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Species diversity and mycotoxin production by members of the *Fusarium tricinctum* species complex associated with Fusarium head blight of wheat and barley in Italy

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Abstract

Fusarium head blight (FHB) is a global cereal disease caused by a complex of *Fusarium* species. In Europe, the main species responsible for FHB are *F. graminearum*, *F. culmorum* and *F. poae*. However, members of the *F. tricinctum* species complex (FTSC) have become increasingly important. FTSC fusaria can synthesize mycotoxins such as moniliformin (MON), enniatins (ENNs) and several other biologically active secondary metabolites that could compromise food quality. In this study, FTSC isolates primarily from Italian durum wheat and barley, together with individual strains from four non-graminaceous hosts, were collected to assess their genetic diversity and determine their potential to produce mycotoxins in vitro on rice cultures. A multilocus DNA sequence dataset (*TEF1*, *RPB1* and *RPB2*) was constructed for 117 isolates from

Italy and 6 from Iran to evaluate FTSC species diversity and their evolutionary relationships. Phylogenetic analyses revealed wide genetic diversity among Italian FTSC isolates. Among previously described FTSC species, *F. avenaceum* (FTSC 4) was the most common species in Italy (56/117 = 47.9%) while *F. tricinctum* (FTSC 3), and *F. acuminatum* (FTSC 2) accounted 11.1% (13/117) and the 8.5% (10/117), respectively. The second most detected species was a new and unnamed *Fusarium* sp. (FTSC 12; 32/117 = 19%) resolved as the sister group of *F. tricinctum*. Collectively, these four phylospecies accounted for 111/117 = 94.9% of the Italian FTSC collection. However, we identified five other FTSC species at low frequencies, including *F. iranicum* (FTSC 6) and three newly discovered species (*Fusarium* spp. FTSC 13, 14, 15). Of the 59 FTSC isolates tested for mycotoxin production on rice cultures, 54 and 55 strains, respectively, were able to produce detectable levels of ENNs and MON. In addition, we confirmed that the ability to produce bioactive secondary metabolites such as chlamydosporol, acuminatopyrone, longiborneol, fungerin and butanolide is widespread across the FTSC.

Keywords

Enniatins, FTSC, *Fusarium avenaceum*, GCPSR, molecular phylogenetics, mycotoxins

Abbreviations

FHB, Fusarium head blight; FTSC, *Fusarium tricinctum* species complex; PDA, potato dextrose agar; CTAB, cetyltrimethylammonium bromide; GCPSR, Genealogical concordance phylogenetic species recognition; *TEF1*, translation elongation factor 1-α; *RPB1*, DNA- directed RNA polymerase II largest subunit; *RPB2*, DNA- directed RNA

polymerase II second largest subunit; FHSC, *F. heterosporum* species complex; LC-MS/MS, liquid chromatography-tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; ESI, electrospray ionization; BEA, beauvericin; ENNs, enniatins; ENNA, enniatin A; ENNA1, enniatin A1; ENNB, enniatin B; ENNB1, enniatin B1; MON, moniliformin; CHL, chlamydosporol; ACU, acuminatopyrone; LONG, longiborneol; FUNG, fungerin; BUT, butanolide; AOD-ol, 2-amino-14,16-dimethyloctadecan-3-ol.

1. Introduction

Fusarium head blight (FHB) is a global cereal disease caused by a complex of *Fusarium* species resulting in high yield losses and reduction in quality mainly due to mycotoxin contamination of grain (McMullen et al., 2012). Geographic distribution of the etiological agents appears to be related to climatic conditions such as temperature and humidity (Backhouse, 2014; Bakker et al., 2018; Vaughan et al., 2016). Although the principal species responsible for FHB in Europe are *F. graminearum*, *F. culmorum* and *F. poae* (Bottalico and Perrone, 2002; Pasquali et al., 2016), members of the *F. tricinctum* species complex (FTSC) have become increasingly important contributors to FHB (Beccari et al., 2016, 2018a). These include several formally named species with Latin binomials (*F. acuminatum*, *F. tricinctum*, *F. avenaceum*, *F. iranicum*, *F. flocciferum*, and *F. torulosum*) (Leslie and Summerell, 2006; Torbati et al., 2019) and several unnamed phylospecies. An informal ad hoc nomenclature using Arabic numerals (e.g. FTSC 1-to-11) was proposed to distinguish the named and unnamed species within this complex (O'Donnell et al., 2018; Stakheev et al., 2016).

Fusarium avenaceum has been reported from the cooler regions of Northern Europe, Canada and Central Europe (Gräfenhan et al., 2013; Karlsson et al., 2017; Kosiak et al., 2003; Logrieco et al., 2002; Uhlig et al., 2007; Yli-Mattila et al., 2004). This species and *F. tricinctum* are saprophytes and plant pathogens of a variety of hosts including barley and wheat (Leslie and Summerell, 2006; Uhlig et al., 2007). However, an increase in their incidence has also been reported recently in warmer regions throughout the world (Beccari et al., 2016, 2017, 2018a,b; Cerón-Bustamante et al., 2018; Cowger et al., 2020; Harrow et al., 2010). *Fusarium acuminatum* has also been recovered from cereals in Canada, Italy and Spain, but at a lower frequency compared to *F. avenaceum* and *F. tricinctum* (Beccari et al., 2018a,b; Gräfenhan et al., 2013; Marín et al., 2012).

Members of the FTSC complex produce several "emerging" mycotoxins (Jestoi, 2008), including moniliformin (MON) and enniatins (ENNs) that may pose a threat to food safety and human health (Covarelli et al., 2015; Gautier et al., 2020; Jestoi, 2008; Kokkonen et al., 2010; Logrieco et al., 2002; Uhlig et al., 2007). Of the 29 ENN analogues characterized to date, A, A1, B and B1 are the most commonly reported due to their widespread occurrence (Liuzzi et al., 2017; Sy-Cordero et al., 2012). ENNs are able to increase oxidative stress, induce cell apoptosis and cause mitochondrial dysfunction in mammals, while a main target of MON is cardiac muscle where its toxicity has been shown to vary among different cell lines (Gautier et al., 2020; Gruber-Dorninger et al., 2017; Jestoi, 2008; Uhlig et al., 2004; Wu et al., 2018). In addition, FTSC taxa have been reported to produce other bioactive secondary metabolites including aurofusarin (AUR), chlamydosporol (CHL), 2-Amino-14,16-dimethyloctadecan-3-ol (AOD-ol), and antibiotic Y (Beccari et al., 2018a, b; Munkvold, 2017; Uhlig et al., 2005).

Because of their morphological similarity, identification of FTSC isolates based exclusively on morphological data poses a daunting challenge even to skilled *Fusarium* taxonomists (Stakheev et al., 2016). For this reason, DNA sequence data from several marker loci have been used to resolve phylogenetic relationship within the FTSC, including *ACL1*, β-tubulin, *ITS* rDNA, *PHO*, *RPB1*, *RPB2*, *TEF1*, but with varying success (Bakker et al., 2018; Gräfenhan et al., 2013; Harrow et al., 2010; Niessen et al., 2012; O'Donnell et al., 2018; Yli-Mattila et al., 2002, 2018). Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor et al., 2000) has become the gold standard for identifying exclusive species level lineages within *Fusarium* that may differ in pathogenicity and production of toxic secondary metabolites (O'Donnell et al., 2018). GCPSR relies on concordance of multigene genealogies to identify evolutionary independent phylospecies, and discordance among gene trees (i.e., recombination among strains within a species) to identify species boundaries.

Although several multilocus molecular phylogenetic studies have been conducted to assess FTSC genetic diversity in Northern Europe (Gräfenhan et al., 2013; Stakheev et al., 2016; Yli-Mattila et al., 2018), similar GCPSR-based studies have not been conducted throughout Italy. Given the increased detection of FTSC species in FHB pathogen surveys within Italy over the past 10 years, the risk posed by toxin contaminated cereals remains to be determined. Therefore, the objectives of this study were to: a) assess the phylogenetic diversity and evolutionary relationships of Italian FTSC isolates recovered from symptomatic wheat and barley via GCPSR-based analyses of multilocus DNA sequence data (*TEF1*, *RPB1* and *RPB2*); and b) determine their ability to produce mycotoxins on rice.

