



Citation: S. Matić, G. Tabone, V. Guarnaccia, M.L. Gullino, A. Garibaldi (2020) Emerging leafy vegetable crop diseases caused by the *Fusarium incarnatum-equiseti* species complex. *Phytopathologia Mediterranea* 59(2): 303-317. DOI: 10.14601/Phyto-10883

Accepted: June 29, 2020

Published: August 31, 2020

Copyright: © 2020 S. Matić, G. Tabone, V. Guarnaccia, M.L. Gullino, A. Garibaldi. This is an open access, peer-reviewed article published by Firenze University Press (<http://www.fupress.com/pm>) and distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Juan A. Navas-Cortes, Spanish National Research Council (CSIC), Cordoba, Spain.

Research Papers

Emerging leafy vegetable crop diseases caused by the *Fusarium incarnatum-equiseti* species complex

SLAVICA MATIĆ^{1,2,*}, GIULIA TABONE¹, VLADIMIRO GUARNACCIA^{1,2}, MARIA LODOVICA GULLINO^{1,2}, ANGELO GARIBALDI¹

¹ AGROINNOVA – Centre of Competence for the Innovation in the Agro-environmental Sector, University of Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy

² Dept. Agricultural, Forestry and Food Sciences (DISAFA), University of Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy.

*Corresponding author: slavica.matic@unito.it

Summary. *Fusarium equiseti*, a member of the *Fusarium incarnatum-equiseti* species complex (FIESC), has recently been reported in Italy as the causal agent of a leaf spot diseases on previously unrecorded plant hosts. This emerging disease has affected leafy vegetable hosts including lettuce, lamb's lettuce, wild rocket, cultivated rocket, spinach and radish, and has caused symptoms that have not been previously described on those plants. Fifty-two fungal isolates obtained from symptomatic plants and different plant organs were identified according to their morphology as belonging to the FIESC. The present study aimed to characterize these isolates by identifying their FIESC phylogenetic species, and to evaluate their pathogenicity and host ranges. Six phylogenetically different species of FIESC were identified using MLST analyses of four loci (*tef1*, *cmdA*, *tub2*, and *IGS*). Most of the isolates were found to belong to *F. compactum* or *F. clavum*, while the other four FIESC species were represented by only a few isolates. All the fungal isolates were capable of inducing leaf spot diseases as single isolates with fulfilling Koch's postulates for these fungi. The intraspecific diversity of the FIESC, the seed-originated isolates of four FIESC identified species, and enhanced range of experimental hosts were observed in the FIESC emerging diseases of these vegetable hosts in Italy. Strict seed inspection procedures, and suitable alteration of environmentally friendly fungicides and biological control agents should achieve efficient management of the FIESC leaf spot diseases on vegetable crops, and prevent further spread of these pathogens to new hosts and new geographical areas.

Keywords. FIESC leaf spot diseases, multi-locus sequence typing, phylogenetic analyses, pathogenicity assays.

INTRODUCTION

Members of the *Fusarium incarnatum-equiseti* species complex (FIESC) are generally associated with diseases of agricultural crops, particularly cereals (Kristensen *et al.*, 2005; Castellá and Cabañes, 2014; Villani *et al.*, 2016; Maryani *et al.*, 2019; Wang *et al.*, 2019). Like many *Fusarium* species com-

plexes, the FIESC contains plant pathogens and species that cause human opportunistic infections, generally of immunocompromised individuals (O'Donnell *et al.*, 2009; Riddell *et al.*, 2010; van Diepeningen *et al.*, 2015; Santos *et al.*, 2019). Species in the FIESC also have the ability to produce mycotoxins, including type A and type B trichothecenes, fusaric acid, and the estrogenic mycotoxin zearalenone, posing potential risks for human and animal health (Langseth *et al.*, 1999; Desjardins, 2006; Goswami *et al.*, 2008; Botha *et al.*, 2014; Villani *et al.*, 2016; Shi *et al.*, 2017; Avila *et al.*, 2019).

FIESC species have pronounced homoplasious morphological characteristics and cryptic speciation (O'Donnell *et al.*, 2009; Avila *et al.*, 2019; Wang *et al.*, 2019). Multi-locus sequence typing (MLST) based on modern taxonomic concepts is therefore necessary for precise identification of FIESC species. The FIESC has been resolved into two clades, the *Equiseti* clade and the *Incaratum* clade, containing more than 40 phylogenetically different species, and was separated from the phylogenetically close *F. camptoceras* species complex (FCAMSC) (O'Donnell *et al.*, 2009; Short *et al.*, 2011; Villani *et al.*, 2016, 2019; O'Donnell *et al.*, 2018; Avila *et al.*, 2019; Hartman *et al.*, 2019; Maryani *et al.*, 2019; Santos *et al.*, 2019; Wang *et al.*, 2019; Xia *et al.*, 2019). Each of these phylogenetic species is assigned an alphanumeric designation, and almost all species have assigned Latin binomial names, with exception of the species FIESC 8, FIESC 22, FIESC 27, FIESC 30, FIESC 31 and FIESC 32 (Xia *et al.*, 2019).

FIESC members are common soil inhabiting fungi which colonize the roots of plants and injured plant tissue. They occur very widely in cool to dry and warm regions (Leslie and Summerell, 2006). FIESC species are sporadic causal agents of plant diseases, including wheat head blight, maize ear and stalk rot, rice bakanae disease, asparagus crown and root rot, and sorghum head blight (Logrieco *et al.*, 2003; Amatulli *et al.*, 2010; Kelly *et al.*, 2017). This complex has also been reported in Italy as an endophyte on chicory and fennel plants (D'Amico *et al.*, 2008). The majority of FIESC reports have been based on morphological observations or sequencing of one gene, without precise species identification by means of MLST. Some MLST studies have reported the presence of different FIESC phylogenetic species on cereal grains, although no data have been provided on the symptomatic status of the grains during sampling (O'Donnell *et al.*, 2009; Villani *et al.*, 2016; Avila *et al.*, 2019). These reports document major presences of *F. clavum* (FIESC 5), *F. flagelliforme* (FIESC 12), *F. equiseti* (FIESC 14a), *F. citri* (FIESC 29), and not yet assigned species (novel FIESC) in cereals from different Euro-

pean countries (Villani *et al.*, 2016; 2019), and *F. hainanense* (FIESC 26) and novel FIESC phylogenetic species in cereals from Brazil (Avila *et al.*, 2019). *Fusarium sulawesiense* (FIESC 16) and *F. tanahbumbuense* (FIESC 24) are predominant species on rice stubble in China, based on the *tef1* phylogeny (Yang *et al.*, 2018).

Unlike the frequent reports of FIESC in cereals, this species complex has recently been reported as the causal agent of different plant diseases in distinct geographical areas. Leaf spot, caused by FIESC, has been observed in different Italian areas on leafy vegetable hosts, including lettuce, lamb's lettuce, cultivated rocket, wild rocket, spinach and radish, grown in open fields and under intensive cultivation (Garibaldi *et al.*, 2011; 2015; 2016a; 2017). Leafy vegetables are highly susceptible to FIESC fungi at high temperatures (25 to 35°C), and the recent increased temperature climate scenario are probably particularly favourable for spread of these pathogens in Italy (Garibaldi *et al.*, 2016b; Gullino *et al.*, 2017a; 2019). There is also risk of a further spread of these pathogens to new geographical areas and hosts as they are transmitted by seeds (Gilardi *et al.*, 2017).

Additional newly described diseases associated with FIESC have also been reported on: onion in Serbia (Ignjatov *et al.*, 2015), bell pepper in Trinidad (Ramdial *et al.*, 2017), mustard and peanut in India (Prasad *et al.*, 2017; Thirumalaisamy *et al.*, 2019), *Nopalea cochenillifera* in Brasil (Santiago *et al.*, 2018), cotton in Pakistan (Chohan and Abid, 2019), banana in Indonesia (Maryani *et al.*, 2019), and various plant hosts in China (Hu *et al.*, 2018; Cao *et al.*, 2019; Wang *et al.*, 2019; Jiang *et al.*, 2019). These reports provide evidence that different FIESC species are the causal agents of plant disease.

The objective of the present study was to perform molecular characterization of fungal isolates identified as FIESC species in previous studies, using morphological observation or single gene sequencing. These isolates have been found to be the causal agents of leaf spot diseases on leafy vegetables originating from six plant hosts. The specific objectives were: (i) to identify the FIESC species of 52 fungus isolates associated with leaf spot diseases, and evaluate their genetic diversity by means of MLST analysis, ii) to evaluate their disease severity through pathogenicity assays, and (iii) to identify any possible new hosts by performing cross inoculation tests.

MATERIALS AND METHODS

Fungus isolates

Fifty-two fungus isolates from the Agroinnova collection (Grugliasco, Italy), previously identified as FIESC

on the basis of their morphological characteristics, were used in this study. Four of the isolates were also identified by *tef1* sequencing as *F. equiseti* (Garibaldi *et al.*, 2011; 2015; 2016a; 2017). Approximately 95% of the isolates maintained in the Agroinnova collection, originating from leafy vegetable hosts, were analyzed during this study. These isolates were collected from six leafy vegetable hosts (lettuce, lamb's lettuce, spinach, wild rocket, cultivated rocket or radish) (Table 1), from 2011 to 2018, from greenhouses in different locations (Northern and Southern Italy).

