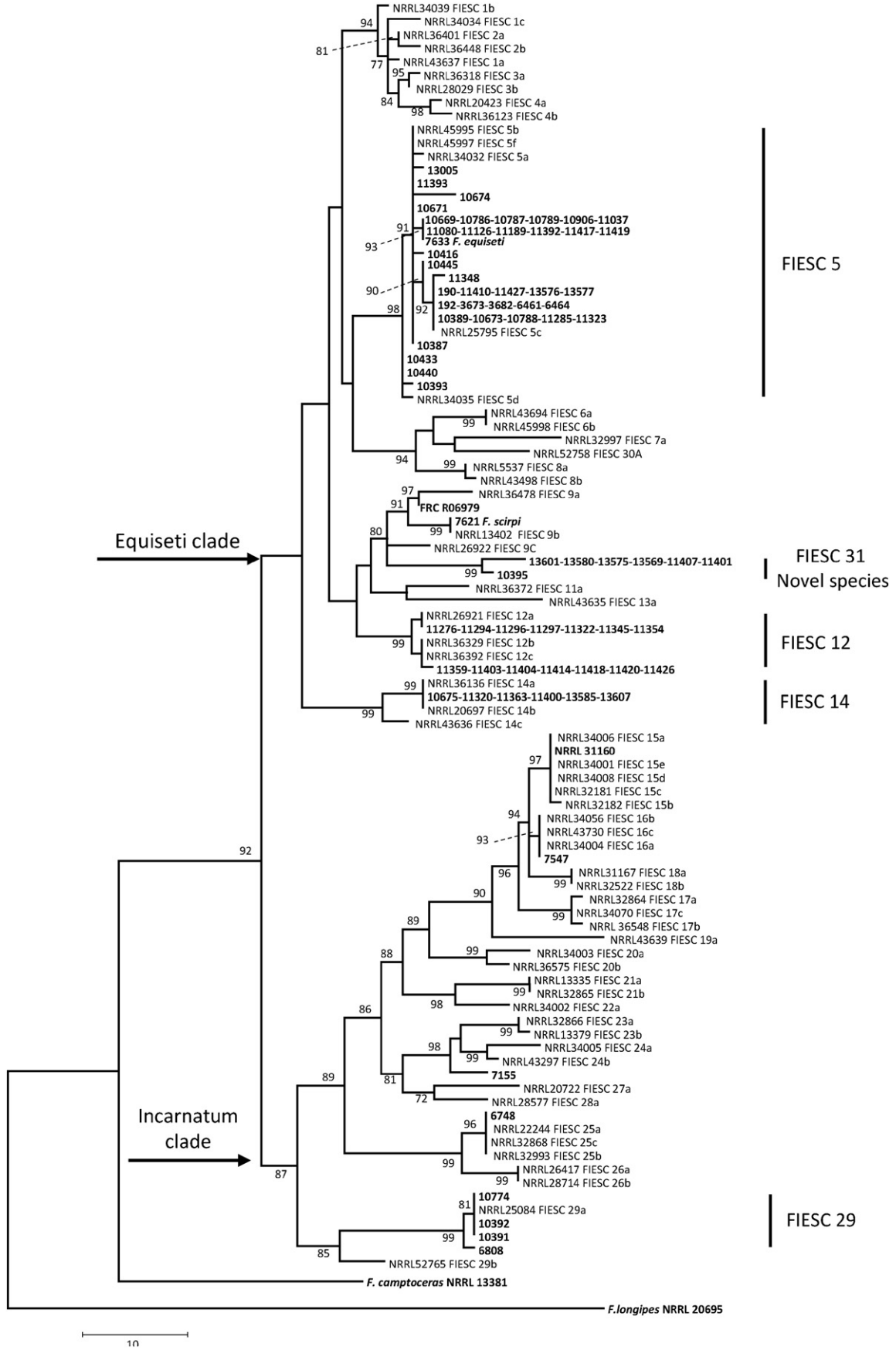

Fig. 1. Phylogenetic tree inferred from partial *TEF1* sequences from members of the *Fusarium incarnatum-equiseti* species complex isolates analyzed in this study. Sequences generated in the present study are in bold, the other sequences were downloaded from Fusarium-ID. The evolutionary history was inferred by using the Maximum Parsimony method. Numbers on branches are bootstrap values based on 500 pseudoreplicates.

mobile phase consisted of (A) water containing 5 mM ammonium acetate and 0.3% acetic acid and (B) methanol containing 5 mM ammonium acetate and 0.3% acetic acid. A linear gradient elution program was applied as follows: initial 10% B, 2 min 10% B, 5 min 33% B, 5.1 min 75% B, 7 min 75% B, 8 min 99% B, 9 min 99% B, 11 min 10% B, and held for a further 3 min for re-equilibration, giving a total run time of 14 min. The injection volume was 10 μL. The MS/MS determination was performed on a Quattro Premier XE tandem quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI + source. The parameters used for ionization were as follows: capillary voltage (3.5 kV), source temperature (120 °C), and desolvation temperature (350 °C). The cone and desolvation gas flow were 50 and 800 L/h, respectively. Analyte confirmation was performed in multiple reaction monitoring (MRM) mode using two product ions. The optimum conditions were set for each mycotoxin by direct infusion. Data acquisition and processing were performed using MassLynx 4.0 (Waters) .

The 28 isolates were grown on PDA for 1 and 2 weeks and then transferred from the plates into 50 mL extraction tubes. The extraction solvent (20 mL), consisted of water/acetonitrile (84/16, *v*/v) was added to each of the tubes. The mixture was shaken for 60 min, and the supernatant was transferred to a new extraction tube and centrifuged at 5000 g for 5 min. A aliquot (5 mL) of the supernatant was collected and dried under nitrogen gas at 40 °C. The residues were then reconstituted using different solutions for LC-TOF-MS and LC-MS/MS analysis, respectively. For LC-TOF-MS, 0.2 mL of water/acetonitrile/methanol (40/30/30) solution was used to dissolve the residues, while for LC-MS/MS, 1 mL of the water solvent (A) containing 5 mM ammonium acetate and 0.3% acetic acid and solvent (B) methanol containing 5 mM ammonium acetate and 0.3% acetic acid (70/30, *v*/v) were used. After vortexing for 2 min followed by centrifugation at 3200 g for 5 min, the reconstituted extracts were filtered (0.22 μm, nylon filter) (Millipore, Billerica, MA, USA) and then centrifuged for 10 min at 10,000 g. The filtrate was collected in LC-MS vials for analysis.