2. Materials and Methods

2.1 Fungal isolates

A total of 123 single-spore isolates belonging to the FTSC were collected and analyzed phylogenetically in this study together with sequences of 17 reference isolates (Supplementary Table S1). Isolates marked with "F" (N = 68) are part of a collection of the Department of Agricultural and Food Science (University of Bologna - *Alma Mater Studiorum*). In addition, 50 "P" strains, isolated from wheat and barley harvested in Italy during the 2017/2018 growing season, were added to the phylogenetic analysis. Finally, six strains from Iran (five "R" and one "F") were included in this study to determine whether they represent FTSC species present in both countries.

These isolates were retrieved from the Department of Agricultural and Food Science, University of Bologna, Bologna, Italy culture collection. P-strains were isolated from 21 durum wheat (*Triticum durum* Desf.) samples from seven different climatic regions in Italy (Fig. 2; Supplementary Table S1). Pathogen surveys were conducted in Northern (Lombardy, Veneto and Emilia Romagna), Central (Abruzzo and Molise) and Southern Italy (Campania and Apulia). P strains were isolated as described by Beccari et al. (2016). In brief, a 50 g sub-sample of kernels from each wheat sample was randomly selected for plating. Kernels were surface sterilized in a water-ethanol (95%)-sodium hypochlorite (7%) solution (82:10:8% vol.) for 2 min and then rinsed twice in sterile water for 1 min with each exchange. After the kernels were blotted dry using sterile paper towels, they were placed on potato dextrose agar (PDA, Biolife Italiana, Milan, Italy) supplemented with streptomycin sulphate (0.16 g/L, Sigma Aldrich) in Petri dishes. The 100 kernels analyzed from each wheat sample were divided evenly among 10 Petri dishes. Petri dishes were incubated at 22 °C in the dark and after 5 days examined for fungal growth using a stereomicroscope. All isolates identified as *Fusarium* were transferred to new PDA plates, incubated at 22 °C in the dark for 7 days

149 and then single-spored. The set of isolates identified morphologically as members of the
150 FTSC, using criteria described by Leslie and Summerell (2006), were subjected to
151 further molecular analysis.
152
153 ***2.2 Genomic DNA extraction, PCR amplification and sequencing***
154 Total genomic DNA was extracted from mycelium of the 123 single-spored isolates
155 after 7 days growth on PDA using a CTAB (cetyltrimethylammonium bromide)
156 protocol (Gardes and Bruns, 1993) with slight modifications. Portions of the following
157 three phylogenetically informative genes were PCR amplified and sequenced to identify
158 the 123-strain set: translation elongation factor 1-*α* (*TEF1*), DNA- directed RNA
159 polymerase II largest (*RPB1*) and second largest (*RPB2*) subunits. Amplicons were
160 obtained using the following primer pairs: EF-1 (5′ ATGGGTAAGGAGGACAAGAC-
161 3′) × EF-2 (5′-GGAAGTACCAGTGATCATG-3′) for *TEF1* (O'Donnell et al., 1998)*;*
162 5f2 (5′-GGGGWGAYCAGAAGAAGGC-3′) × 11ar (5-′
163 GCRTGGATCTTRTCRTCSACC-3′) for *RPB2* (Liu et al., 1999; Reeb et al., 2004);
164 and Amp3f (5'-GAYTACATCTTCAAYCGTCAGCC-3') × Amp3r (5'-
165 GTTCTTGGAHGACACACCRGCG-3') for *RPB1*. Because the primers reported in
166 literature were not able to successfully amplify the *RPB1* gene across the entire FTSC,
167 novel PCR primers were designed in this study by aligning *RPB1* sequences of 16
168 FTSC isolates and two closely related species, *F. graminum* NRRL 20692 and *F.*
169 *heterosporum* NRRL 20693 from the *F. heterosporum* species complex (FHSC). In
170 addition, all isolates were screened for their potential to produce ENNs by PCR
171 amplification of a portion of the *Esyn1* gene, which encodes a nonribosomal peptide
172 synthetase that synthesizes enniatin (Kulik et al., 2007).

Each 25 μL PCR reaction contained 50 mM $MgSO_4$, 2 mM of each deoxynucleoside triphosphate, 0.6 mM of each primer pair, 1 U of *Taq* High Fidelity polymerase (Invitrogen) and 50 ng of genomic DNA. Reactions were carried out using the following PCR cycling conditions: EF-1/EF-2 primer pairs, 96 °C 2 min, 94 °C 30 s, 56 °C 30 s, 68 °C 60 s (35 cycles), 68 °C 60 s, followed by a 4 °C soak; for the 5f2/11ar and Amp3f/Amp3r primer pairs, 96 °C 2 min, 94 °C 30 s, 56 °C 30 s, 68 °C 60 (40 cycles), 68 °C 60 s, followed by a 4 °C soak; and for the ESYA1/ESYA2 primer pair, 96 °C 2min, 96 °C 30 s, 58 °C 30 s, 68 °C 60 s (35 cycles), 68 °C 60 s, followed by a 4 °C soak. Amplicons and a 1-10 kb ladder (Invitrogen) were separated by electrophoresis in a 1.5% agarose gel that was run at 80 V for 1 h, stained with ethidium bromide and then visualized over a UV transilluminator. Once amplicons were purified with MultiScreen-PCR_{96} filter plates (Millipore), they were sequenced using a Big Dye Terminator Sequencing kit ver. 3.1 (Applied Biosystems) and then analyzed with an ABI 3730 DNA Analyzer (Applied Biosystems). ABI chromatograms were edited using Sequencher (ver. 5.0, Gene Codes, Ann Arbor, MI), exported as fasta files that were aligned using MUSCLE (Edgar, 2004). Sequences were used to conduct BLASTn queries of *Fusarium MLST* (https://fusarium.mycobank.org/) and NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) to obtain preliminary identifications of the 123 FTSC isolates (O'Donnell et al., 2015).

2.3 Phylogenetic analyses

Aligned sequences of the 123 FTSC isolates collected in this study were combined with those from 17 FTSC reference strains (Supplementary Table S1) (O'Donnell et al., 2018; Torbati et al., 2019) and then analyzed via maximum likelihood bootstrapping (ML-BS) using IQ-TREE 1.6.12 (Nguyen et al., 2015) (http://www.iqtree.org/). Once

198 the best-fit model of molecular evolution was determined for *TEF1* (TIMe+G4), *RPB1*
199 (TNe+G4) and *RPB2* (TNe+I+G4), using ModelFinder (Kalyaanamoorthy et al., 2017)
200 based on the Bayesian information criterion (BIC) scores (Chernomor et al., 2016), a
201 combined partitioned ML-BS analysis was conducted with IQ-TREE ver. 1.6.12.
202 Statistical support for the branches was evaluated by conducting a ML-BS bootstrap
203 analysis of 5000 replicates. Sequences of two outgroup species from the FHSC, *F.*
204 *graminum* NRRL 20692 and *F. heterosporum* NRRL 20693, were chosen for rooting
205 the trees, and sequences of *F. nurragi* NRRL 36452, the closest sister lineage to the
206 FTSC (Geiser et al., 2021), were also included in the analyses.
207
208 ***2.4 Production of mycotoxins and other biologically active secondary metabolites in***
209 ***vitro***
210 Based on results of the phylogenetic analyses, 59 isolates that included representatives
211 of 10 FTSC phylospecies were selected to evaluate their ability to synthesize
212 mycotoxins and several bioactive secondary metabolites in vitro on rice cultures. After
213 strains were grown on V8 juice agar plates (20% V8 juice, 0.3% $CaCO_3$, 2% agar) for 7
214 days at 28 °C, two 5 mm diam plugs from each plate were placed in dram vials
215 containing autoclaved rice (4.4 g + 1.8 mL of water). After the rice cultures were
216 incubated for 10-12 days in the dark at 25 °C, they were extracted with 10 mL ethyl
217 acetate for 30 min with shaking. One mL of each extract was transferred to a 1-dram
218 vial and dried under nitrogen with heat. Once the dried extracts were resuspended in
219 100 µL ethyl acetate, they were analyzed with an Agilent 5873 GC-MS fitted with a
220 HP-5MS column (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness)
221 and a 5973-mass spectrometer with an electron impact source. Samples were injected at
222 150 °C, the temperature was held for 1 min and then the column was heated at 30

°C/min to 280 °C and then held for 7.7 min. Individual peaks in chromatograms were examined and compounds were identified based on retention time, comparison of ion fragmentation patterns with a NIST library, and a library prepared with purified standards. Under these conditions, butanolide (BUT) was detected at 3.10 min, longiborneol (LONG) at 3.94 min, acuminatopyrone (ACU) at 5.23 min, chlamydosporol (CHL) at 5.43 min, and fungerin (FUNG) at 5.85 min.