DNA extraction from fungi, PCR and sequencing

Total DNA was extracted using the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek) according to the manufacturer's protocol. One hundred mg of fresh fungal mycelium grown on PDA plates was used for each isolate. Portions of the following genes were PCR amplified: translation elongation factor 1 α (*tef1*; O'Donnell *et al.*, 1998), calmodulin (*cmdA*; Carbone and Kohn, 1999; Groenewald *et al.*, 2013), β -tubulin (*tub2*; Glass and Donaldson, 1995), and the intergenic spacer region of the rDNA (IGS; Appel and Gordon, 1995). All the primer sets used for PCR are listed in Supplementary Table 1. The PCR products were purified using a QIAquick PCR purification kit (Qiagen) in accordance with the manufacturer's instructions, and were sequenced in both directions at the BMR Genomics Centre (Padua, Italy). The obtained sequences were deposited in the NCBI GenBank database under the following accession numbers: MK922189-MK922238 for *tef1*, MK937861-MK937912 for *cmdA*, MN078811-MN078862 for *tub2*, and MN078863-MN078914 for IGS (Table 1).

Sequence analyses of isolates

The *tef1* sequences of 52 isolates were aligned with the sequences available at the Fusarium-ID database, and all *tef1* sequences shared high similarity (99-100%) with the FIESC species (Geiser *et al.*, 2004). To place the studied isolates within the correct phylogenetic species, phylogenetic analyses were performed on a concatenated dataset of two loci (*tef1* and *cmdA*) including 59 reference sequences of 33 distinct FIESC phylogenetic species (Table 1; O'Donnell *et al.*, 2009; 2012; Villani *et al.*, 2016; Gebru *et al.*, 2019; Maryani *et al.*, 2019; Torbati *et al.*, 2019; Wang *et al.*, 2019; Xia *et al.*, 2019). The additional phylogenetic analyses consisted of the single locus and concatenated sequences (*tef1*, *cmdA*, *tub2* and

IGS), which were performed with 52 study isolates and eight FIESC reference strains (*F. clavum* ITEM 11348, *F. flagelliforme* ITEM 11294, *F. equiseti* CS581 and ITEM 11363, *F. irregulare* NRRL 31160, *F. citri* MOD1 FUNGI17 and ITEM 10392, and novel FIESC ITEM 11401). This was to confirm the assignment of the study isolates to the determined phylogenetic species. The eight reference strains were the only strains having the IGS sequence available at NCBI database, beside the *tef1*, *cmdA*, and *tub2* sequences, due to availability of their whole-genome sequence data (Gebru *et al.*, 2019; Villani *et al.*, 2019). Few additional reference strains were included in the *tub2* phylogenetic analyses (Table 1). *Fusarium concolor* (NRRL 13459) sequences were used as the outgroup.

Phylogenetic analyses were carried out on the bases of the Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI). The MP analysis was carried out using Phylogenetic Analysis Using Parsimony (PAUP. v. 4.0b10; Swofford, 2003) for the concatenated dataset of *tef1* and *cmdA*. Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. Tree bisection-reconnection was used, with the branch swapping option set on 'best trees' only, with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated for parsimony, and the bootstrap analyses (Hillis and Bull, 1993) were based on 1000 replications. The BIs for all the analyses were conducted using MrBayes v. 3.2.5 (Ronquist *et al.*, 2012) to generate a phylogenetic tree under optimal criteria per partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The heating parameter was set to 0.2 and trees were sampled every 1000 generations. The analyses ceased once the average standard deviation of split frequencies was < 0.01. The best evolutionary model for each partition was determined using MrModeltest v. 2.3 (Nylander, 2004), and incorporated into the analysis. The ML analyses were performed with 1000 bootstrap replications, using MEGA software 7 (Kumar *et al.*, 2016) for the single locus analyses.

Morphological characterization of isolates

Monoconidium cultures, stored as conidium suspensions in a 30% glycerol solution at -80°C, were used for each isolate. The cultures were then grown on potato dextrose agar plates (PDA, Merck®) amended with streptomycin (Applichem) at 50 mg L⁻¹. Microscope

Table 1. List of *Fusarium* isolates used in the study with their corresponding origin, plant host and isolation source.

No	Isolate	Host	Locality	Country	Tissue	Clade	Species	Species complex	<i>tefi</i>	<i>cmdA</i>	<i>tub2</i>	IGS
1	Feq 12/14	Lettuce	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	KT149290*	MK937861	MN078811	MN078863
2	Zarina	Lettuce	Veneto	Italy	Stem	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922189	MK937862	MN078812	MN078864
3	LBV Feq1	Lettuce	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922190	MK937863	MN078813	MN078865
4	LBV Feq2	Lettuce	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922191	MK937864	MN078814	MN078866
5	LBV Feq3	Lettuce	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922192	MK937865	MN078815	MN078867
6	LBV Feq4	Lettuce	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922193	MK937866	MN078816	MN078868
7	LBV Feq5	Lettuce	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922194	MK937867	MN078817	MN078869
8	LBV Feq6	Lettuce	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. ipomoeae</i>	FIESC 1	MK922195	MK937868	MN078818	MN078870
9	LBV Feq6R	Lettuce	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922196	MK937869	MN078819	MN078871
10	LBV Feq7	Lettuce	Unknown	Italy	Seed	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	MK922197	MK937870	MN078820	MN078872
11	LBV Feq7R	Lettuce	Unknown	Italy	Seed	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	MK922198	MK937871	MN078821	MN078873
12	LBV Feq8	Lettuce	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922199	MK937872	MN078822	MN078874
13	LBV Feq8R	Lettuce	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922200	MK937873	MN078823	MN078875
14	Feq A	Lamb's lettuce	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922201	MK937874	MN078855	MN078876
15	Feq B	Lamb's lettuce	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922202	MK937875	MN078824	MN078877
16	Feq C	Lamb's lettuce	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922203	MK937876	MN078825	MN078878
17	Feq 7/10	Cultivated rocket	Piedmont	Italy	Leaf	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922204	MK937877	MN078826	MN078879
18	5A, Feq 1/15	Wild rocket	Campania	Italy	Leaf	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	MK922205	MK937878	MN078827	MN078880
19	Feq 1/14	Wild rocket	Campania	Italy	Leaf	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	MK922206	MK937879	MN078828	MN078881
20	Feq 2/14 c1	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922207	MK937880	MN078856	MN078882
21	Feq 2/14 c3	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. lacertarum</i>	FIESC 4	MK922208	MK937881	MN078857	MN078883
22	Feq 2/14 c6	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. longifundum</i>	FIESC 11	MK922209	MK937882	MN078858	MN078884
23	Feq 6/14	Wild rocket	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922210	MK937883	MN078829	MN078885
24	Feq 7/14 M	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922211	MK937884	MN078830	MN078886
25	Feq 8/14 M	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922212	MK937885	MN078831	MN078887
26	Feq 9/14 M	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922213	MK937886	MN078832	MN078888
27	Feq 13/14	Wild rocket	Campania	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922214	MK937887	MN078833	MN078889
28	Feq 16/14 M	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922215	MK937888	MN078834	MN078890
29	Feq 21/14.7	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922216	MK937889	MN078835	MN078891
30	Feq 21/14.8	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK922217	MK937890	MN078836	MN078892
31	Feq 21/14.9	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922218	MK937891	MN078837	MN078893
32	Feq 21/14.12	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922219	MK937892	MN078838	MN078894
33	Feq 28/14 M	Wild rocket	Veneto	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922220	MK937893	MN078859	MN078895
34	Feq5	Wild rocket	Unknown	Italy	Seed	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922221	MK937894	MN078839	MN078896
35	Feq 1A	Spinach	Lombardy	Italy	Root	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK922222	MK937895	MN078860	MN078897

(Continued)

Table 1. (Continued).