3. Results

3.1. Identification of cereal isolates as members of the FIESC

Initial assessment of 69 isolates of *Fusarium* recovered from cereals grown in Canada, Italy, Mexico, The Netherlands, Spain and Turkey (Table 1) indicated that they were morphologically similar to *F. equiseti* and related species within the FIESC. To determine whether these isolates were members of FIESC, we conducted a BLASTn analysis of the *TEF1* sequence from each isolate against the Fusarium-ID database (Geiser et al., 2004). The analysis indicated that the *TEF1* sequences were most similar to members of the FIESC.

To further resolve the identity of the 69 isolates, we conducted a phylogenetic analysis with *TEF1* sequences from the isolates as well as from previously defined phylogenetic species within the FIESC (O'Donnell et al., 2009, 2012). This analysis indicated 65 isolates were nested within the *Equiseti* clade and only four isolates grouped with the *Incarnatum* clade (Fig. 1). The evolutionary history was inferred using the Maximum Parsimony (MP) and Maximum Likelihood methods (data not shown) and the bootstrap support from analysis of the individual genes and combined gene sequences are shown in Table 5. MP Tree #1 out of 74 most-parsimonious trees (length = 427) is shown (Fig. 1). The consistency index is (0.58), the retention index is (0.91), and the composite index is 0.57 (0.53) for all sites and parsimony-informative sites (in parentheses). For the isolates resolved within the *Equiseti* clade, 58 were further resolved into three previously defined unnamed phylogenetic species: FIESC 5, FIESC 12 and FIESC 14 (Fig. 1). The four isolates resolved within the *Incarnatum* clade were identified as phylogenetic species FIESC 29 (Fig. 1). The majority of the isolates examined here were identified as FIESC 5. Most of these isolates were recovered from wheat in Italy, but some were recovered from other hosts and/or geographic origins. The 14 FIESC 12 isolates were all recovered from oats in Canada. Isolates identified as FIESC 14 (n = 6) were recovered from multiple hosts and locations: two isolates were from maize in The Netherlands, three were from oats in Canada, and one was from wheat in Spain. The four FIESC 29 isolates also had multiple origins: one was from rice and two were from wheat in Italy, and one was from maize in Turkey.

In the *TEF1* phylogeny, seven isolates (10395, 11401, 11407, 13569, 14575, 13580 and 13601) nested within the *Equiseti* clade appeared to represent a novel phylogenetic species that was strongly supported by bootstrapping (bootstrap value 100). In order to better assess the species status of this putatively novel lineage, we conducted phylogenetic analyses of portions of *CAL1* and *RPB2* (Supplementary Fig. S1; Supplementary Fig. S2) and a 4-gene combined data set (Fig. 2). Maximum Parsimony tree #1 out of 21 most-parsimonious trees (length = 851) is shown (Fig. 1). The consistency index is (0.54), the retention index is (0.89), and the composite index is 0.63 (0.48) for all sites and

Table 5
Summary sequence data and tree statistics for the three loci and the combined data set obtained with Maximum Likelihood and Maximum Parsimony.

Gene	size	No. of PICs[a]	Consistency index	Retention index	MPT length[b]	Phylogenetic species	ML[c]	MP[d]
TEF1a	601	79	0.58	0.91	851	FIESC 5	97	98
						FIESC 12	99	99
						FIESC 14	100	99
						FIESC 29	100	99
						Novel/FIESC 31	100	99
CAL1	652	59	0.58	0.94	335	FIESC 5	83	70
						FIESC 12	90	82
						FIESC 14	93	<70
						FIESC 29	95	97
						Novel/FIESC 31	99	98
RPB2	790	55	0.56	0.93	286	FIESC 5	90/99	90/98
						FIESC 12	99	98
						FIESC 14	81	78
						FIESC 29	99	100
						Novel/ FIESC 31	71	78
Combined data set	2454	255	0.53	0.89	427	FIESC 5	74	99
						FIESC 12	100	100
						FIESC 14	100	100
						FIESC 29	100	100
						Novel/ FIESC 31	100	100

[a] number of Parsimony Informative Characters (PIC).
[b] length (number of steps) of most parsimonious tree.
[c] maximum likelihood bootstrap value.
[d] maximum parsimony bootstrap value.