HPLC-MS analysis was performed using a Dionex Ultimate U3000 liquid chromatography system coupled to a QExactive high resolution mass spectrometer equipped with an electrospray ionization (ESI) source (ThermoFisher Scientific). ENNs and 2-Amino-14,16-dimethyloctadecan-3-ol (AOD-ol) were separated using a Phenomenex Kinetex 2 mm x 50 mm XB-C18 100A column (2.6 µm particle size, 100 Å pore size). Elution of the metabolites was accomplished in a binary gradient flow of mobile phase A [water / acetic acid (99.7: 0.3 v/v)] and mobile phase B [methanol / acetic acid (99.7: 0.3 v/v)], in which the injection volume was 10 µL. The gradient of 20-95% mobile phase B over 5 min was delivered at a flow rate of 0.6 mL/min. The HPLC flow was coupled to the mass spectrometer operated in positive mode utilizing the following parameters: 320 °C capillary temperature, 310 °C heater temperature, and spray voltage of 4.00 kV for positive ESI. For analysis of MON the HPLC utilized a gradient of mobile phase A [water / formic acid (99.1: 0.1 v/v)] and mobile phase B [methanol / formic acid (99.1: 0.1 v/v)]; injection volume: 10 µL, HPLC column: Waters XBridge 4.6 mm x 150 mm BEH-C18 column (5 µm particle size, 130 nm pore size). The gradient of 5-95% mobile phase B over 5 min was delivered at a flow rate of 0.8 mL/min. The HPLC flow was coupled to the mass spectrometer operated in negative mode utilizing the following parameters: 320 °C capillary temperature, 310 °C heater temperature, and spray voltage of -4.00 kV for negative ESI. For both positive and

248 negative mode experiments the mass spectrometer was operated in full MS mode (m/z
249 range 150/2000 and 70,000 resolution). Quantification and identification of each
250 metabolite were performed by comparison to purified standards. Instrument operation
251 and data processing were done using Xcalibur data acquisition and interpretation
252 software (ThermoFisher Scientific). Limits of quantitation for enniatin A (ENNA),
253 enniatin A1 (ENNA1), enniatin B (ENNB), enniatin B1 (ENNB1), AOD-ol and MON
254 were 1 ng/µL.
255
256 **3. Results**
257
258 **3.1 Phylogenetic analyses**
259
260 Partial *TEF1* sequences of the 117 Italian and six Iranian isolates recovered in our
261 pathogen surveys were used to conduct BLASTn queries of *Fusarium MLST* and NCBI
262 GenBank to obtain preliminary species level identifications. In addition, maximum
263 likelihood bootstrap (ML-BS) analyses of the three individual partitions, which
264 contained sequences of 17 reference strains, identified the optimal model of molecular
265 evolution as TIM2e+G4 for *TEF1* [Supplementary Fig. S1: 684 bp alignment, 138
266 parsimony informative characters (PICs)] TNe+G4 for *RPB1* (Supplementary Fig. S2:
267 1606 bp alignment, 233 PIC) and TNe+I+G4 for *RPB2* (Supplementary Fig. S3: 1693
268 bp alignment, 256 PIC). These three models were used to conduct a partitioned ML-BS
269 analysis of the combined 3-locus dataset (Fig. 3: 3983 bp alignment, 628 PIC). ML-BS
270 analyses of the three individual and combined datasets resolved the 117 Italian and 6
271 Iranian isolates, respectively, as 9 and 4 FTSC species (Fig. 3, Table 1). Of the 10 *F.*
272 *tricinctum* clade species represented by 2 or more stains, *TEF1* strongly supported 8/10

as reciprocally monophyletic (94-100% ML-BS), *RPB1* all 10 (86-100% ML-BS), *RPB2* 8/10 (75-100% ML-BS), and the combined dataset all 10 (99-100% ML-BS). Although *F. gamsii* (FTSC 1) and *F. avenaceum* (FTSC 4) in analyses of *TEF1* and *F. iranicum* (FTSC 6) and *Fusarium* sp. (FTSC 14) in the ML-BS *RPB2* phylogeny were not supported as genealogically exclusive, their monophyly was not contradicted. Four phylospecies accounted for 111/117 (94.9%) of the FTSC from Italy (Fig. 2), and these included in descending prevalence: *F. avenaceum* (FTSC 4, N = 56), *Fusarium* sp. (FTSC 12, N = 32), *F. tricinctum* (FTSC 3, N = 13), and *F. acuminatum* (FTSC 2, N = 10). Except for *F. tricinctum*, which was only found in Northern and Central Italy, isolates of the other three species from wheat (N = 78) were collected in Northern, Central and Southern Italy. The same four species were recovered from barley, but in low numbers ranging from 1-to-10, and of the 17 total, 2 and 15, respectively, were from Northern and Central Italy. Five other FTSC species were present in the Italian collection, and these included two isolates of *Fusarium* sp. (FTSC 13), one from *Malvus domestica* (apple wood) and *Buxus sempervirens* (boxwood), and singletons of the following 4 FTSC: *F. iranicum* (FTSC 6) and *Fusarium* spp. (FTSC 11, 14 and 15). The 6 environmental Iranian strains that were typed included *F. acuminatum* (FTSC 2) from water and sediment and one isolate of *F. gamsii* (FTSC 1) from foam and *Fusarium* sp. (FTSC 14) from decaying vegetation (Fig. 2). Analyses of the three individual and combined dataset suggest that *Fusarium petersiae* from Dutch soil (Lombard, 2017) is a later synonym of *F. flocciferum* (Fig. 2, Supplementary Figs. 1-3).

3.2 Production of mycotoxins and other biologically active secondary metabolites in vitro

All 123 analyzed strains tested positive for the presence of *Esyn1*, confirming their potential ability to synthesize this class of secondary metabolites. In addition, based on phylogenetic analyses, 59 isolates comprising 10 FTSC phylospecies were selected to assess their ability to produce mycotoxins and other secondary metabolites in vitro on rice (Table 2). Forty-three isolates were from wheat, 10 from barley, four non-graminaceous hosts, and one each from decaying vegetation and foam (Supplementary Table S1). At least one isolate of all 10 species was able to produce quantitatively detectable levels of one or more enniatin; however, ENNs were not detected in three strains of *Fusarium* sp. (FTSC 12) from wheat and barley and *Fusarium* sp. (FTSC 14) from *Ligustrum*. Although ENNA was only produced by one isolate of three phylospecies, ENNA1 was detected in one or more isolate of 9 species (39/59 = 66.1%), and ENNB (54/59 = 91.5%) and ENNB1 (51/59 = 86.4%) in all 10 species. The highest total ENN production by a strain of the four most common species recovered from Italian wheat and barley (Table 2) was 701.8 μg/kg in *F. avenaceum* F1503 (FTSC 4, all 23 produced), 594.1 μg/kg in *Fusarium* sp. F1509 (FTSC 12, 12/15 produced), 853.5 μg/kg in *F. acuminatum* F1389 (FTSC 2, all 9 produced), and 498.8 μg/kg in *F. tricinctum* F1460 (FTSC 3, all 5 produced).