No	Isolate	Host	Locality	Country	Tissue	Clade	Species	Species complex	<i>tefl</i>	<i>cmdA</i>	<i>tub2</i>	IGS
36	Feq 1AR	Spinach	Lombardy	Italy	Root	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK9222223	MK937896	MN078861	MN078898
37	Feq 2	Spinach	Lombardy	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK9222224	MK937897	MN078862	MN078899
38	Feq 3	Spinach	Lombardy	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK9222225	MK937898	MN078840	MN078900
39	Feq 3R	Spinach	Lombardy	Italy	Leaf	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK9222226	MK937899	MN078841	MN078901
40	Feq 52A	Spinach	Lombardy	Italy	Root	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK9222227	MK937900	MN078842	MN078902
41	Feq 11/14	Spinach	Piedmont	Italy	Stem	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK9222228	MK937901	MN078843	MN078903
42	Feq 11/14R	Spinach	Piedmont	Italy	Stem	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK9222229	MK937902	MN078844	MN078904
43	B63	Spinach	-	Italy	-	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK9222230	MK937903	MN078845	MN078905
44	SPI	Spinach	-	Italy	Stem	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK9222231	MK937904	MN078846	MN078906
45	Feq 1/18	Spinach	Piedmont	Italy	Stem	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK9222232	MK937905	MN078847	MN078907
46	Feq 2/18	Spinach	Piedmont	Italy	Stem	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK9222233	MK937906	MN078848	MN078908
47	Feq 3/18	Spinach	Piedmont	Italy	Stem	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK9222234	MK937907	MN078849	MN078909
48	Feq 4/18	Spinach	Piedmont	Italy	Stem	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK9222235	MK937908	MN078850	MN078910
49	Feq 5/18	Spinach	Piedmont	Italy	Stem	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK9222236	MK937909	MN078851	MN078911
50	Feq 7/18	Spinach	Piedmont	Italy	Stem	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MK9222237	MK937910	MN078852	MN078912
51	Feq 8/18	Spinach	Piedmont	Italy	Root	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	MK9222238	MK937911	MN078853	MN078913
52	IT26	Radish	Piedmont	Italy	Leaf	<i>Equiseti</i>	<i>F. ipomoeae</i>	FIESC 1	KY688192	MK937912	MN078854	MN078914
53	CBS 135762	<i>Miscanthus giganteus</i>	-	USA	-	<i>Equiseti</i>	<i>F. ipomoeae</i>	FIESC 1	MN170478	MN170344	-	-
54	CBS 140909	Tomato	Vladistok	Russia	Fruit	<i>Equiseti</i>	<i>F. ipomoeae</i>	FIESC 1	MN170479	MN170345	-	-
55	NRRL 43637	Dog	-	PA, USA	-	<i>Equiseti</i>	<i>F. ipomoeae</i>	FIESC 1	GQ505564	GQ505575	-	-
56	NRRL 36401	Cotton	Maputo	Mozambique	-	<i>Equiseti</i>	<i>F. duofalcatisporum</i>	FIESC 2	GQ505651	GQ505563	-	-
57	NRRL 36448 ^T	Common bean	Nile Province	Sudan	Seed	<i>Equiseti</i>	<i>F. duofalcatisporum</i>	FIESC 2	GQ505652	GQ505564	-	-
58	NRRL 36323 ^T	Cotton yarn	-	England	-	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	GQ505648	GQ505560	-	-
59	NRRL 28029	Human	-	CA, USA	Eye	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	GQ505602	GQ505514	-	-
60	NRRL 36318	Unknown	-	Unknown	-	<i>Equiseti</i>	<i>F. compactum</i>	FIESC 3	GQ505646	GQ505558	-	-
61	NRRL 36123	Unknown	-	Unknown	-	<i>Equiseti</i>	<i>F. lacertarum</i>	FIESC 4	GQ505643	GQ505555	-	-
62	NRRL 20423 ^T	Lizard	-	India	Skin	<i>Equiseti</i>	<i>F. lacertarum</i>	FIESC 4	GQ505593	GQ505505	-	-
63	ITEM 10393	Wheat	-	Italy	-	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	LN901566	LN901586	-	-
64	CBS 126202 ^T	Soil	Maltahohe	Namibia	-	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MN170456	MN170322	-	-
65	CBS 131015	<i>Phalaris minor</i>	Aziz abad	Iran	-	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MN170458	MN170324	-	-
66	CBS 131448	<i>Secale montanum</i>	Parsabad	Iran	-	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	MN170459	MN170325	-	-
67	NRRL 34037	Human	-	CO, USA	Abscess	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	GQ505638	GQ505550	-	-
68	NRRL 34032	Human	-	TX, USA	Abscess	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	GQ505635	GQ505547	-	-
69	ITEM 7633	-	-	-	-	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	-	-	LN901628	-

(Continued)

Table 1. (Continued).

No	Isolate	Host	Locality	Country	Tissue	Clade	Species	Species complex	<i>tefl</i>	<i>cmdA</i>	<i>tub2</i>	IGS
70	ITEM 10787	Wheat	-	Spain	-	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	-	-	LN901620	-
71	ITEM 10789	Wheat	-	Spain	-	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	-	-	LN901621	-
72	ITEM 11348	Oat	-	Canada	-	<i>Equiseti</i>	<i>F. clavum</i>	FIESC 5	QGE000000000	QGE000000000	QGE000000000	QGE000000000
73	NRRL 43638 ^T	Manatee	-	FL, USA	-	<i>Equiseti</i>	<i>F. brevicaudatum</i>	FIESC 6	GQ505665	GQ505576	-	-
74	NRRL 32997	Human	-	CO, USA	Toenail	<i>Equiseti</i>	<i>F. arcuatisporum</i>	FIESC 7	GQ505624	GQ505536	-	-
75	NRRL 5537	Fescue hay	-	MO, USA	-	<i>Equiseti</i>	<i>Fusarium</i> sp.	FIESC 8	GQ505588	GQ505500	-	-
76	NRRL 36478 ^{NT}	Pasture soil	-	Australia	-	<i>Equiseti</i>	<i>F. scirpi</i>	FIESC 9	GQ505654	GQ505566	-	-
77	ITEM 7621	-	-	-	-	<i>Equiseti</i>	<i>F. scirpi</i>	FIESC 9	-	-	LN901632	-
78	NRRL 26922	Soil	-	France	-	<i>Equiseti</i>	<i>F. neoscirpi</i>	FIESC 9n	GQ505601	GQ505513	-	-
79	CBS 131777 ^T	<i>Triticum</i> sp.	-	Iran	-	<i>Equiseti</i>	<i>F. croceum</i>	FIESC 10	MN170463	MN170329	-	-
80	CBS 131788	<i>Triticum</i> sp.	-	Iran	-	<i>Equiseti</i>	<i>F. croceum</i>	FIESC 10	MN170464	MN170330	-	-
81	NRRL 36372	Air	-	Netherlands	-	<i>Equiseti</i>	<i>F. longifundum</i>	FIESC 11n	GQ505649	GQ505561	-	-
82	NRRL 36269	<i>Pinus nigra</i>	-	Croatia	Seedling	<i>Equiseti</i>	<i>F. flagelliforme</i>	FIESC 12	GQ505645	GQ505557	-	-
83	NRRL 36392	Unknown plant	-	Germany	Seedling	<i>Equiseti</i>	<i>F. flagelliforme</i>	FIESC 12	GQ505650	GQ505562	-	-
84	ITEM 11294	Oat	-	Canada	-	<i>Equiseti</i>	<i>F. flagelliforme</i>	FIESC 12	LN901571	QHHI0000000	QHHI0000000	QHHI0000000
85	ITEM 11296	Oat	-	Canada	-	<i>Equiseti</i>	<i>F. flagelliforme</i>	FIESC 12	-	-	LN901622	-
86	NRRL 43635 ^T	Horse	-	NE, USA	-	<i>Equiseti</i>	<i>F. gracilipes</i>	FIESC 13	GQ505662	GQ505573	-	-
87	NRRL 26419 ^T	Soil	-	Germany	-	<i>Equiseti</i>	<i>F. equiseti</i>	FIESC 14a	GQ505599	GQ505511	-	-
88	NRRL 36136	Unknown	-	Unknown	-	<i>Equiseti</i>	<i>F. equiseti</i>	FIESC 14a	GQ505644	GQ505556	-	-
89	ITEM 10675	-	-	-	-	<i>Equiseti</i>	<i>F. equiseti</i>	FIESC 14a	-	-	LN901623	-
90	ITEM 13585	Maize	-	Netherlands	-	<i>Equiseti</i>	<i>F. equiseti</i>	FIESC 14a	-	-	LN901624	-
91	ITEM 11363	Oat	-	Canada	-	<i>Equiseti</i>	<i>F. equiseti</i>	FIESC 14a	LN901574	QGE000000000	QGE000000000	QGE000000000
92	CS581	Wheat	-	Australia	-	<i>Equiseti</i>	<i>F. equiseti</i>	FIESC 14a	MTPY000000000	MTPY000000000	MTPY000000000	MTPY000000000
93	NRRL 43636	Dog	-	TX, USA	-	<i>Equiseti</i>	<i>F. toxicum</i>	FIESC 14b	GQ505663	GQ505574	-	-
94	CBS 219.63	Soil	-	Germany	-	<i>Equiseti</i>	<i>F. toxicum</i>	FIESC 14b	MN170507	MN170373	-	-
95	CBS 119880	Unknown	-	Unknown	-	FIESC	<i>F. serpentinum</i>	Novel	MN170499	MN170365	-	-
96	CBS 150.25	Unknown	-	Unknown	-	FIESC	<i>F. cateniforme</i>	Novel	MN170451	MN170317	-	-
97	CBS 123.73	Unknown	-	Unknown	-	FIESC	<i>F. longicaudatum</i>	Novel	MN170481	MN170347	-	-
98	NRRL 31160	Human lung	-	TX, USA	-	<i>Incarnatum</i>	<i>F. irregularare</i>	FIESC 15	GQ505607	GQ505519	QGE000000000	QGE000000000
99	ITEM 7547	<i>Musa sapientium</i> var. <i>robusta</i>	-	Bahamas	-	<i>Incarnatum</i>	<i>F. sulawesiense</i>	FIESC 16	-	-	LN901629	-
100	NRRL 32522	Human	-	USA, IL	Diabetic cellulitis	<i>Incarnatum</i>	<i>F. luffae</i>	FIESC 18	GQ505612	GQ505524	-	-
101	NRRL 43639 ^T	Manatee	-	FL, USA	-	<i>Incarnatum</i>	<i>F. multiceps</i>	FIESC 19	GQ505666	GQ505577	-	-
102	NRRL 34003	Human	-	TX, USA	Sputum	<i>Incarnatum</i>	<i>F. caatingaense</i>	FIESC 20	GQ505627	GQ505539	-	-

(Continued)

Table 1. (Continued).