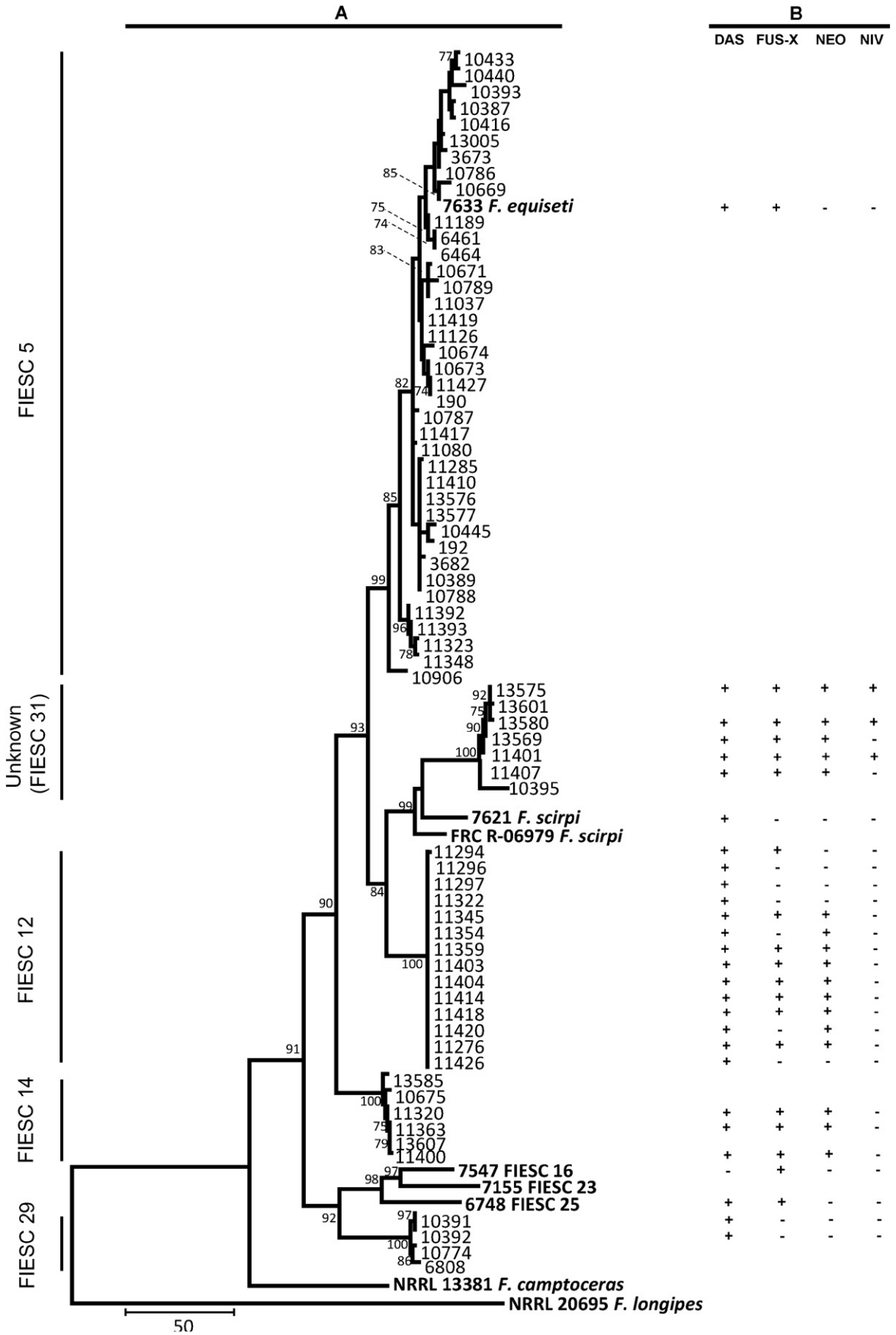

Fig. 2. Phylogenetic tree derived from combined DNA sequences of *CAL1, RPB2, TEF1* and *TUB2* and chemical profile. A). The evolutionary history was inferred using the Maximum Parsimony method. Numbers on branches indicate bootstrap values based on 500 pseudoreplicates. Reference strains are in bold. B) Chemical profile of 29 FIESC isolates examined by LC MS/MS.

parsimony-informative sites (in parentheses). *TUB2* sequence data were not available for all reference FIESC strains, so a tree showing the relationship of TUB2 sequences from the cereal isolates to those of reference FIESC strains is not presented. Bootstrap analysis of *CAL1* and *RPB2* and of the four-gene dataset also supported grouping of cereal isolates into FIESC 5, FIESC 12, FIESC 14 or FIESC 29 (81–100% bootstrap) (Supplemental Figs. S1 and S2). Bootstrap analysis of *CAL1* (98% bootstrap), *RPB2* (78% bootstrap), and the combined 4-gene dataset (100% bootstrap) support the genealogical exclusivity of the novel lineage, which we designate as phylogenetic species FIESC 31. Although analysis of the combined *CAL1*, *RPB2*, *TEF1* and *TUB2* dataset did not include reference sequences for FIESC 5, FIESC 12, FIESC 14 and FIESC 29, bootstrap analysis of the combined dataset provided high levels of support (100% bootstrap) for grouping the cereal isolates in to distinct clades (Fig. 2), which almost certainly correspond to FIESC 5, FIESC 12, FIESC 14 and FIESC 29 based on comparison of the four-gene tree (Fig. 2) to single-gene trees that included reference sequences (Figs. S1 and S2).

The analysis of intra-species variability for FIESC 5 (39 isolates) was overlooked, selecting only 1 strain (species reference ITEM 7633 with formal name *F. equiseti*), as representative of the group, widely analyzed by Marın et al., 2012; Kosiak et al., 2005; Jurado et al., 2006. Further analysis with different approaches were conducted on remnant 30 isolates, with slight variations in the number for each different approach.

3.2. MALDI-TOF MS analysis

Fig. 4 shows the dendrogram resulting from MALDI-TOF MS analysis of 25 selected isolates from our collection of cereal isolates as well as reference strains of six species: *F. equiseti* ITEM 7633 and ITEM 15511, *F. scirpi* ITEM 7621 and ITEM 15556, ITEM 7547, and ITEM 6748. In the dendrogram, the similarity between individual spectra is expressed as the relative or absolute number of matching mass signals, based on the presence or absence of each ion peak in the spectra. Each spectrum is built summing all ion peaks that appeared in 1000 shots, and differences between replicates are due to peaks with low intensity that did not appear in all replicates.

The dendrogram showed low percentage of mass similarity for some replicates (ITEM 6748, ITEM 15511, and ITEM 10392), but this low number of similar peaks was enough to differentiate the strains. In this analysis, the following distribution for the field isolates was obtained: FIESC 25 (ITEM 6748) and FIESC 16 (ITEM 7547) show clear separation based on the proteomic profile. Moreover, good clustering of strains was obtained for strains from the same haplotype, all isolates within the novel species, FIESC 31, and most isolates within FIESC 12. For the other haplotypes, only two or three isolates were tested. Except within FIESC 14, all isolates within the same haplotype clustered together. If different strains from a certain species produce different metabolites (with different intracellular proteins), it will reduce the similarity between different strains, resulting in the clustering of strains of the same phylogenetic species into different clades. Even so, it was possible to clearly differentiate FIESC 31, corroborating data obtained by other means.

3.3. LC-(TOF-) MS(/MS) analysis

Untargeted LC-TOF-MS(/MS) analysis originally revealed the possible presence of eight mycotoxins (15-ADON, 3-ADON, CHA, DAS, FUS-X, MPA, NEO, and NIV), however, targeted LC-MS/MS with reference standards could not confirm all of them. The chemical profile of 28 of the FIESC isolates is shown in Fig. 2. DON, 3-ADON, 15-ADON, DON-3G, CHA, and MPA were absent in all of the isolates analyzed. DAS was produced by all of the isolates, except the FIESC 16 (ITEM 7547). NEO was detected in all of the FIESC 31 and FIESC 14 isolates, in 9 of 14 FIESC 12 isolates, while it was not detected in *F. scirpi* reference strain ITEM 7621, *F. equiseti* reference strain ITEM 7633, and reference strains ITEM 6748 and ITEM 7547, or FIESC 29 isolates (ITEM 10391 and ITEM 10392). FUS-X was produced by 19 of the 28 isolates tested. NIV was produced by only 3 isolates, all of which were FIESC 31 (ITEM 11401, 13575 and 13580). Eighteen isolates produced both type A (NEO/DAS) and type B (NIV/FUS-X) trichothecenes, ITEM 7547 produced only type B (FUS-X) trichothecenes and 9 isolates produced only type A (DAS/NEO) trichothecenes.