Detectable levels of MON were recorded in 55/59 isolates (Table 2), but not in three isolates of *Fusarium* sp. (FTSC 12) and the single isolate of *F. iranicum* (FTSC 6) tested. MON production by strains of the four most common species recovered from Italian wheat and barley ranged from 207.7-803.9 μg/kg in *F. avenaceum* (FTSC 4, all 23 produced), 12.4-322.2 μg/kg in *Fusarium* sp. (FTSC 12, 12/15 produced), 55.7-417.8 μg/kg in *F. acuminatum* (FTSC 2, all 9 produced), and 71.6-702.8 μg/kg in *F. tricinctum* (FTSC 3, all 5 produced). Similarly, detectable levels of AOD-ol were recorded in 52/59 isolates (Table 2). The ability to produce AOD-ol appears to be

322 distributed broadly across the FTSC (Kim et al., 2020). A qualitative screen of the rice
323 culture extracts revealed that some of the 59 FTSC isolates could produce several other
324 secondary metabolites. These included CHL production by 26 isolates representing 6
325 species, ACU by 20 isolates comprising four species, LONG by 13 isolates from three
326 species, FUNG by 23 strains representing eight species, and BUT by 8 isolates from
327 five of the species (Table 2).
328

329 **4. Discussion and Conclusions**
330

331 The present research is the first to assess FTSC species diversity associated with FHB
332 of wheat and barley in Italy employing phylogenetic species recognition based on
333 concordance of three independent gene genealogies, which indicate genetic isolation
334 among the cryptic evolutionary independent phylospecies (GCPSR, Taylor et al., 2000),
335 and to experimentally test the ability of 10 FTSC phylospecies to produce mycotoxins
336 and other biologically active secondary metabolites in vitro. Consistent with the
337 findings of a recent GCPSR-based study of toxigenic fusaria (O'Donnell et al., 2018),
338 our pathogen survey of wheat and barley revealed that nine FTSC species were
339 represented among the 117 isolates from Italy, including four unnamed phylospecies
340 new to science (i.e., *Fusarium* spp. FTSC 12 - 15). To aid in identifying the Italian
341 collection, sequences of 17 isolates comprising 11 FTSC species were included as a
342 reference (O'Donnell et al., 2009, 2012, 2018). Because nine of the 15 FTSC species
343 included in this study lack Latin binomials, the named and unnamed species were
344 distinguished informally using Arabic numbers (i.e., FTSC 1 - 15). An ad hoc
345 nomenclature using Arabic numbers was also employed in prior studies of other
346 species-rich complexes in *Fusarium* (Laraba et al., 2021; O'Donnell et al., 2008, 2009,

2012), given that GCPSR studies have consistently revealed morphological species recognition greatly underestimates species diversity within this genus. As reported here, published multilocus molecular phylogenetic analyses of FTSC pathogen collections have consistently encountered novel species diversity (Cerón-Bustamante et al., 2018; Gräfenhan et al., 2013; Harrow et al., 2010; Niessen et al., 2012; O'Donnell et al., 2018; Ponts et al., 2020; Torbati et al., 2019), strongly suggesting the FTSC comprises well over 15 phylospecies. Some of this species level diversity, however, has been misinterpreted as infraspecific variation within *F. avenaceum* (Yli-Mattila et al., 2002). Recent taxonomic advances within the FTSC include formal descriptions of two species from *Agaricus bisporus* in Iran as *F. gamsii* (FTSC 1) and *F. iranicum* (FTSC 6) (Torbati et al., 2019); however, *F. petersiae* from Dutch soil (Lombard, 2017) is treated here as illegitimate because it appears to be a later synonym of *F. flocciferum* (FTSC 7) based on our phylogenetic analyses and those of Ponts et al. (2020). Future molecular systematic advances within the FTSC need to take advantage of the rich genomic resources that should be exploited to develop additional phylogenetically informative marker loci needed to infer robust GCPSR-based hypotheses of FTSC species diversity (Kim et al., 2020; Lysøe et al., 2014).

Our maximum likelihood bootstrap analyses of DNA sequence from portions of three phylogenetically informative genes (i.e., *TEF1*, *RPB1* and *RPB2*), and the combined 3-locus 140 isolate dataset, strongly supported the monophyly of the 10 FTSC phylospecies represented by two or more strains, employing the highly conservative criteria of genealogical concordance and nondiscordance under GCPSR (Dettman et al., 2003) (Table 1). These analyses revealed that 4/11 FTSC species accounted for 111/117 (94.9%) of the FHB-associated collection from Italian wheat and barley. *F. avenaceum* (FTSC 4) was the predominant species comprising close to half of

the Italian collection (N 56/117 = 47.9%), followed by the newly discovered phylospecies *Fusarium* sp. FTSC 12 (N 32/117 = 27.4%), *F. tricinctum* FTSC 3 (N 13/117 = 11.1%), and *F. acuminatum* FTSC 2 (N 10/117 = 8.5%). As reported here, *F. avenaceum* was one of the most common FTSC species recovered in prior pathogen surveys of durum wheat in Italy (Beccari et al., 2018a, 2018b, 2020). While *F. avenaceum* has generally been reported from the cooler region of Northern Europe and Canada (Gräfenhan et al., 2013; Stakheev et al., 2016; Uhlig et al., 2007; Xu et al., 2008; Yli-Mattila et al., 2004), it was recently recovered in FHB pathogen surveys in Mexico (Cerón-Bustamante et al., 2018), Brazil (Moreira et al., 2020), North Carolina, U.S. (Cowger et al., 2020), and New Zealand (Harrow et al., 2010). Although members of the *F. graminearum* species complex (FGSC), especially *F. graminearum*, are the most important and aggressive FHB pathogens on small grain cereals in warm and temperate regions worldwide (Astolfi et al., 2011; Bottalico and Perrone, 2002; Garmendia et al., 2018; Ji et al., 2019; Reynoso et al., 2011; Ward et al., 2008) a recent putative "shift" in the *Fusarium* community composition was observed involving species thus far considered secondary invaders such as *F. poae* and members of the FTSC (Beccari et al., 2016, 2017, 2018b; Covarelli et al., 2015a). The increased presence of *F avenaceum* and other closely related species might be due to climate change and/or agricultural practices including fungicide applications (Cowger et al., 2020; Decleer et al., 2018; Gräfenhan et al., 2013; Karlsson et al., 2017; Tini et al., 2020; Vogelgsang et al., 2019). Our working hypothesis is that when climatic conditions are unfavorable for *F. graminearum*, secondary invaders such as *F. avenaceum* become more competitive (Beccari et al., 2017; Cerón-Bustamante et al., 2018; Gräfenhan et al., 2013; Vogelgsang et al., 2019; Xu and Nicholson, 2009). In

addition, infection timing during anthesis might help explain the increased presence of *F. avenaceum* (Beccari et al., 2019).

The novel unnamed taxon *Fusarium* sp. FTSC 12, which was supported in our analyses as sister to *F. tricinctum*, was the second most common FTSC species recovered from Italian wheat and barley, comprising slightly more than one-quarter of our Italian pathogen collection (N 32/117 = 27.4%). As reported here for *F. tricinctum*, the ability of *Fusarium* sp. FTSC 12 to produce mycotoxins such as ENNs and MON was observed in 28/32 isolates tested. These data suggest that this newly discovered species could contribute to mycotoxin contamination of Italian cereals, compromising wheat and barley quality. The available data suggests that this novel phylospecies may have been reported as *F. tricinctum* in prior phylogenetic studies of the FTSC (Niessen et al., 2012; Ponts et al., 2020).

Only 8.5% (10/117) of the Italian isolates were identified as *F. acuminatum*, consistent with prior results that found this species was present at low frequencies on wheat and malting barley in Italy (Beccari et al., 2018a, 2018b). *Fusarium acuminatum* was recently recovered from wheat in Spain, Canada, and North Carolina in the U.S. where it was reported as a minor contaminant except in North Carolinian wheat fields (Cowger et al., 2020; Gräfenhan et al., 2013; Marín et al., 2012). Cowger et al. (2020), however, reported that it accounted for approximately half of the North Carolinian FTSC isolates (122/249 = 49%), suggesting that it can become a significant contaminant under favorable conditions. Future studies are also needed to determine whether endophytic fusaria in native plants near cultivated areas serve as a reservoir of pathogen diversity, given the report that 50% of the endophytes present in symptomless wild grasses in Minnesota were members of the FTSC (Lofgren et al., 2018).