No	Isolate	Host	Locality	Country	Tissue	Clade	Species	Species complex	<i>tefl</i>	<i>cmdA</i>	<i>tub2</i>	IGS
103	NRRL 34002	Human	-	TX, USA	Ethmoid sinus	<i>Incarnatum</i>	<i>Fusarium</i> sp.	FIESC 22	GQ505626	GQ505538	-	-
104	NRRL 32866	Human	-	TX, USA	Cancer patient	<i>Incarnatum</i>	<i>F. incarnatum</i>	FIESC 23	GQ505615	GQ505527	-	-
105	ITEM 7155	-	-	-	-	<i>Incarnatum</i>	<i>F. incarnatum</i>	FIESC 23	-	-	LN901630	-
106	ITEM 6748	<i>Sorghum</i> sp.	-	Unknown	-	<i>Incarnatum</i>	<i>F. nanum</i>	FIESC 25	-	-	LN901631	-
107	NRRL 26417	Unknown plant	-	Cuba	Leaf litter	<i>Incarnatum</i>	<i>F. hainanense</i>	FIESC 26	GQ505598	GQ505510	-	-
108	CBS 635.76	Bermuda grass	-	New Zealand	-	<i>Incarnatum</i>	<i>F. coffeatum</i>	FIESC 28	MNI120755	MNI120696	-	-
109	CBS 621.87	<i>Medicago sativa</i>	-	Denmark	-	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	MNI170452	MNI170318	-	-
110	CBS 678.77	Soil	-	Japan	-	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	MNI170453	MNI170319	-	-
111	LC6896 ^T	<i>Citrus reticulata</i>	Hunan	China	Leaf	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	MK289617	MK289668	-	-
112	MOD1 FUNGH17	Peanut	Washington	DC, USA	-	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	RBJE00000000	RBJE00000000	RBJE00000000	RBJE00000000
113	CBS 130905	<i>Triticum</i> sp.	-	Iran	-	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	MNI170454	MNI170320	-	-
114	ITEM 10392	Wheat	-	Italy	-	<i>Incarnatum</i>	<i>F. citri</i>	FIESC 29	LN901576	LN901592	LN901625	QH HH00000000
115	CBS 143595	Ganoderma sp	Moghan-Ardabil	Iran	-	<i>Incarnatum</i>	<i>F. persicinum</i>	FIESC 29/30	LT970778	LT970731	-	-
116	CBS 143596	<i>Stereum irsutum</i>	Moghan-Ardabil	Iran	-	<i>Incarnatum</i>	<i>F. persicinum</i>	FIESC 29/30	LT970779	LT970732	-	-
117	CBS 102394	Cashew	-	El Salvador	-	<i>Equiseti</i>	<i>F. mucidum</i>	FIESC 30	MNI170484	MNI170350	-	-
118	CBS 102395 ^T	Cashew	-	El Salvador	-	<i>Equiseti</i>	<i>F. mucidum</i>	FIESC 30	MNI170485	MNI170351	-	-
119	ITEM 11401	Oat	-	Canada	-	<i>Equiseti</i>	<i>Fusarium</i> sp.	Novel	LN901578	LN901594	LN901626	QHKN00000000
120	ITEM 13580	Maize	-	Netherlands	-	<i>Equiseti</i>	<i>Fusarium</i> sp.	Novel	-	-	LN901627	-
121	LC12158	<i>Musa nana</i>	Guangdong	China	Leaf	<i>Incarnatum</i>	<i>F. humuli</i>	FIESC 33	MK289592	MK289645	-	-
122	CBS 131382 ^T	<i>Oryza australiensis</i>	-	Australia	-	<i>Incarnatum</i>	<i>F. fasciculatum</i>	Novel	MNI170473	MNI170339	-	-
123	NRRL 13459 ^T	Plant debris	-	South Africa	-	FCONSC	<i>F. concolor</i>	FCONSC	GQ505674	GQ505585	-	-

^aThe accession numbers from reference isolates from the previous studies are indicated in bold (O'Donnell *et al.*, 2009; Villani *et al.*, 2016; Gebru *et al.*, 2019; Maryani *et al.*, 2019; Torbati *et al.*, 2019; Wang *et al.*, 2019; Xia *et al.*, 2019). ^T = ex-type strain; ^{NT} = neotype strain. FIESC: *Fusarium incarnatum-equiseti* species complex, FCONSC: *Fusarium concolor* species complex.

observations of conidial shape and size for 52 isolates were carried out as described by Leslie and Summerell (2006).

Pathogenicity assays

Seedlings of five different plant hosts were used for the pathogenicity assays: spinach cv. Crocodile (Rijk Zwaan), lettuce cv. Gentilina (Maraldi sementi), lamb's lettuce cv. Palace (Meilland Richardier), cultivated rocket cv. Rucola coltivata (La Semiorto), and wild rocket cv. Frastagliata Mazzocchi (Casalpusterlengo). Seeds were sown in 2 L capacity plastic pots containing a sterilized mixture of 80% peat and 20% perlite, at nine seeds/pot. The pots were arranged in a randomized block design with three replicates. The resulting seedlings were grown in a greenhouse at 22 to 24°C until inoculation.

Fifty-two isolates of FIESC were grown on PDA supplemented with 50 mg L⁻¹ of streptomycin sulfate for 10 d at 22°C and a photoperiod of 12 h. Three-week-old plants were artificially inoculated by spraying the above ground plant organs with spore suspensions of 1 × 10⁵ conidia mL⁻¹ (1 mL of suspension per pot). The plants were left for 5 d in a chamber enclosed by transparent polyethylene film, to achieve 100% relative humidity. The plants were then maintained under growth chamber conditions of 25°C (day), 23°C (night), 12 h photoperiod and with twice daily watering. Re-isolations were performed from the leaves of non-inoculated plants and plants inoculated with FIESC isolates, and by observing macro- and micro-molecular characteristics of re-isolated fungi from the inoculated plants, completion of Koch's postulates was assessed.

Disease severity (percentage of affected leaf area) was assessed 7 d post-inoculation (dpi) using a 0-5 diagrammatic scale (Garibaldi *et al.*, 2016b), where: 0 = absence of symptoms; 1 = up to 5% leaf area affected; 2 = 6-10%; 3 = 11-25%; 4 = 26-50%; and 5 = 51 -100% leaf area affected. Small, circular and brown leaf spot symptoms were searched for and recorded on 100 leaves per pot. Disease severity (DS) was calculated as $DS = \Sigma(\text{no. of leaves} \times X_{0-5}) / (\text{no. of recorded leaves})$, using an approximate class midpoint (X_{0-5}): $X_0 = 0$, $X_1 = 5\%$, $X_2 = 10\%$, $X_3 = 25\%$, $X_4 = 50\%$ and $X_5 = 75\%$.

Statistical analyses used in this study were carried out using SPSS software (version 24.0, SPSS Inc.). Differences in disease severity between FIESC isolates were analyzed by a one-way ANOVA followed by the Turkey HSD used for mean separation when ANOVA results were significant ($P < 0.05$).

RESULTS

Molecular identification of pathogen species and phylogenetic analyses

Phylogenetic analyses were carried out, after alignment of the *tefl* sequences of 52 isolates with those available at the Fusarium-ID database resulting in high similarity (99–100%) of all isolates with the FIESC species. A total of 1174 characters (*cmdA*: 1–573, *tefl*: 580–1174) were included in the two locus phylogenetic analyses, 295 characters were parsimony-informative, 195 were variable and parsimony-uninformative, and 678 were constant. A maximum of 1000 equally most parsimonious trees were saved (Tree length = 1169, CI = 0.592, RI = 0.845, and RC = 0.5). Bootstrap support values from the parsimony analyses are plotted on the Bayesian phylogenies in Figure 1.

In the Bayesian analysis, the *cmdA* partition had 168 unique site patterns and the *tefl* partition had 278 unique site patterns. The analysis ran for 3,555,000 generations, resulting in 7112 trees of which 5334 trees were used to calculate the posterior probabilities included in the Figure 1. The consensus tree obtained from the Bayesian analysis agreed with the tree topology obtained from the MP analyses, and Bayesian posterior probability values were mainly in accordance with the ML bootstrap values (Figure 1).