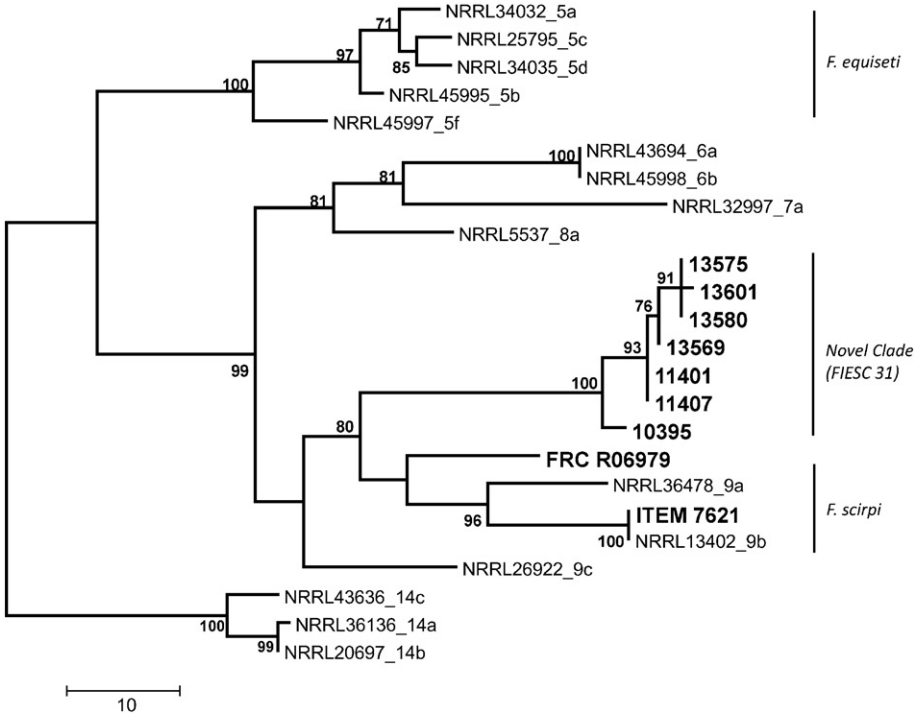

Fig. 3. Phylogenetic tree generated by Maximum Parsimony from combined DNA sequences of *CAL1*, *RPB2*, and *TEF1* fragments for FIESC31 isolates and the FIESC isolates downloaded from the Fusarium-ID database. Isolates sequenced in this study are in bold. Numbers on branches are bootstrap values based on 500 pseudoreplicates. Bootstrap values ≥70% were considered significant. Consistency index (CI) = 0.77, retention index (RI) = 0.92.

3.4. Analysis of trichothecene biosynthetic genes

Because isolates from cereals produced trichothecenes, we analyzed variation in select *TRI* genes in a subset of isolates from our collection. The subset consisted of 26 isolates that represented the range of phylogenetic diversity within the collection. We amplified and sequenced positions of *TRI1*, *TRI4*, *TRI5*, *TRI8* and *TRI11*, as well as the putative *TRI* Gene F. Six strains did not yield amplicons for one or more of the targeted *TRI* genes (Supplementary Table S1). As a result, these strains were excluded from phylogenetic analysis of the combined *TRI*-gene dataset (Fig. 5). Trees generated from individual *TRI* genes using Maximum Parsimony analysis were also provided as supplementary material (Supplementary Figs. S3-S8).

The phylogenetic tree inferred using Maximum Parsimony analysis of the combined data set of the selected *TRI* genes was concordant with the species phylogeny derived from housekeeping gene sequences (Fig. 5). For example, the *TRI* phylogeny resolved the *Equiseti* and *Incarnatum* clades. In addition, the *TRI* phylogeny resolved isolates from different phylogenetic species (FIESC 5, FIESC 12, FIESC 14, FIESC 29 and FIESC 31) into separate and well-supported clades. Finally, the *TRI* phylogeny resolved FIESC 12 and FIESC 31 isolates as well as a FIESC 9/*F. scirpi* reference strain into a clade independent of the FIESC 5 and FIESC 14 isolates (Fig. 5, Supplementary Figs. S3-S8).

4. Discussion

In the present study, we used for the first time a polyphasic approach to characterize a collection of FIESC isolates recovered from cereals grown in Western Europe, North America and Turkey. The primary focus of this research was to expand our knowledge of FIESC fungi occurring on crops, and in particular on worldwide cereals. Published studies on this group used only a single approach, focusing on phylogenetics relationships and species identification (O'Donnell et al., 2009, 2012; Castellá and Cabañes, 2014) and overlooking mycotoxin production, or alternatively focused on mycotoxin production and overlooked ascertaining phylogenetic species identity (Marín et al., 2012; Kosiak et al., 2005).

Within the FIESC, DNA-based phylogenetic analysis resolved most of the isolates into four previously described phylogenetic species. Over half of the isolates (55%) were from the same species, FIESC 5. Our study identified a group of seven isolates that constitute a novel species lineage within FIESC. In phylogenetic analyses with the four housekeeping genes, either separately or in combination, these seven isolates were resolved as a well-supported clade that was distinct from all previously reported species within the FIESC (Figs. 1–3, Supplementary Fig. S1; Supplementary Fig. S2). The seven isolates were also resolved as an exclusive and well-supported clade in phylogenetic analysis of *TRI* genes (Fig. 5, Supplementary Figs. S3-S8), and as a distinct clade in the MALDI-TOF analysis (Fig. 4). Furthermore, three of the seven isolates exhibited a trichothecene production profile (chemotype) that was distinct from all other isolates examined in that they produced NIV in addition to DAS, NEO and FUS-X (Fig. 2). Based on these data, we propose that the seven isolates constitute a novel phylogenetic species of FIESC, which we designate as FIESC 31.

Data obtained from MLST, MALDI-TOF MS, and untargeted LC-TOF-MS(/MS) and targeted LC-MS/MS analysis provided tools for better defining biological and toxicological significance of species and haplotypes of FIESC. Moreover, species-specific patterns of proteins in the MALDI-TOF analysis confirmed the identification of the phylogenetic species, thereby providing another method to distinguish between members of FIESC and that can be used in combination with MLST data. On the other hand, the mycotoxin production profiles were variable among isolates, and were not consistent within haplotypes, phylogenetic species or groups of closely related species (Fig. 2).