The present research adds to a growing number of studies that have shown that members of the FTSC are able to produce significant levels of MON and ENN mycotoxins (Bottalico and Perrone, 2002; Kokkonen et al., 2010; O'Donnell et al., 2018; Orlando et al., 2019; Pereira et al., 2020; Schütt et al., 1998; Sørensen et al., 2009). These mycotoxins are defined as "emerging" because maximum levels have not been established by the European Union (EU) and elsewhere (EFSA, 2018, 2014) and because they are not monitored (Gruber-Dorninger et al., 2017). Moreover, while *F. avenaceum* has been reported to produce beauvericin (BEA) (Logrieco et al., 2002; Morrison et al., 2002), this mycotoxin was not detected in the current and several other surveys of the FTSC (Cerón-Bustamante et al., 2018; Covarelli et al., 2015a; Jestoi et al., 2004; O'Donnell et al., 2018; Uhlig et al., 2006, 2007; Vogelgsang et al., 2008). However, because BEA and ENNs are produced by the same biosynthetic pathway, cultural conditions may not have been optimal for BEA production in the aforementioned studies (Sørensen and Giese, 2013). Moreover, because comparative genomic analyses have shown toxins and other bioactive secondary metabolites are frequently not produced even when the gene clusters that encode them are intact (Kim et al., 2020), future studies are warranted to assess whether FTSC species can produce BEA.

Extending the finding of prior studies that established *F. avenaceum* and *F. tricinctum* produce significant levels of MON and ENNs (Beccari et al., 2020; Fredlund et al., 2013; Orlando et al., 2019; Uhlig et al., 2006), our analyses revealed that all but one of the 10 FTSC phylospecies we analyzed produced significant levels of these toxins in vitro on rice. Therefore, it should be assumed that all members of the FTSC possess the genetic potential to contaminate cereals with MON and ENNs until proven otherwise. Until recently, these secondary metabolites were thought to be a concern

primarily for Northern European countries (Ivanova et al., 2006); however, Santini et al. (2012) reported not only high levels of ENNs on grain-based foods within the Mediterranean, but also the resistance of these compounds to food processing. In addition to MON and ENNs, GC-MS analyses of rice culture extracts reported here revealed that several FTSC species possess the ability to produce lesser-known secondary metabolites in vitro such as the lactone chlamydosporol (Sørensen et al., 2009), heterocyclic ketone acuminatopyrone, sesquiterpene alcohol longiborneol, antifungal alkaloid fungerin, and lactone butanolide (Beccari et al., 2018a, 2018b). Although limited data is available concerning acute and chronic toxicity, CHL was reported to be toxic to human cells (Solfrizzo et al., 1994) and ACU toxic to mouse cells, human fibroblasts and chick embryos (Solfrizzo et al., 1994; Solfrizzo and Visconti, 1996). Kim et al. (2020) characterized the gene cluster responsible for production of AOD-ol and discovered that this cluster is widely distributed in the FTSC. Early studies showed AOD-ol to be cytotoxic in a variety of assays (Uhlig et al., 2008). More recently, it has been shown that AOD-ol induced a transient accumulation of vacuoles in the cells of the HepG2 human model liver cell line (Solhaug et al., 2020).

In conclusion, the present study significantly increases our knowledge of FTSC species diversity and mycotoxin potential associated with FHB-symptomatic wheat and barley in Italy. Because MON and ENN toxin levels in cereals and other food and feed are currently not regulated by the European Food Safety Authority, the toxin data reported here should provide a robust framework for improving our understanding of the risk they pose to human health and food security (EFSA, 2014, 2018). Towards this end, in-depth toxicological studies are urgently needed to inform science-based regulatory decisions.

DISCLAIMER

The mention of company names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture (USDA) over other companies or similar products not mentioned. USDA is an equal opportunity provider and employer.

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874 **Table 1:** Loci sequenced and maximum likelihood bootstrap support for species

875 monophyly.

876

***Fusarium* species (FTSC #[a])**	***TEF1***	***RPB1***	***RPB2***	**Combined**
PIC/bp - % PIC[b]	138/684 - 20.2	233/1606 - 14.5	256/1693 - 15.1	628/3983 - 15.8
F. gamsii (FTSC 1)	52	100	100	100
F. acuminatum (FTSC 2)	99	100	99	100
F. tricinctum (FTSC 3)	99	100	87	100
F. avenaceum (FTSC 4)	69	99	100	100
Fusarium sp. (FTSC 5)	NA[c]	NA	NA	NA
F. iranicum (FTSC 6)	94	96	<[d]	99
F. flocciferum (FTSC 7)	100	86	97	100
Fusarium sp. (FTSC 8)	NA	NA	NA	NA
F. torulosum (FTSC 9)	NA	NA	NA	NA
Fusarium sp. (FTSC 10)	NA	NA	NA	NA
Fusarium sp. (FTSC 11)	100	100	88	100
Fusarium sp. (FTSC 12)	98	100	75	100
Fusarium sp. (FTSC 13)	98	100	100	100
Fusarium sp. (FTSC 14)	94	99	<	100
Fusarium sp. (FTSC 15)	NA	NA	NA	NA

877

878 [a] The FTSC # represents an informal ad hoc nomenclature used to distinguish taxa because only seven of

879 the 15 species included in this study have Latin binomials.

880 [b] PIC/bp = parsimony informative characters per base pair.

881 [c] NA = not applicable for five species represented by single strains where monophyly cannot be assessed.

882 [d] < = monophyly neither supported nor contradicted by bootstrapping.

883

884 **Table 2:** Mycotoxins and other bioactive secondary metabolites produced by FTSC isolates representing the phylogenetic diversity sampled in

885 this study.

886

Isolate #[a]	Species (FTSC #[b])	CHL[c]	ACU[c]	LONG[c]	FUNG[c]	BUT[c]	ENNA[d]	ENNA1[d]	ENNB[d]	ENNB1[d]	MON[d]	AOD-ol[d]
R83	*F. gamsii* (FTSC 1)	-[e]	+[e]	-	-	-	< LOQ	4.9	8	1.1	16.1	< LOQ
F1389	*F. acuminatum* (FTSC 2)	+	+	-	-	-	1.1	187.5	318.5	346.4	63.1	5.9
F1392	*F. acuminatum* (FTSC 2)	+	+	-	-	-	< LOQ	80.6	236.4	211	55.7	186.3
F1468	*F. acuminatum* (FTSC 2)	-	-	-	-	-	< LOQ	2.8	6.4	6.9	72.6	2139.3
F1471	*F. acuminatum* (FTSC 2)	+	-	-	-	-	< LOQ	23.4	109.4	81.7	417.8	182.8
F1541	*F. acuminatum* (FTSC 2)	+	-	-	-	-	< LOQ	32.3	102.9	92.8	276.8	208.3
P82b	*F. acuminatum* (FTSC 2)	+	-	-	-	-	< LOQ	77.1	197.9	187.5	169	226.7
P92a	*F. acuminatum* (FTSC 2)	+	+	-	+	-	< LOQ	30.6	94.5	82.2	304.8	127.1
P454a	*F. acuminatum* (FTSC 2)	+	-	-	-	-	< LOQ	188.9	283.3	304	218.6	< LOQ
P2214b	*F. acuminatum* (FTSC 2)	+	+	-	-	+	< LOQ	41.9	119.3	116.1	366.4	337.5
F1281	*F. tricinctum* (FTSC 3)	-	-	+	-	-	< LOQ	63.8	110	122.2	702.8	269.6
F1458	*F. tricinctum* (FTSC 3)	-	-	-	+	-	< LOQ	22.1	91.6	72	345.8	104.1
F1460	*F. tricinctum* (FTSC 3)	+	-	-	+	-	2.6	110.6	186	199.6	162.6	193.2