Phylogenetic analyses based on concatenated *tefl* and *cmdA* gene sequences of the study isolates and reference isolates of 33 FIESC phylogenetic species grouped 23 isolates with *F. clavum*, 21 with *F. compactum*, four with *F. citri*, two with *F. ipomoeae*, one with *F. lacertarum*, and one isolate with *F. longifundum* (Figure 1). All the tested isolates, except four of *F. citri* (LBY Feq7 and LBY Feq7R from lettuce, and Feq 1/14 and Feq 5A from wild rocket), grouped in the *Incarnatum* clade, were included in the *Equiseti* clade.

Phylogenetic analyses were then performed for four single-locus sequences. The results of the *tefl*, *cmdA* and IGS analyses were in agreement, and the isolates were grouped into six phylogenetically distinct species. Conversely, the *tub2* phylogenetic tree did not permit clear separation of all the FIESC species (Supplementary Figure 1A), in contrast to the IGS region (Supplementary Figure 1B).

Concatenated phylogenetic analyses for all four loci (*tefl*, *cmdA*, *tub2* and IGS) were carried out, and the results obtained were similar with those of the previous phylogenetic analyses, whereby the study isolates were grouped into six phylogenetically distinct FIESC species. The bootstrap score supporting this analysis was greater, indicating better separation of the phylogenetic species

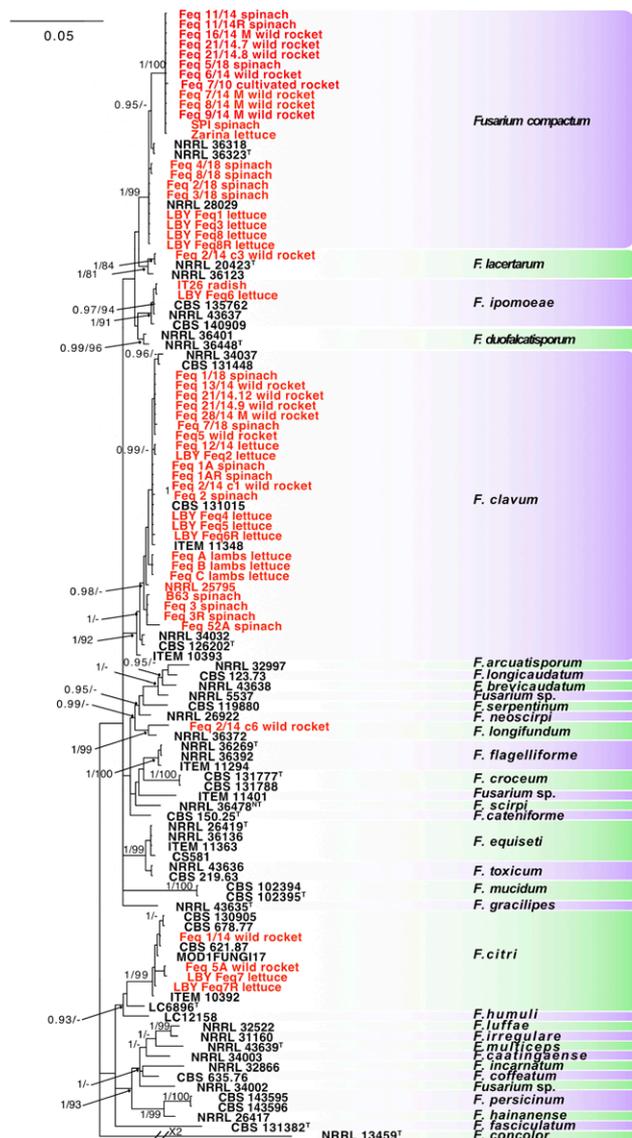


Figure 1. Consensus phylogram of 5334 trees resulting from a Bayesian analysis of the combined *tefl* and *cmdA* sequences from different isolates of the *Fusarium incarnatum-equiseti* species complex (FIESC). The isolate designation, host affiliation and fungal species of the 52 isolates used in this study are shown in red. Bayesian posterior probability (> 0.95) and bootstrap support values (> 70%) are shown at the nodes. The tree was rooted to *Fusarium concolor* (NRRL 13459). Ex-type and neotype strains are indicated with ^T and ^{NT}, respectively.

(bootstrap value = 100% for the *Equiseti* clade and 79% for the *Incarnatum* clade; Supplementary Figure 2).

Intraspecific molecular diversity was observed in isolates from this study in which distinct phylogenetic subgroups were demonstrated; two for *F. compactum* (bootstrap values = 99%), three for *F. clavum* (bootstrap values = 99 and 91%) and one for *F. citri* (bootstrap value =

90%) (Supplementary data, Figure 2). No clustering was observed within the study isolates on the basis of plant host. For geographical sampling location, *F. compactum* was identified among isolates from Northern Italy (the Piedmont and Veneto regions), *F. clavum* was found among isolates from Northern and Southern regions, while *F. citri* was represented by only in two isolates from Southern Italy (the Campania region). Additionally, these three species all included seed-originated isolates from unknown locations (Table 1).

When plant host/pathogen range was considered, wild rocket was a natural host for *F. compactum*, *F. clavum*, *F. citri*, *F. lacertarum* and *F. longifundum*, lettuce a host for *F. compactum*, *F. clavum*, *F. citri* and *F. ipomoeae*, and spinach a host for *F. compactum* and *F. clavum*. The other studied plant hosts were not representative due to limited number of fungal isolates.

Morphological species identification

The size and shape of conidia were similar among all study isolates, and they corresponded to those described for FIESC (Leslie and Summerell, 2006; Wang *et al.*, 2019). The macroconidia measured 25 to 35 × 3 to 5 μm, with 3-5 septa. They were spindle-shaped with slight curvature, and frequent apical tapering, which had almost a needle form in *F. compactum*. No macroconidia were observed in the isolates of the species identified by phylogeny as *F. clavum*. When present, the microconidia were non-septate, and ellipsoidal to ovoid.

Pathogenicity assays

The initial symptoms on inoculate plants consisted of tiny, brown spots on leaves of all five plant species (lettuce, lamb's lettuce, cultivated rocket, wild rocket, and spinach) at 4 dpi. Necrotic spots then enlarged, sometimes surrounded by yellow halos, and the plants progressed to wilt at 14 dpi (Supplementary Figure 3). All tested isolates of six FIESC species (*F. clavum*, *F. compactum*, *F. citri*, *F. ipomoeae*, *F. lacertarum* and *F. longifundum*) reproduced the leaf necrosis symptoms on their corresponding isolation hosts, but they were also capable for infections of experimental hosts causing specific symptoms, with exceptions of *F. compactum* Feq C and *F. clavum* Feq 13/14 isolates on lamb's lettuce (Supplementary Table 2).

One-way ANOVA tests on disease severity data between different FIESC isolates on inoculated plant species showed statistically significant differences between the isolates (Figure 2). FIESC isolates were more