To date, studies on genetic diversity within the FIESC have included single nuclear gene sequences (Kristensen et al., 2005; Marín et al., 2012; Castellá and Cabañes, 2014) or multilocus sequencing approaches (O'Donnell et al., 2009). Our polyphasic approach was applied to isolates from agricultural sources, naturally occurring in fields. The MLST analysis was not only a valuable tool for identifying a novel phylogenetic species within the complex, but also allowed us to assign isolates to four phylogenetic species (FIESC 5, FIESC 12, FIESC 14, and FIESC 29) previously described by O'Donnell et al. (2009, 2012). It is noteworthy that 94% of the cereal isolates examined here were members of the *Equiseti* clade; whereas only 6% were members of the *Incarnatum* clade. Kosiak et al. (2005) described two distinct populations of *F. equiseti*, Type 1 and Type 2, isolated from Norwegian cereals. These isolates were later identified by Castellá and Cabañes (2014) as FIESC 14 and FIESC 5, respectively. In our study, the most frequently occurring species was FIESC 5, which is in agreement with an analysis of FIESC isolates from Spanish wheat (Castellá and Cabañes, 2014) and an analysis of FIESC isolates from Sardinian soil (Balmas et al., 2010). Thus, three studies indicate that FIESC 5 is a dominant species associated with cereals or soil from cereal fields.

To our knowledge, the current study represents the first report of the occurrence of FIESC 5, FIESC 12, and FIESC 14 in Canada. In addition, as far as we are aware, this study is the first report of the occurrence of FIESC 12 on cereals; previously it was reported only from human tissues in Germany (O'Donnell et al., 2009). The four FIESC 29 isolates originated from regions with warmer climates (Italy and Mexico), similar to those isolated from wheat reported by Castellá and Cabañes' (2014). It remains to be determined whether FIESC 29 occurs more frequently in warmer than in cooler climates.

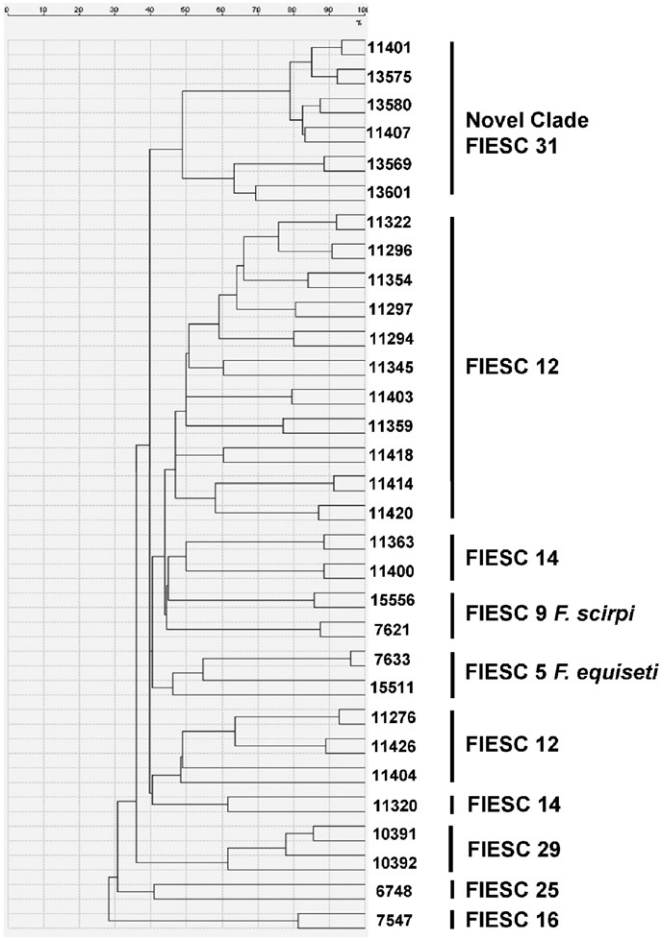

Fig. 4. Dendrogram based on single-linkage cluster analysis of mass spectra of 25 FIESC isolates obtained by MALDI-TOF MS analysis. Distances were measured as percentage of mass similarity.