F1502	*F. tricinctum* (FTSC 3)	-	-	-	+	-	< LOQ	15	88.2	60	550.4	302.7
P325b	*F. tricinctum* (FTSC 3)	-	-	-	+	+	< LOQ	9.8	25	24.6	71.6	47.6
F1275	*F. avenaceum* (FTSC 4)	+	+	+	-	-	< LOQ	< LOQ	16.2	1.6	286.5	259.8
F1436	*F. avenaceum* (FTSC 4)	-	-	+	-	-	< LOQ	57.6	296.1	227.2	348.9	< LOQ
F1444	*F. avenaceum* (FTSC 4)	-	-	+	-	-	< LOQ	4.5	28.8	136.2	420	75.3
F1479	*F. avenaceum* (FTSC 4)	-	-	+	-	-	< LOQ	2.1	80.5	17.6	462.8	629.1
F1480	*F. avenaceum* (FTSC 4)	-	-	+	-	-	< LOQ	< LOQ	13.8	1.7	287.6	227.7
F1481	*F. avenaceum* (FTSC 4)	+	+	+	-	+	< LOQ	< LOQ	41.6	8.4	303.5	233.3
F1486	*F. avenaceum* (FTSC 4)	+	+	+	-	-	< LOQ	< LOQ	48.5	8.4	331.4	< LOQ
F1503	*F. avenaceum* (FTSC 4)	+	+	-	+	-	2.5	117.5	306.9	274.9	469.4	86.8
F1533	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	9	130.2	50	426.3	218.0
F1565	*F. avenaceum* (FTSC 4)	-	-	-	-	+	< LOQ	< LOQ	73.5	9.9	611.4	577.5
P8C	*F. avenaceum* (FTSC 4)	+	-	-	-	-	< LOQ	< LOQ	12	1.8	365.8	221.8
P78d	*F. avenaceum* (FTSC 4)	-	-	-	-	-	< LOQ	10.7	117	45.2	772.5	244.2
P88a	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	< LOQ	30.2	3.8	376.7	193.7
P89a	*F. avenaceum* (FTSC 4)	+	+	+	-	-	< LOQ	2.4	61.3	7.2	558.4	165.8
P91a	*F. avenaceum* (FTSC 4)	+	-	-	-	-	< LOQ	<LOQ	97.4	10.8	482.9	160.4

P101a	*F. avenaceum* (FTSC 4)	-	-	-	-	-	< LOQ	10.3	214.8	77.4	503.1	421.4
P208a	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	< LOQ	3.2	< LOQ	319.6	290.0
P440a	*F. avenaceum* (FTSC 4)	+	+	+	-	+	< LOQ	< LOQ	1.3	< LOQ	445.6	335.3
P507c	*F. avenaceum* (FTSC 4)	-	-	-	-	-	< LOQ	< LOQ	33.1	5.6	362.9	154.3
P514a	*F. avenaceum* (FTSC 4)	+	-	+	-	-	< LOQ	6	160.4	48.9	207.7	320.6
P2149a	*F. avenaceum* (FTSC 4)	-	-	-	-	+	< LOQ	1.5	69.1	1.4	803.9	165.8
P2164a	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	< LOQ	63.7	9.3	338.6	66.6
P2221b	*F. avenaceum* (FTSC 4)	+	+	-	-	-	< LOQ	< LOQ	6.9	< LOQ	283.8	313.5
P64d	*F. iranicum* (FTSC 6)	+	-	-	+	-	< LOQ	60.8	2.7	20	< LOQ	406.4
P2289a	*Fusarium* sp. (FTSC 11)	-	-	-	+	-	< LOQ	< LOQ	74.3	12.6	159.5	6.2
F444	*Fusarium* sp. (FTSC 12)	+	-	+	+	-	< LOQ	3.1	11.3	9.6	138	< LOQ
F445	*Fusarium* sp. (FTSC 12)	+	+	-	+	-	< LOQ	< LOQ	1.5	1.5	12.4	238.2
F1036	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	15.2	59.2	43.5	171.5	239.0
F1093	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	78.4	12.4	136	176.5	194.8
F1233	*Fusarium* sp. (FTSC 12)	+	-	+	+	-	< LOQ	< LOQ	< LOQ	< LOQ	27.6	119.1
F1456	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	23.2	80.4	64	187.4	228.6
F1459	*Fusarium* sp. (FTSC 12)	+	+	-	+	-	< LOQ	47.9	128.7	115.6	277.2	85.7

F1463	*Fusarium* sp. (FTSC 12)	-	-	-	-	-	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	16.8
F1465	*Fusarium* sp. (FTSC 12)	+	+	-	+	-	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	76.4
F1509	*Fusarium* sp. (FTSC 12)	-	-	-	+	-	< LOQ	136.2	220.5	237.4	256	< LOQ
F1539	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	123.4	192.4	207.1	89.1	310.4
P37a	*Fusarium* sp. (FTSC 12)	-	-	-	-	-	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	14.7
P80a	*Fusarium* sp. (FTSC 12)	-	-	-	+	+	< LOQ	31.2	82.6	66.6	94.1	14.7
P129a	*Fusarium* sp. (FTSC 12)	+	-	-	+	-	< LOQ	27.8	64.8	62.3	< LOQ	471.6
P2283a	*Fusarium* sp. (FTSC 12)	+	+	-	+	-	< LOQ	48.7	142.2	125.4	322.2	6.4
F1501	*Fusarium* sp. (FTSC 13)	-	-	-	+	-	< LOQ	16.5	96.6	67.2	584.1	19.1
R972	*Fusarium* sp. (FTSC 14)	-	-	-	-	+	< LOQ	36.1	110.9	97.5	295.8	< LOQ
F1540	*Fusarium* sp. (FTSC 14)	-	-	-	-	-	< LOQ	< LOQ	< LOQ	< LOQ	574.5	333.0
P78c	*Fusarium* sp. (FTSC 15)	+	-	-	+	-	< LOQ	13	64.8	46.5	483.3	35.3

[a]Isolate collection maintained at the Department of Agricultural and Food Science, University of Bologna, Bologna, Italy.

[b]An informal ad hoc nomenclature employing Arabic numerals was used to distinguish taxa because six of the 10 species tested for mycotoxin production in vitro lack Latin binomials.

[c]Mycotoxins and other secondary metabolites analyzed by GC/MS: CHL = chlamydosporol; ACU = acuminatopyrone; LONG = longiborneol; FUNG = fungerin; BUT = butenolide.

[d]Mycotoxins and other secondary metabolites analyzed by LC-MS: ENNA = enniatin A; ENNA1 = enniatin A1; ENNB = enniatin B; ENNB1 = enniatin B1; MON = moniliformin; AODol = 2-Amino-14,16-dimethyloctadecan-3-ol. "< LOQ" indicates below method limit of quantitation (1 ng/µl)