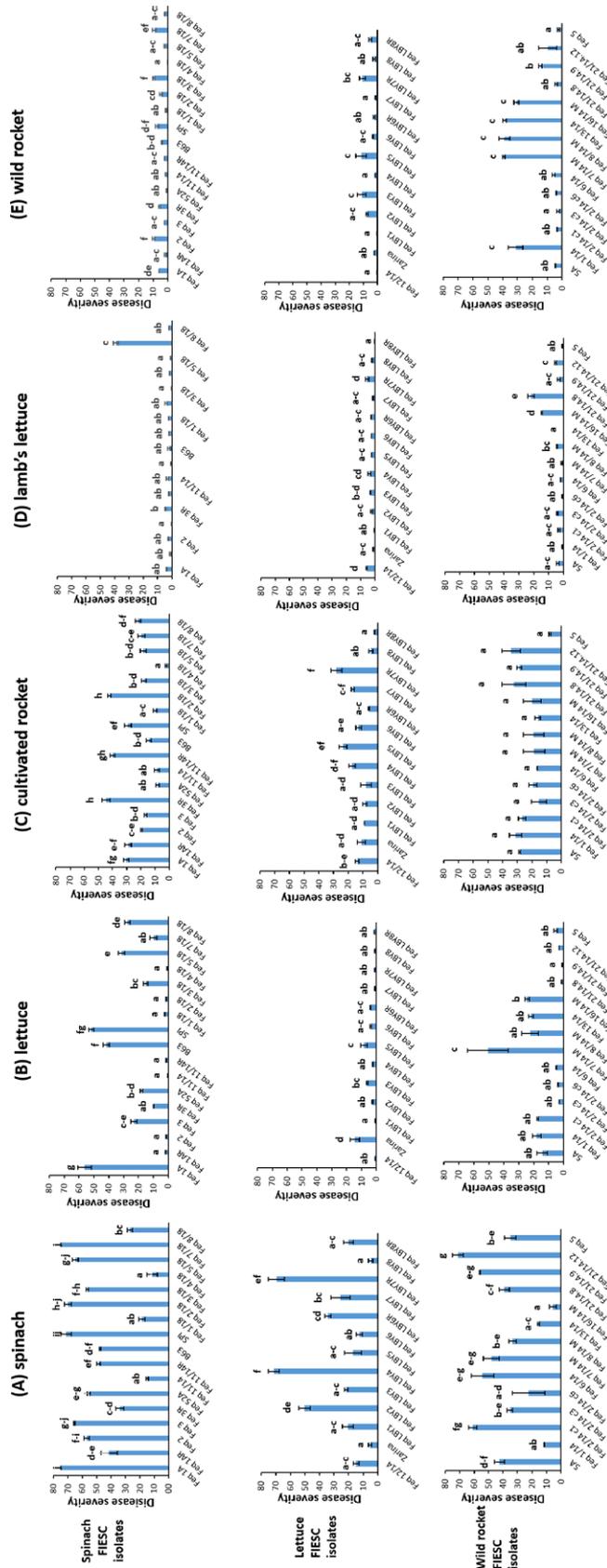


Figure 2. Mean disease severities (and standard deviations) on artificially inoculated leafy vegetable hosts 1 week after inoculation with different isolates of the *Fusarium incarnatum-equiseti* species complex (FIESC). The following FIESC species and their corresponding isolates were used in simple and cross-pathogenicity assays: *F. clavum* (Feq 1A, Feq 1AR, Feq 2, Feq 3, Feq 3R, Feq 52A, B63, Feq 1/18, Feq 7/18) and *F. compactum* (Feq 11/14, Feq 11/14R, SPI, Feq 2/18, Feq 3/18, Feq 4/18, Feq 5/18, Feq 8/18) from spinach; *F. clavum* (Feq 12/14, LBV Feq2, LBV Feq4, LBV Feq5, LBV Feq6R), *F. compactum* (Zarina, LBV Feq1, LBV Feq3, LBV Feq8, LBV Feq8R), *F. ipomoeae* (LBV Feq6) and *F. citri* (LBV Feq7, LBV Feq7R) from lettuce; *F. clavum* (Feq 2/14 c1, Feq 13/14, Feq 21/14.9, Feq 21/14.12, Feq5), *F. compactum* (Feq 6/14, Feq 7/14 M, Feq 8/14 M, Feq 16/14 M, Feq 21/14.7, Feq 21/14.8), *F. citri* (5A, Feq 1/14), *F. lacertarum* (Feq 2/14 c3) and *F. longifundum* (Feq 2/14 c6) from wild rocket. The plant hosts inoculated with the FIESC isolates were: (A) spinach, (B) lettuce, (C) cultivated rocket, (D) lamb's lettuce and (E) wild rocket. The values present the means and standard deviations of 9 replicates.

harmful to plant vigor on experimental hosts, including lamb's lettuce and cultivated rocket, compared to the original hosts of isolation. Thus, the most aggressive isolates were found on alternative hosts: *F. clavum* Feq3R on cultivated rocket (mean DS = 44.8%), and *F. clavum* Feq 7/18 on lamb's lettuce (mean DS = 37.9%). For spinach and wild rocket, the most aggressive isolates were from the original plant host: *F. clavum* Feq 1A and Feq 7/18 on spinach (mean DS = 75.0%), and *F. compactum* Feq 7/14 M on wild rocket (mean DS = 39.4%) (Figure 2). The most virulent species on most leafy vegetable hosts was *F. clavum*.

In general, the most susceptible vegetable hosts were spinach and cultivated rocket, followed by the lettuce, while lamb's lettuce and wild rocket were not greatly affected. The greatest plant mortality after inoculation was for spinach (> 70%), while only occasional inoculated plants of the other plant hosts died after inoculations (data not shown).

DISCUSSION

Fifty-two fungus isolates originating from diverse geographical locations in Northern and Southern Italy, isolated in different years over an 8 year range, from six different leafy vegetable hosts, and different isolation sources (seeds and other plant tissues) have been characterized in this research as members of FIESC. These fungi have also been shown to be causal agents of leaf spot diseases of leafy vegetables.

The single locus (*tef1*, *cmdA* or IGS) phylogenetic analyses identified six phylogenetically distinct species. These were *F. clavum*, *F. compactum*, *F. citri*, *F. ipomoeae*, *F. lacertarum* and *F. longifundum*. The most frequently isolated fungi were *F. clavum* and *F. compactum*. This confirmed the *tef1* barcoding marker as the most informative for the separation of FIESC species (O'Donnell *et al.*, 2009). The *cmdA* and IGS loci were phylogenetically informative, allowing separation of 52 isolates into six FIESC species. The *btub* gene was the least discriminative locus, probably due to the presence of paralogous or xenologous *btub* sequences (O'Donnell *et al.*, 2009). The *tef1* gene cannot provide precise species identification within FIESC in some cases (O'Donnell *et al.*, 2009; Villani *et al.*, 2016; Torbati *et al.*, 2019), and the use of MLST analyses is recommended, due to its higher resolution capacity and sensitivity. Use of MLST in the present study allowed increased sensitivity in the bootstrap score support between two clades, based on concatenated two-locus (*tef1* and *cmdA*) and four-locus (*tef1*, *cmdA*, *tub2* and IGS) sequences.

The greater resolution of MLST mirrored the results of previous FIESC multi-locus studies based on *cmdA*, *tef1*, *tub2* and *rpb2* genes (Villani *et al.*, 2016), and on *cmdA*, *tef1*, *rpb1* and *rpb2* genes (Wang *et al.*, 2019). However, for improved FIESC comparisons, it will be useful to include additional molecular markers, such as *rpb1* and *rpb2*. In this study, robust molecular analyses (the use of MLST and the Fusarium ID database) made it possible to improve the species identification in four isolates, assigning them as *F. compactum* (Feq 7/10 and Feq 9/14 M), *F. ipomoeae* (IT26), and *F. clavum* (Feq 12/14), instead of *F. equiseti*, identified previously by single locus sequencing (Garibaldi *et al.*, 2011; 2015; 2016a; 2017).

All the isolates examined in this study were also identified morphologically as FIESC. However, it was not possible to identify precise species based on morphological observations, because of their high cryptic speciation. This has been reported for previous FIESC morphological descriptions (Leslie and Summerell, 2006; O'Donnell *et al.*, 2009; Avila *et al.*, 2019; Wang *et al.*, 2019).

Identification of the presence of FIESC on wild rocket in Italy was based exclusively on the ITS sequencing of one isolate (Garibaldi *et al.*, 2015), but the use of MLST in the present study has permitted identification of four FIESC species within 17 wild rocket isolates. These were *F. compactum*, *F. lacertarum*, *F. citri* and *F. longifundum*. Furthermore, this study reports, for the first time, that four FIESC species, *F. ipomoeae*, *F. compactum*, *F. citri* and *F. clavum* were the causal agents of the leaf spot disease of lettuce, whereas *F. ipomoeae* caused this disease on radish. This study is also the first report of *F. clavum* on lamb's lettuce, and of *F. compactum* and *F. clavum* on spinach.

The diversity of FIESC in leafy vegetable hosts was shown in the present study, and six phylogenetically distinct species were differentiated among the 52 isolates. *Fusarium compactum* and *F. clavum* were the most commonly isolated species, with the greatest number of isolates of the different phylogenetic subgroups, comprising different hosts and isolation sources (leaves, stems, roots and seeds). This is consistent with the results of previous FIESC studies (Marín *et al.*, 2012; Castellá and Cabañes, 2014; Villani *et al.*, 2016; Wang *et al.*, 2019; Xia *et al.*, 2019; Avila *et al.*, 2019) on other plant hosts in different geographical locations. Comparing the FIESC prevalence in this and the study of Villani *et al.* (2016), *F. clavum* and *F. citri* were present in cereals and leafy vegetables, in Italy, while *F. compactum* was found exclusively in vegetable hosts. Furthermore, *Fusarium compactum* was previously identified from plant hosts originating from England (O'Donnell *et al.*, 2009). The six FIESC phylogenetic species identified in the present study were

single isolates from individual plants, and all caused leaf spot diseases on test plants as single isolates. Additional research involving greater numbers of fungal isolates, single hosts and fields are necessary to evaluate if mixed infections of FIESC species could also be involved in leaf spot diseases.

The recent emergence of FIESC on leafy vegetable hosts in Italy could be related to changes in pathogenicity of this fungal complex. These fungi may have shifted from endophytic or sporadic and weak plant pathogenic status to principal plant pathogens, as has been reported in other fungi (Sacristán and García-Arenal, 2008; Bamisile *et al.*, 2018). Moreover, its natural plant host range has extended, and different phylogenetic species are able to cause similar leaf spot symptoms on individual plant host species. This host expansion is likely to continue, since each identified FIESC species was able to infect leafy vegetable species different of original host of isolation. This has also been recently reported for other necrotrophic fungal pathogens of *Paramyrothecium*, *Albifimbria* and *Alternaria* (Matic *et al.*, 2019; 2020).