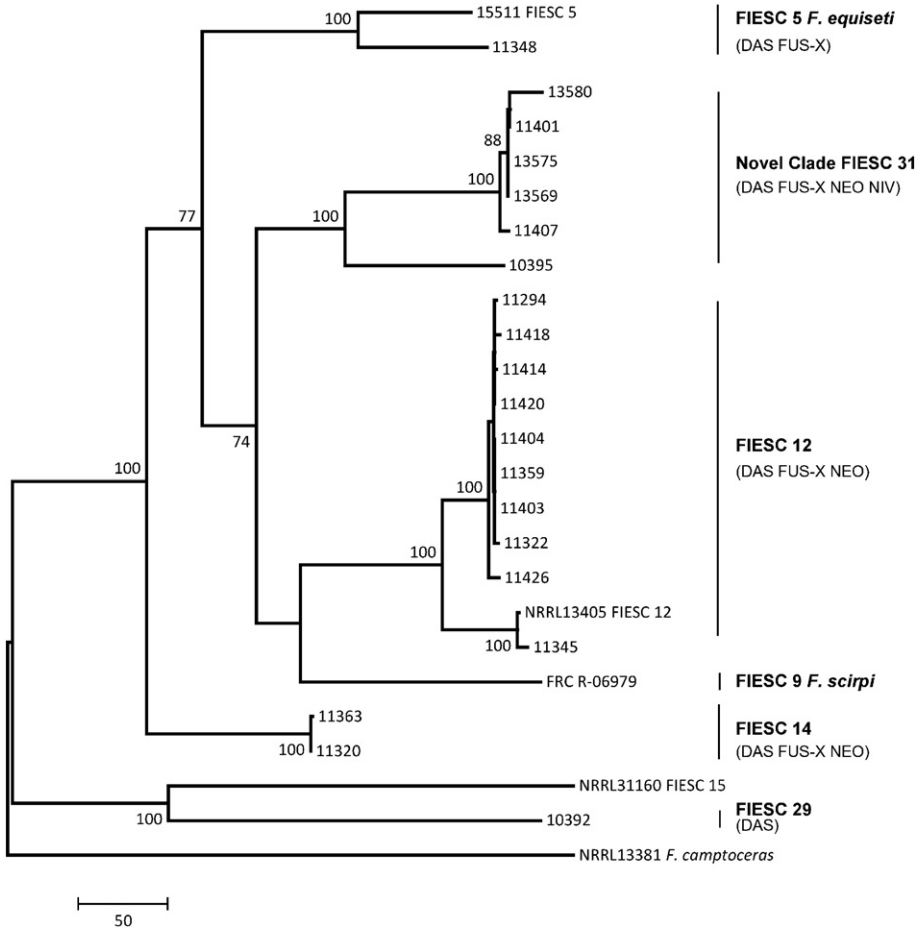

Fig. 5. Phylogenetic tree based on the combined sequences (4557 bp) for six TRI cluster genes (*TRI1*, *TRI4*, *TRI5*, *TRI8*, *TRI11*, and Gene F). Data was obtained from 20 FIESC strains. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 6 most-parsimonious trees (length = 1983) is shown. Consistency index (CI) = 0.58, Retention index (RI) = 0.79. Numbers on branches indicate bootstrap values based on 500 pseudoreplicates.

Although members of FIESC are considered moderately aggressive on cereals, some of them have been reported to produce toxins, including both type A and type B trichothecenes. However, reports on trichothecene production in the FIESC present an inconsistent picture that suggests production is variable among and/or within species. For example, Kosiak et al. (2005) reported production of high levels of type A trichothecenes (e.g. DAS and NEO), significant levels of NIV and FUS-X (type B), but no DON or 3/15-ADON (type B) in an analysis of isolates identified as *F. equiseti* collected from Norwegian cereals. In contrast, Marın et al. (2012) reported production of DON as well as NIV, NEO and DAS in an analysis of *F. equiseti* strains collected from cereals from multiple locations around the world. These differences in mycotoxin profile could partly be attributed to the influence of the conditions under which the isolates were grown for toxin production/detection. In the study of Kosiak et al. (2005), isolates were grown for two weeks in darkness on YES medium, while wheat was used as substrate by Marın et al. (2012) and the cultures were incubated for four weeks under fluorescence light. The differences could also be due to the fact that most of *F. equiseti* isolates analyzed by Kosiak et al. (2005) belong to FIESC 14 (*F. equiseti* Type I, as reported by Castellá and Cabanes, 2014) population, while the study of Marın et al. (2012) included mainly FIESC 5 (*F. equiseti* Type II, as reported by Castella et al. 2014) *F. equiseti* isolates. Results reported in the current study (isolates were grown on PDA for two weeks) are more consistent with those of Kosiak et al. (2005) in that we detected production of DAS and NEO as well as the type B trichothecenes FUS-X and NIV, but we did not detect production of DON, 3-ADON, and 15-ADON. Interestingly, the majority of *F. equiseti* isolates included in our study originated in Canada and most probably belong to FIESC 14 (Type I) based on the study of Marın et al. (2012). This finding suggests that most of the Canadian isolates reported as *F. equiseti* are FIESC 14 (Type I), while FIESC 5 (Type II) includes mainly strains that occur in Southern Europe. All isolates (except ITEM 7547) that we examined for trichothecenes produced DAS, 68% of the isolates produced FUS-X, 58% produced NEO, and 10% produced NIV. About 30% of the isolates produced only type A (DAS/NEO), while over 65% produced both type A (DAS/NEO) and B trichothecenes (FUS-X/NIV). NIV production was detected only in FIESC 31. Therefore, the results of our trichothecene production analysis provide further evidence that members of FIESC produce both type A and B trichothecenes. However, we found no evidence for production of 8-aceylated type A trichothecenes such as T-2 toxin and HT-2 toxin. As far as we are aware, this is only the second report (Marın et al., 2012) that has combined an MS-based determination of trichothecene chemotype and phylogenetic-based determination of species identity of FIESC isolates.

Recent studies (Proctor et al., 2009; McCormick et al., 2013) reported that the synteny of several *TRI* genes within the *TRI* cluster in the FIESC is different than in other trichothecene-producing lineages of *Fusarium*, such as the well studied species *F. graminearum* and *F. sporotrichioides*. In the FIESC, *TRI1* and *TRI101* are located in the trichothecene cluster; *TRI12* is absent; the *TRI3-TRI7-TRI8* region of the cluster is in the opposite orientation and located at the opposite end of the cluster; and there is a transcription factor gene (Gene F) located between *TRI5* and *TRI6*. It is not clear whether the modified arrangement of the trichothecene cluster is related to the variability in trichothecene production within the FIESC and to production of both type A and type B

trichothecenes. However, variation in trichothecene production as well as production of both type A and B trichothecenes has also been reported in *F. poae* (Thrane et al., 2004), a species that is more closely related to and has a trichothecene cluster more similar to the cluster in *F. graminearum* and *F. sporotrichioides* (O'Donnell et al., 2013).

In the current study, the *TRI*-gene phylogeny was highly concordant with the phylogeny derived from housekeeping gene sequences. However, there was poor correlation between trichothecene production profile and phylogenetic relationships within the *TRI*-gene phylogeny (Fig. 5, Supplementary Table S1; Supplementary Figs. S3-S8). Furthermore, the *TRI*-gene phylogeny suggests that FIESC 31 isolates are more closely related to FIESC 12, while the housekeeping gene phylogeny suggests that FIESC 31 is more closely related to FIESC 9 (Figs. 1, 2). The lack of amplification products most likely resulted from a primer sequence issue or some other technical problem, because two strains that did not yield a TRI5 amplicon produced trichothecenes (ITEM 10391 and ITEM 11297), and yet TRI5 is essential for trichothecene production (Alexander et al., 2009).

The proteomic profiles obtained by MALDI-TOF MS corroborated the MLST analysis, in that different phylogenetic species were resolved into different clades in both analyses. However, the correlation was not perfectly concordant because some isolates in the same phylogenetic species (according to the DNA analysis) were resolved into different clades in the MALDI-TOF MS analysis. In particular, among isolates identified as FIESC 12, ITEM 11276, ITEM 11426 and ITEM 11404 formed a cluster that was more closely related to the FIESC 14 isolate ITEM 11320. Although the MALDI-TOF data were consistent with the DNA-based phylogenetic analysis with respect to resolving FIESC 31 isolates as an exclusive lineage, the two analyses differed in placement of FIESC 31 within the *Equiseti* clade. In the MALDI-TOF analysis, FIESC 31 isolates formed a sister group to all other isolates within the *Equiseti* clade, whereas multigene species phylogenies suggest that FIESC 31 was most closely related to FIESC 9 (*F. scirpi*) and FIESC 12 (Figs. 2, 3).