[e] +/- = detected / not detected

Figure 1

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Figure 2

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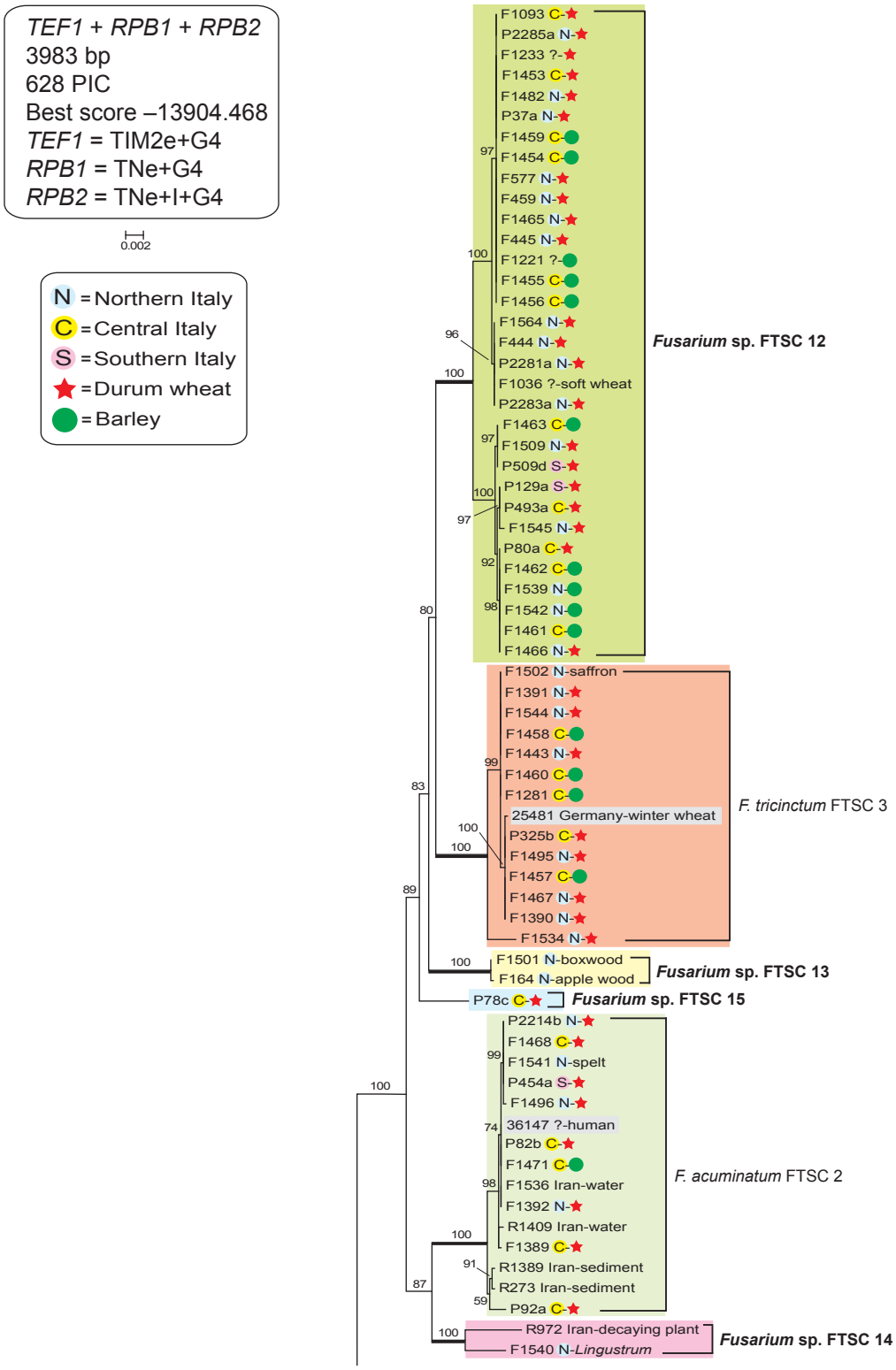

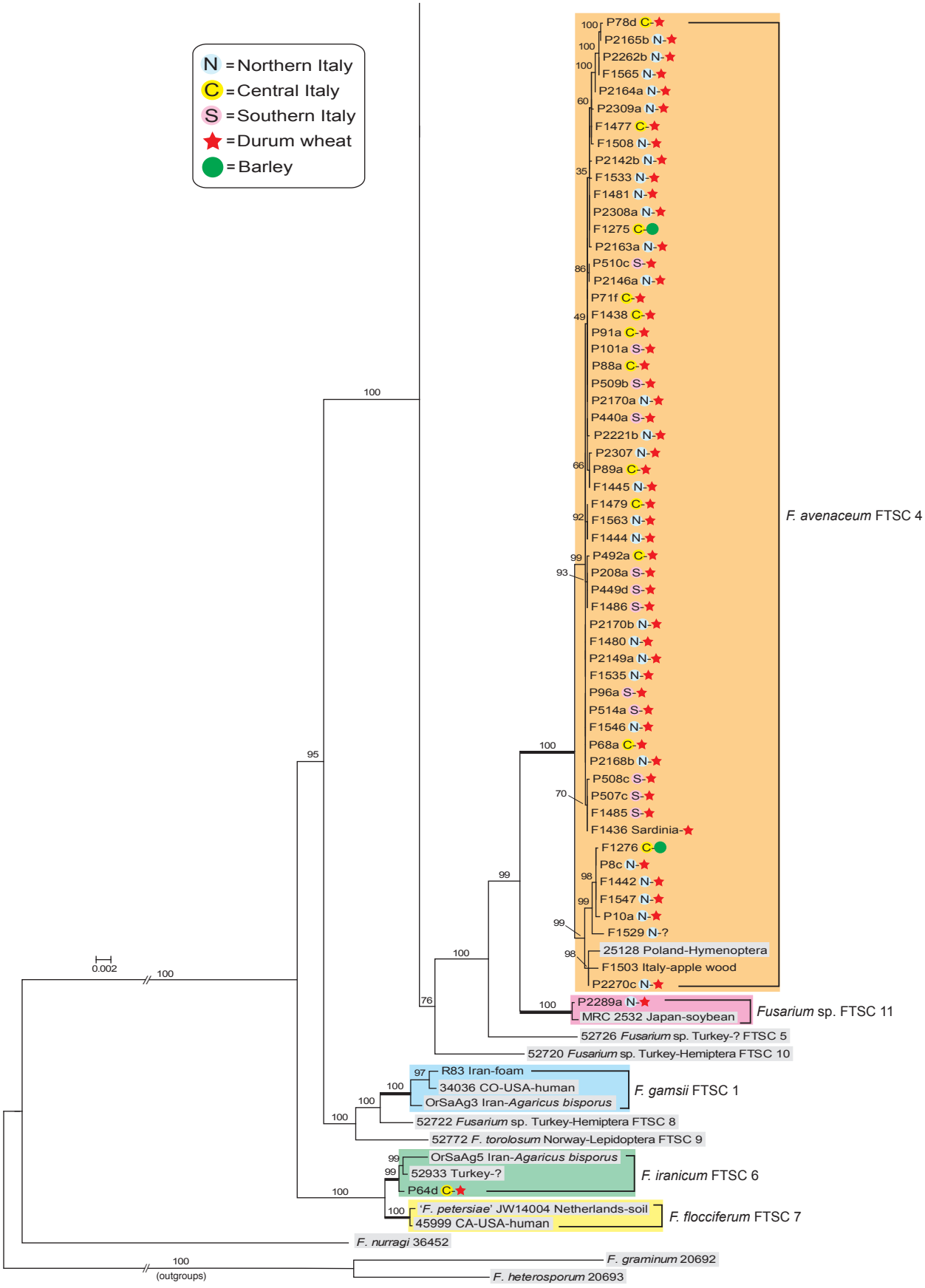
N = Northern Italy
C = Central Italy
S = Southern Italy
= Durum wheat
= Barley
P78d C-
P2165b N-
P2262b N-
F1565 N-
P2164a N-
P2309a N-
F1477 C-
F1508 N-
P2142b N-
F1533 N-
F1481 N-
P2308a N-
F1275 C-
P2163a N-
P510c S-
P2146a N-
P71f C-
F1438 C-
P91a C-
P101a S-
P88a C-
P509b S-
P2170a N-
P440a S-
P2221b N-
P2307 N-
P89a C-
F1445 N-
F1479 C-
F1563 N-
F1444 N-
P492a C-
P208a S-
P449d S-
F1486 S-
P2170b N-
F1480 N-
P2149a N-
F1535 N-
P96a S-
P514a S-
F1546 N-
P68a C-
P2168b N-
P508c S-
P507c S-
F1485 S-
F1436 Sardinia-
F1276 C-
P8c N-
F1442 N-
F1547 N-
P10a N-
F1529 N-?
25128 Poland-Hymenoptera
F1503 Italy-apple wood
P2270c N-
F. avenaceum FTSC 4
P2289a N-
MRC 2532 Japan-soybean
Fusarium sp. FTSC 11
52726 Fusarium sp. Turkey-? FTSC 5
52720 Fusarium sp. Turkey-Hemiptera FTSC 10
R83 Iran-foam
34036 CO-USA-human
OrSaAg3 Iran-Agaricus bisporus
F. gamsii FTSC 1
52722 Fusarium sp. Turkey-Hemiptera FTSC 8
52772 F. torolosum Norway-Lepidoptera FTSC 9
OrSaAg5 Iran-Agaricus bisporus
52933 Turkey-?
P64d C-
F. iranicum FTSC 6
'F. petersiae' JW14004 Netherlands-soil
45999 CA-USA-human
F. flocciferum FTSC 7
F. nurragi 36452
F. graminum 20692
F. heterosporum 20693
(outgroups)
0.002

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Figure captions

Fig. 1: Map of regions in Italy where pathogen surveys were conducted.