The emergence of these fungi on new hosts could be associated with environmental changes. Gullino *et al.* (2017a) reported increases in disease incidence and severity caused by *F. equiseti* on wild rocket and radish, as a result of increases in average temperatures and CO₂ concentrations. Furthermore, additional necrotrophic pathogens which cause similar leaf spot diseases, including *Alternaria alternata*, *Paramyrothecium* spp., *Albifimbria verrucaria*, *Plectosphaerella cucumerina* and *Allophoma tropica*, have emerged on vegetables in Italy. All of these fungi are favoured by elevated temperature or CO₂ (Gullino *et al.*, 2014; 2017b; Siciliano *et al.*, 2017; Bosio *et al.*, 2017; Matic *et al.*, 2019). FIESC emergence was observed on the plants, and in undisturbed soils of the grassland biome. The recent poisoning of animals, caused by feeding with FIESC-infected grass (containing different species of FIESC), and associated with environmental changes, has been reported (Botha *et al.*, 2014; Jacobs *et al.*, 2018).

Another epidemiological aspect that should be taken into consideration is the ability of FIESC to be transmitted by seeds of wild rocket, onion, bean, and other hosts (Ignjatov *et al.*, 2015; Marcenaro and Valkonen, 2016; Gilardi *et al.*, 2017; Gullino *et al.*, 2019). The globalization of seed markets and inefficient seed health inspections, along with climate changes, may be additional causes of the recent outbreaks of seed-borne fungal pathogens.

Epidemiological facet which may also be of crucial importance is the polyphagousness of FIESC. This low host specificity may have assisted the emergence

of FIESC, along with seed transmission and climatic changes. Low host specificity has also allowed different plant- and animal-originating isolates of FIESC to be grouped within the same fungus haplotypes (Ramdial *et al.*, 2017).

In summary, the present study has shown that the emerging leaf spot diseases of leafy vegetable hosts in Italy are caused by members of FIESC. The polyphagous nature of this species complex, which affects plant hosts of different monocot and dicot families, is an important disease management consideration. Rigorous seed inspection measures, and suitable choice of eco-sustainable fungicides and biological control agents should also be implemented. These measures, together with crop rotation, the use of resistant cultivars and the alternation of products, may lead to efficient management of FIESC leaf spot diseases on vegetable crops.

ACKNOWLEDGMENTS

The research reported in this paper was funded from the European Union Horizon 2020 research and innovation program under grant agreement numbers: 634179 “Effective Management of Pests and Harmful Alien Species-Integrated Solutions” (EMPHASIS), and 633999 “EU-CHINA Lever for IPM Demonstration” (EUCLID). The authors thank Dr G. Gilardi (Agroinnova, Grugliasco (TO), Italy) for isolating the FIESC strains, and Marguerite Jones for language revision of the manuscript of this paper.

LITERATURE CITED

- Amatulli M.T., Spadaro D., Gullino M.L., Garibaldi A., 2010. Molecular identification of *Fusarium* spp. associated with bakanae disease of rice in Italy and assessment of their pathogenicity. *Plant Pathology* 59: 839–844.
- Appel D.J., Gordon T.R., 1995. Intraspecific variation within populations of *Fusarium oxysporum* based on RFLP analysis of the intergenic spacer region of the rDNA. *Experimental Mycology* 19: 120–128.
- Avila C.F., Moreira G.M., Nicolli C.P., Gomes L.B., Abreu L.M., ... Del Ponte E.M., 2019. *Fusarium incarnatum-equiseti* species complex associated with Brazilian rice: Phylogeny, morphology and toxigenic potential. *International Journal of Food Microbiology* 306: 108267.
- Bamisile B.S., Dash C.K., Akutse K.S., Keppanan R., Wang L., 2018. Fungal Endophytes: Beyond Herbivore Management. *Frontiers in Microbiology* 9: 544.

- Botha C.J., Truter M., Jacobs A., 2014. *Fusarium* species isolated from *Pennisetum clandestinum* collected during outbreaks of kikuyu poisoning in cattle in South Africa. *Onderstepoort Journal of Veterinary Research* 81: e1–e8.
- Bosio P., Siciliano I., Gilardi G., Gullino M.L., Garibaldi A., 2017. Verrucaridin A and roridin E produced on rocket by *Myrothecium roridum* under different temperatures and CO₂ levels. *World Mycotoxin Journal* 10: 229–236.
- Cao P., Li C., Xiang W., Wang X., Zhao J., 2019. First report of *Fusarium incarnatum-equiseti* species complex causing fruit rot on muskmelon (*Cucumis melo*) in China. *Plant Disease* 103: 1768.
- Carbone I., Kohn L.M., 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91: 553–556.
- Castellá G., Cabañes F.J. 2014. Phylogenetic diversity of *Fusarium incarnatum-equiseti* species complex isolated from Spanish wheat. *Antonie Van Leeuwenhoek* 106: 309–317.
- Chohan S., Abid M., 2019. First report of *Fusarium incarnatum-equiseti* species complex associated with boll rot of cotton in Pakistan. *Plant Disease* 103: 151.
- D'Amico M., Frisullo S., Cirulli M., 2008. Endophytic fungi occurring in fennel, lettuce, chicory, and celery d commercial crops in southern Italy. *Mycological Research* 112: 100–107.
- Desjardins A.E., 2006. *Fusarium* mycotoxins. Chemistry, genetics, and biology. APS Press, St. Paul, MN, USA.
- Garibaldi A., Gilardi G., Bertoldo C., Gullino M.L., 2011. First report of leaf spot of rocket (*Eruca sativa*) caused by *Fusarium equiseti* in Italy. *Plant Disease* 95: 1315.
- Garibaldi A., Gilardi G., Ortu G., Gullino M.L., 2015. First report of leaf spot of wild rocket (*Diploptaxis tenuifolia*) caused by *Fusarium equiseti* in Italy. *Plant Disease* 99: 118.
- Garibaldi A., Gilardi G., Ortu G., Gullino M.L., 2016a. First report of leaf spot of lettuce (*Lactuca sativa*) caused by *Fusarium equiseti* in Italy. *Plant Disease* 100: 531.
- Garibaldi A., Gilardi G., Berta F., Gullino M.L., 2016b. Temperature and leaf wetness affect the severity of leaf spot on lettuce and wild rocket incited by *Fusarium equiseti*. *Phytoparasitica* 44: 681–687.
- Garibaldi A., Gilardi G., Matic S., Gullino M.L., 2017. Occurrence of *Fusarium equiseti* on radish (*Raphanus sativus*) seedlings in Italy. *Plant Disease* 101: 1548.
- Gebbru S.T., Mammel M.K., Gangiredla J., Tournas V.H., Lampel K.A., Tartera C., 2019. Draft Genome sequences of 12 isolates from 3 *Fusarium* species recovered from moldy peanuts. *Microbiology Resource Announcements* 8: e01642–18.
- Geiser D.M., del Mar Jiménez-Gasco M., Kang S., Makalowska I., Veeraraghavan N., ...O'Donnell K., 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. *European Journal of Plant Pathology* 110: 473–479.
- Gilardi G., Pintore I., Gullino M.L., Garibaldi A., 2017. Occurrence of *Fusarium equiseti* as a contaminant of *Diploptaxis tenuifolia* seeds. *Journal of Plant Pathology* 99: 245–248.
- Glass N.L., Donaldson G., 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* 61: 1323–1330.
- Goswami R.S., Dong Y., Punja Z.K., 2008. Host range and mycotoxin production by *Fusarium equiseti* isolates originating from ginseng fields. *Canadian Journal of Plant Pathology* 30: 155–160.
- Groenewald J.Z., Nakashima C., Nishikawa J, Shin H.D., Park, J. H., ... Crous P.W., 2013. Species concepts in *Cercospora*: spotting the weeds among the roses. *Studies in Mycology* 75: 115–170.
- Gullino M.L., Gilardi G., Garibaldi A., 2014. Seed-borne pathogens of leafy vegetable crops. In: *Global Perspectives on the Health of Seeds and Plant Propagation Material* (M. L. Gullino, G. Munkvold, ed.) Springer, Dordrecht, the Netherlands, 47–53.
- Gullino M.L., Gilardi G., Garibaldi A., 2017a. Effect of a climate change scenario on *Fusarium equiseti* leaf spot on wild rocket and radish under phytotron simulation. *Phytoparasitica* 45: 293–298.
- Gullino M.L., Gilardi G., Garibaldi A., 2017b. Evaluating severity of leaf spot of lettuce, caused by *Allophoma tropica*, under a climate change scenario. *Phytopathologia Mediterranea* 56: 235–241.
- Gullino M.L., Gilardi G., Garibaldi A., 2019. Ready-to-Eat Salad Crops: A Plant Pathogen's Heaven. *Plant Disease* 103: 2153–2170.
- Hartman G.L., McCormick S.P., O'Donnell K., 2019. Trichothecene-Producing *Fusarium* Species Isolated from Soybean Roots in Ethiopia and Ghana and their Pathogenicity on Soybean. *Plant Disease* 103: 2070–2075.
- Hillis D.M., Bull J.J., 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* 42: 182–192.
- Hu J., Ren H.Y., Gao T., Yang J.Y., Li J., ...Yang G.W., 2018. First report of *Fusarium* patch on *Festuca arundinacea* caused by *Fusarium incarnatum-equiseti* species complex (FIESC 1) in China. *Plant Disease* 102: 1035.