In conclusion, our polyphasic analysis of diversity of FIESC from cereal resolved five phylogenetic species, including a novel species, FIESC 31. The analysis has provided the first evidence for the occurrence of FIESC 12 in cereals, FIESC 29 in maize and rice, FIESC 14 in oat and maize, and FIESC 5 in oat, maize, rice, soil, and barley. Furthermore, the data provide evidence that trichothecene production is variable within the FIESC. Further studies are required to determine the genetic basis for this variability.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2016.06.023.

Acknowledgment

This work was supported by the EU project EC KBBE-2007-222690-2 MYCORED.

References

Alexander, N.J., Proctor, R.H., McCormick, S.P., 2009. Genes, gene clusters, and biosynthesis of trichothecenes and fumonisins in *Fusarium*. Toxin Rev. 28, 198–215.

Balmas, V., Migheli, Q., Scherm, B., Garau, P., O'Donnell, K., Ceccherelli, G., Kang, S., Geiser, D.M., 2010. Multilocus phylogenetics show high levels of endemic fusaria inhabiting Sardinian soils Tyrrhenian Islands. Mycologia 102, 803–812. http://dx.doi.org/10.3852/09-201.

Brown, D.W., McCormick, S.P., Alexander, N.J., Proctor, R.H., Desjardins, A.E., 2001. A genetic and biochemical approach to study trichothecene diversity in Fusarium sporotrichioides and Fusarium graminearum. Fungal Genet. Biol. 32 (2), 121–133.

Castellá, G., Cabañes, F.J., 2014. Phylogenetic diversity of *Fusarium incarnatum-equiseti* species complex isolated from Spanish wheat. Antonie Van Leeuwenhoek 106 (2), 309–317.

Desjardins, A.E., 2006. *Fusarium* mycotoxins, chemistry, genetics, and biology. American Phytopathological Society APS Press, St. Paul Minnesota, USA.

European Commission, 2015. Agricultural and rural development, short-term outlook. http://ec.europa.eu/agriculture/markets-and-prices/short-term-outlook/index_en.htm- (Accessed 10/07/2015).

FAOSTAT, 2013. Food and agriculture Organization of the United Nations, FAO statistical database. http://faostat.fao.org/site/291/default.aspx (Accessed 19/06/2013).

Lambotte, Fautrey, 1894. Rev. Mycol. (Toulouse) 16 (n. 63), 111.

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39, 783–791.

Geiser, D.M., del Mar Jiménez-Gasco, M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T.J., Zhang, N., Kuldau, G.A., O'Donnell, K., 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. Eur. J. Plant Pathol. 110, 473–479.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Int. J. Food Microbiol. 61, 1323–1330.

Grovey, J.F., 2007. The trichothecenes and their biosynthesis. In: Herz, W., Falk, H., Kirby, G.W. (Eds.), Progress in the Chemistry of Organic Natural Products. Springer-Verlag, Vienna, pp. 63–130.

Jurado, M., Vázquez, C., Patiño, B., González-Jaén, M.T., 2005. PCR detection assay for the trichothecene-producing species *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium poae*, *Fusarium equiseti* and *Fusarium sporotrichioides*. Syst. Appl. Microbiol. 28, 562e568.

Jurado, M., Vázquez, C., Callejas, C., González-Jaén, M.T., 2006. Occurrence and variability of mycotoxigenic *Fusarium* species associated to wheat and maize in the south of Spain. Mycotoxin Res. 22, 87e91.

Kosiak, E.B., Holst-Jensen, A., Rundberget, T., Gonzalez-Jaen, M.T., Torp, M., 2005. Morphological, chemical and molecular differentiation of *Fusarium equiseti* isolated from Norwegian cereals. Int. J. Food Microbiol. 99, 195–206.

Kristensen, R., Torp, M., Kosiak, B., Holst-Jensen, A., 2005. Phylogeny and toxigenic potential is correlated in Fusarium species as revealed by partial translation elongation factor 1 alpha gene sequences. Mycol. Res. 109, 173–186. http://dx.doi.org/10.1017/S0953756204002114.

Leslie, J.F., Summerell, B.A., 2006. *Fusarium* laboratory workshops, a recent history. Mycotoxin Res. 22, 73–74.

Liu, Y.L., Whelen, S., Hall, B.D., 1999. Phylogenetic relationships among ascomycetes, evidence from an RNA polymerase II subunit. Mol. Bio. Evol. Soc. 16, 1799–1808.

Ma, L.J., Geiser, D.M., Proctor, R.H., Rooney, A.P., O'Donnell, K., Trail, F., Gardiner, D.M., Manners, J.M., Kazan, K., 2013. Fusarium Pathogenomics. Annu. Rev. Microbiol. 67, 399–416.

Maier, F.J., Miedaner, T., Hadeler, B., Felk, A., Salomon, S., Lemmens, M., Kassner, H., Schäfer, W., 2006. Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (Tri5) gene in three field isolates of different chemotype and virulence. Mol. Plant Pathol. 7, 449–461.

Marın, P., Moretti, A., Ritieni, A., Jurado, M., Vazquez, C., Gonzalez, J.M.T., 2012. Phylogenetic analysis and toxigenic profiles of *Fusarium equiseti* and *Fusarium acuminatum* isolated from cereals from southern Europe. Food Microbiol. 31, 229–237.

Marinach-Patrice, C., Lethuillier, A., Marly, A., Brossas, J.Y., Gene, J., Symoens, F., Datry, A., Guarro, J., Mazier, D., Hennequin, C., 2009. Use of mass spectrometry to identify clinical *Fusarium* isolates. Clin. Microbiol. Infect. 15, 634–642.

McCormick, S.P., 2013. Microbial detoxification of mycotoxins. J. Chem. Ecol. 39, 907–918.

McCormick, S.P., Alexander, N.J., Proctor, R.H., 2013. Trichothecene triangle: toxins, genes, and plant disease. Phytochemicals, Plant Growth, and the Environment. Springer, pp. 1–17.

Moretti, A., Logrieco, A., Bottalico, A., Ritieni, A., Fogliano, V., Randazzo, G., 1996. Diversity in beauvericin and fusaproliferin production by different populations of *Gibberella fujikuroi Fusarium* section Liseola. Sydowia 48, 44–56.