- Ignjatov M., Milošević D., Nikolić Z., Tamindžić G., Gvozdanović-Varga J., ... Popović T., 2015. First report of *Fusarium* sp. FIESC 3 on onion seed in Serbia. *Plant Disease* 99: 1277.
- Jacobs A., Mojela L., Summerell B., Venter E., 2018. Characterisation of members of the *Fusarium incarnatum-equiseti* species complex from undisturbed soils in South Africa. *Antonie van Leeuwenhoek* 111: 1999–2008.
- Jiang S., Jin Y., Jiang X., 2019. First report of *Stachys sieboldii* angular leaf spot caused by *Fusarium incarnatum-equiseti* species complex in Guizhou Province, China. *Journal of Plant Pathology* 101: 1225–1226.
- Kelly L.A., Tan Y.P., Ryley M.J., Aitken E.A.B., 2017. *Fusarium* species associated with stalk rot and head blight of grain sorghum in Queensland and New South Wales, Australia. *Plant Pathology* 66: 1413–1423.
- 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. *Mycological Research* 109: 173–186.
- Kumar S., Stecher G., Tamura K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution* 33: 1870–1874.
- Langseth W., Bernhoft A., Rundberget T., Kosiak B., Gareis M., 1999. Mycotoxin production and cytotoxicity of *Fusarium* strains isolated from Norwegian cereals. *Mycopathologia* 144: 103–113.
- Leslie J.F., Summerell B.A., 2006. The *Fusarium* Laboratory Manual, 1st edn. Blackwell Publishing, Ames, Iowa.
- Logrieco A., Bottalico A., Mulé G., Moretti A., Perrone G., 2003. Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. *European Journal of Plant Pathology* 109: 645–667.
- Marcenaro D., Valkonen J.P.T., 2016. Seedborne Pathogenic Fungi in Common Bean (*Phaseolus vulgaris* cv. INTA Rojo) in Nicaragua. *PLoS One* 11: e0168662.
- Marín P., Moretti A., Ritieni A., Jurado M., Vázquez C., González-Jaén M.T., 2012. Phylogenetic analyses and toxigenic profiles of *Fusarium equiseti* and *Fusarium acuminatum* isolated from cereals from Southern Europe. *Food Microbiology* 31: 229–237.
- Maryani N., Sandoval-Denis M., Lombard L., Crous P.W., Kema G.H.J. 2019. New endemic *Fusarium* species hitch-hiking with pathogenic *Fusarium* strains causing Panama disease in small-holder banana plots in Indonesia. *Persoonia* 43: 48–69.
- Matic S., Gilardi G., Gullino M.L., Garibaldi A., 2019. Emergence of leaf spot disease on leafy vegetable and ornamental crops caused by *Paramyrothecium* and *Albifimbria* species. *Phytopathology* 109: 1053–1061.
- Matic S., Tabone G., Garibaldi A., Gullino M.L., 2020. *Alternaria* leaf spot caused by *Alternaria* species: an emerging problem on ornamental plants in Italy. *Plant Disease*. DOI: 10.1094/PDIS-02-20-0399-RE.
- Nylander J.A.A., 2004. MrModeltest v. 2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.
- O'Donnell K., Kistler H.C., Cigelnik E., Ploetz R.C., 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Sciences of the United States of America* 95: 2044–2049.
- O'Donnell K., Sutton D.A., Rinaldi M.G., Gueidan C., Crous P.W., Geiser D.M., 2009. Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F. equiseti* and *F. chlamydsosporum* species complexes within the United States. *Journal of Clinical Microbiology* 47: 3851–3861.
- O'Donnell K., Humber R.A., Geiser D.M., Kang S., Park B., ... 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: 427–445.
- O'Donnell K., McCormick S.P., Busman M., Proctor R.H., Ward T.J., ... Rheeder J.P., 2018. Marasas et al. 1984 “Toxigenic *Fusarium* Species: Identity and Mycotoxicology” revisited. *Mycologia* 110: 1058–1080.
- Prasad L., Kamil D., Singh N., Singh O.W., Yadava D.K., Prameela Devi T. 2017. First report of *Fusarium equiseti* causing stem and root rot on *Brassica juncea* in India. *Journal of Plant Pathology* 99: 799–818.
- Ramdlal H., Latchoo R.K., Hosein F.N., Rampersad S.N., 2017. Phylogeny and haplotype analysis of fungi within the *Fusarium incarnatum-equiseti* species complex. *Phytopathology* 107: 109–120.
- Riddell J.IV., Woodside K.J., Leavitt M.A., Newton D.W., Punch J.D., 2010. *Fusarium incarnatum/equiseti* hemodialysis graft infection. *Infectious Disease Reports* 2: e14.
- Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Höhna S., ... Huelsenbeck J.P., 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539–542.
- Santiago M.F., Santos A.M.G., Inácio C.P., Neto A.C.L., Assis T.C., ... Laranjeira D., 2018. First report of

- Fusarium lacertarum* causing cladode rot in *Nopalea cochenellifera* in Brazil. *Journal of Plant Pathology* 100: 611.
- Santos A.C.D.S., Trindade J.V.C., Lima C.S., Barbosa R.D.N., da Costa, A.F., ... de Oliveira N.T., 2019. Morphology, phylogeny, and sexual stage of *Fusarium caatingaense* and *Fusarium pernambucanum*, new species of the *Fusarium incarnatum-equiseti* species complex associated with insects in Brazil. *Mycologia* 111: 244–259.
- Sacristán S., García-Arenal F., 2008. The evolution of virulence and pathogenicity in plant pathogen populations. *Molecular Plant Pathology* 9: 369–384.
- Shi W., Tan Y., Wang S., Gardiner D.M., De Saeger S., Liao Y., ... Wu A., 2017. Mycotoxigenic potentials of *Fusarium* species in various culture matrices revealed by mycotoxin profiling. *Toxins* 9: 6.
- 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. *Journal of Clinical Microbiology* 49: 4264–4272.
- Siciliano I., Bosio P., Gilardi G., Gullino M.L., Garibaldi A., 2017. Verrucaridin A and roridin E produced on spinach by *Myrothecium verrucaria* under different temperatures and CO₂ levels. *Mycotoxin Research* 33: 139–146.
- Swofford D.L., 2003. PAUP*. Phylogenetic analysis using parsimony (*and other methods), v. 4.0b10. Sinauer Associates, Sunderland, Massachusetts.
- Torbati M., Arzanlou M., Sandoval-Denis M., Crous P.W., 2019. Multigene phylogeny reveals new fungicolous species in the *Fusarium tricinctum* species complex and novel hosts in the genus *Fusarium* from Iran. *Mycological Progress* 18: 119–133.
- Thirumalaisamy P.P., Dutta R., Jadon K.S., Nataraja M.V., Padvi R.D., ... Yusufzai S., 2019. Association and characterization of the *Fusarium incarnatum-F. equiseti* species complex with leaf blight and wilt of peanut in India. *Journal of General Plant Pathology* 85: 83–89.
- van Diepeningen A.D., Feng P., Ahmed S., Sudhadham M., Bunyaratavej S., de Hoog G.S., 2015. Spectrum of *Fusarium* infections in tropical dermatology evidenced by multilocus sequencing typing diagnostics. *Mycoses* 58: 48–57.
- Villani A., Moretti A., De Saeger S., Han Z., Di Mavungu J.D., ... Susca A., 2016. A polyphasic approach for characterization of a collection of cereal isolates of the *Fusarium incarnatum-equiseti* species complex. *International Journal of Food Microbiology* 234: 24–35.
- Villani A., Proctor R.H., Kim H.S., Brown, D.W., Logrieco, A.F., ... Susca A., 2019. Variation in secondary metabolite production potential in the *Fusarium incarnatum-equiseti* species complex revealed by comparative analysis of 13 genomes. *BMC genomics* 20: 314.
- Wang M.M., Chen Q., Diao Y.Z., Duan W.J., Cai L., 2019. *Fusarium incarnatum-equiseti* complex from China. *Persoonia* 43: 70–89.
- Xia J.W., Sandoval-Denis M., Crous P.W., Zhang X.G., Lombard L., 2019. Numbers to names - restyling the *Fusarium incarnatum-equiseti* species complex. *Persoonia* 43: 186–221.
- Yang M., Zhang H., Kong X., van der Lee T., Waalwijk C., ... Feng, J., 2018. Host and Cropping System Shape the *Fusarium* Population: 3ADON-Producers Are Ubiquitous in Wheat Whereas NIV-Producers Are More Prevalent in Rice. *Toxins* (Basel): 10: 115.