Moretti, A., Susca, A., Mulé, G., Logrieco, A.F., Proctor, R.H., 2013. Molecular biodiversity of mycotoxigenic fungi that threaten food safety. Int. J. Food Microbiol. 167, 57–66.

O'Donnell, K., Cigelnik, E., Nirenberg, H.I., 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90, 465–493.

O'Donnell, K., Sarver, B.A., Brandt, M., Chang, D.C., Noble-Wang, J., Park, B.J., Sutton, D.A., Benjamin, L., Lindsley, M., Padhye, A., Geiser, D.M., Ward, T.J., 2007. Phylogenetic diversity and microsphere array-based genotyping of human pathogenic fusaria, including isolates from the multistate contact lens-associated US keratitis outbreaks of 2005 and 2006. J. Clin. Microbiol. 45 (7), 2235–2248.

O'Donnell, K., Sutton, D.A., Rinaldi, M.G., Gueidan, C., Crous, P.W., Geiser, D.M., 2009. A novel multi-locus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F. equiseti* and *F. chlamydosporum* species complexes within the U.S. J. Clin. Microbiol. 47, 3851–3861.

O'Donnell, K., Sutton, D.A., Rinaldi, M.G., Sarver, B.A.J., Balajee, S.A., Schroers, H.J., Summerbell, R.C., Robert, V.A.R., Crous, P.W., Zhang, N., Aoki, T., Jung, K., Park, J., Lee, Y.H., Kang, S., Park, B., Geiser, D.M., 2010. Internet-accessible DNA sequence database for identifying fusaria from human and animal infections. J. Clin. Microbiol. 48, 3708e3718.

O'Donnell, K., Humber, R.A., Geiser, D.M., Kang, S., Park, B., Robert, V.A., Crous, P.W., Johnston, P.R., Aoki, T., Rooney, A.P., Rehner, S.A., 2012. Phylogenetic diversity of insecticolous fusaria inferred from multilocus DNA sequence data and their molecular identification via FUSARIUM-ID and FUSARIUM MLST. Mycologia 104, 427e445.

O'Donnell, K., Nirenberg, H.I., Aoki, T., Cigelnik, E., 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex, detection of additional phylogenetically distinct species. Mycoscience 41, 61–78.

O'Donnell, K., Rooney, A.P., Proctor, R.H., Brown, D.W., McCormick, S.P., Ward, T.J., Frandsen, R.J., Lysøe, E., Rehner, S.A., Aoki, T., Robert, V.A., Crous, P.W., Groenewald, J.Z., Kang, S., Geiser, D.M., 2013. Phylogenetic analyses of RPB1 and RPB2 support a middle cretaceous origin for a clade comprising all agriculturally and medically important Fusaria. Fungal Genet. Biol. 52, 20–31.

Proctor, R.H., Brown, D.W., Plattner, R.D., Desjardins, A.E., 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genet. Biol. 38, 237–249.

Proctor, R.H., McCormick, S.P., Alexander, N.J., Desjardins, A.E., 2009. Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus *Fusarium*. Mol. Microbiol. 74, 1128–1142.

Ranque, S., Normand, A.C., Cassagne, C., Murat, J.B., Bourgeois, N., Dalle, F., Gari-Toussaint, M., Fourquet, P., Hendrickx, M., Piarroux, R., 2014. MALDI-TOF mass spectrometry identification of filamentous fungi in the clinical laboratory. Mycoses 57, 135–140. http://dx.doi.org/10.1111/myc.12115.

Rocha, O., Ansari, K., Doohan, F.M., 2005. Effects of trichothecene mycotoxins on eukaryotic cells, a review. Food Addit. Contam. 22 (4), 369–378.

Rodrigues, P., Santos, C., Venâncio, A., Lima, N., 2011. Species identification of *Aspergillus* section Flavi isolates from Portuguese almonds using phenotypic, including MALDI-TOF ICMS, and molecular approaches. J. Appl. Microbiol. 111, 877–892.

Saccardo, P.A., 1886. Sylloge Hyphomycetum. Sylloge Fungorum 4, 1–807.

Santos, C., Ventura, J.A., Costa, H., Fernandes, P.M.B., Lima, N., 2015. MALDI-TOF MS to identify the pineapple pathogen *Fusarium guttiforme* and its antagonist *Trichoderma asperellum* on decayed pineapple. Trop. Plant Pathol. http://dx.doi.org/10.1007/s40858-015-0027-7 (On-line).

Short, D.P.G., O'Donnell, K., Zhang, N., Juba, J.H., Geiser, D.M., 2011. Widespread occurrence of diverse human pathogenic types of the fungus *Fusarium* detected in plumbing drains. J. Clin. Microbiol. 49, 4264–4272.

Silva, F.C., Chalfoun, S.M., Batista, L.R., Santos, C., Lima, N., 2015. Use of a polyphasic approach including MALDI-TOF MS for identification of *Aspergillus* section Flavi strains isolated from food commodities in Brazil. Ann. Microbiol. http://dx.doi.org/10.1007/s13213-015-531 1050-0 (on-line).

Susca, A., Mulè, G., Mastropasqua, L., Paciolla, C., 2015. Biodiversità molecolare di Fusarium semitectum isolati da Hylocereus undatus e altri ospiti. Inf. Bot. Ital. 47 (2), 291–313.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5, molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL 541 W, improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Thrane, U., Adler, A., Clasen, P., Galvano, F., Langseth, W., Lew, H., Logrieco, A., Nielsen, K.F., Ritieni, A., 2004. Diversity in metabolite production by *Fusarium langsethiae*, *Fusarium poae*, and *Fusarium sporotrichioides*. Int. J. Food Microbiol. 95, 257–266.

van Diepeningen, A.D., Brankovics, B., Iltes, J., van der Lee, T.A.J., Waalwijk, C., 2015. Diagnosis of *Fusarium* infections, approaches to identification by the clinical mycology laboratory. Curr. Fungal Infect. Rep. 9, 135–143.

Ward, T.J., Bielawski, J.P., Kistler, H.C., Sullivan, E., O'Donnell, K., 2002. Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. Proc. Natl. Acad. Sci. 99, 9278–9283.

Watanabe, M., Yonezawa, T., Sugita-Konishi, Y., Kamata, Y., 2013. Utility of the phylotoxigenic relationships among trichothecene-producing *Fusarium* species for predicting their mycotoxin-producing potential. Food Addit. Contam. A 30 (8), 1370–1381.