Fig. 2: Maximum likelihood (ML) phylogeny inferred from combined *TEF1*+ *RPB1* and *RPB2* data set (3983 bp) of Italian (n = 117) and Iranian (n = 6) *Fusarium tricinctum* species complex (FTSC) isolates. The ML analysis included the optimal model of molecular evolution for each partition as follows: TIM2e + G4 (*TEF1*), TNe + G4 (*RPB1*) and TNe + I + G4 (*RPB2*). Putatively novel FTSC species 12 to 15 are highlighted in bold. The 11 Italian plus Iranian phylospecies are distinguished using a different colour. Reference species are highlighted in grey. Bootstrap values (% based on 5000 pseudoreplications) are shown on branches.

Supplementary data
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Supplementary Materials

Species diversity and mycotoxin production by members of the *Fusarium tricinctum* species complex associated with *Fusarium* head blight of wheat and barley in Italy

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Supplementary Table S1: Histories of strains included in the present study (ID strain, Geographic origin, host, cultivar and year of isolation)

Strain #[a]	Geographic Origin[b]	Host	Cultivar[c]	Year of isolation
F164	Emilia Romagna (Northern Italy)	Apple wood *Malus domestica*	Unknown	Unknown
F444	Emilia Romagna (Northern Italy) - Bologna	Durum wheat *Triticum durum*	Duillio	2006
F445	Emilia Romagna (Northern Italy) - Bologna	Durum wheat *Triticum durum*	Levante	2006
F459	Emilia Romagna (Northern Italy)- Bologna	Durum wheat *Triticum durum*	Neodur	2006
F577	Emilia Romagna (Northern Italy) - Bologna	Durum wheat *Triticum durum*	Neodur	2006
F1036	Italy	Soft wheat *Triticum aestivum*	Unknown	2009
F1093	Umbria (Central Italy)	Durum wheat *Triticum durum*	Unknown	2009
F1221	Italy	Barley *Hordeum vulgare*	Unknown	2012
F1233	Italy	Barley *Hordeum vulgare*	Unknown	2012
F1275	Umbria (Central Italy) - Perugia	Barley *Hordeum vulgare*	Naturel	2014
F1276	Umbria (Central Italy) - Perugia	Barley *Hordeum vulgare*	Naturel	2014
F1281	Umbria (Central Italy) - Perugia	Barley *Hordeum vulgare*	Naturel	2014
F1389	Umbria (Central Italy) - Perugia	Durum wheat *Triticum durum*	Iride	2015
F1390	Emilia Romagna (Northern Italy) - Bologna	Durum wheat *Triticum durum*	Odisseo	2015
F1391	Emilia Romagna (Northern Italy) - Piacenza	Durum wheat *Triticum durum*	Orobel	2015
F1392	Emilia Romagna (Northern Italy) - Jolanda Di Savoia	Durum wheat *Triticum durum*	Obelix	2015
F1436	Sardinia	Durum wheat *Triticum durum*	Karalis	2016
F1438	Umbria (Central Italy)	Durum wheat *Triticum durum*	Colorado	2015
F1442	Emilia Romagna (Northern Italy) - Bologna	Durum wheat *Triticum durum*	Odisseo	2015
F1443	Emilia Romagna (Northern Italy) - Bologna	Durum wheat *Triticum durum*	Orobel	2016
F1444	Emilia Romagna (Northern Italy)	Durum wheat - *Triticum durum*	Orobel	2016
F1445	Emilia Romagna (Northern Italy)	Durum wheat - *Triticum durum*	Orobel	2016

F1453	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2016
F1454	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2016
F1455	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2016
F1456	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2016
F1457	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2014
F1458	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2014
F1459	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2014
F1460	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2013
F1461	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2014
F1462	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2014
F1463	Umbria (Central Italy)	Barley *Hordeum vulgare*	Quench	2014
F1465	Emilia Romagna (Northern Italy) - Reggio Emilia	Durum wheat *Triticum durum*	Odisseo	2016
F1466	Emilia Romagna (Northern Italy) - Modena	Durum wheat *Triticum durum*	Odisseo	2016
F1467	Emilia Romagna (Northern Italy) - Modena	Durum wheat *Triticum durum*	Odisseo	2016
F1468	Umbria (Central Italy)	Durum wheat - *Triticum durum*	Unknown	2010
F1471	Umbria (Central Italy)	Barley *Hordeum vulgare*	Unknown	2016
F1477	Umbria (Central Italy)	Durum wheat *Triticum durum*	Unknown	2014
F1479	Umbria (Central Italy)	Durum wheat *Triticum durum*	Unknown	2009
F1480	Emilia Romagna (Northern Italy)	Durum wheat *Triticum durum*	Unknown	2009
F1481	Emilia Romagna (Northern Italy)	Durum wheat *Triticum durum*	Unknown	Unknown
F1482	Emilia Romagna (Northern Italy)	Durum wheat *Triticum durum*	Cesare	2017
F1485	Apulia (Southern Italy)	Durum wheat *Triticum durum*	Unknown	Unknown
F1486	Apulia (Southern Italy)	Durum wheat *Triticum durum*	Unknown	Unknown
F1495	Emilia Romagna (Northern Italy) - Ravenna	Durum wheat *Triticum durum*	Tyrex	2017
F1496	Emilia Romagna (Northern Italy) - Ravenna	Durum wheat *Triticum durum*	Tyrex	2017
F1501	Emilia Romagna (Northern Italy)	Boxwood *Buxus sempervirens*	Unknown	2017
F1502	Emilia Romagna (Northern Italy)	Saffron *Crocus sativus*	Unknown	2017
F1503	Italy	Apple wood *Malus domestica*	Unknown	2017
F1508	Emilia Romagna (Northern Italy)	Durum wheat *Triticum durum*	Rusticano	2018
F1509	Emilia Romagna (Northern Italy)	Durum wheat *Triticum durum*	Saragolla	2018
F1529	Emilia Romagna (Northern Italy) - Piacenza	Unknown	Athoris	2018
F1533	Emilia Romagna (Northern Italy) Reggio Emilia	Durum wheat *Triticum durum*	Levante	2018
F1534	Emilia Romagna (Northern Italy) Reggio Emilia	Durum wheat *Triticum durum*	Levante	2018
F1535	Emilia Romagna (Northern Italy) - Parma	Durum wheat *Triticum durum*	Monastir	2018
F1536	Iran	Water	NA	2018
F1539	Emilia Romagna (Northern Italy) - Bologna	Barley *Hordeum vulgare*	Ketos	2018

F1540	Emilia Romagna (Northern Italy) - Bologna	*Ligustrum* sp.	Unknown	2018
F1541	Emilia Romagna (Northern Italy) - Bologna	Spelt *Triticum monococcum*	Unknown	2018
F1542	Emilia Romagna (Northern Italy) -Bologna	Barley *Hordeum vulgare*	Ketos	2018
F1544	Liguria (Northern Italy) - Albaro	Durum wheat *Triticum durum*	Jubilar	2018
F1545	Emilia Romagna (Northern Italy) - Bologna	Durum wheat *Triticum durum*	Consort	2018
F1546	Emilia Romagna (Northern Italy) - Bologna	Durum wheat *Triticum durum*	Unknown	2018
F1547	Emilia Romagna (Northern Italy) - Bologna	Durum wheat *Triticum durum*	Marco Aurelio	2018
F1563	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	Oliver	2018
F1564	Lombardy (Northern Italy) - Mantova	Durum wheat *Triticum durum*	Oliver	2018
F1565	Lombardy (Northern Italy) - Mantova	Durum wheat *Triticum durum*	PR22D66	2018
P8c	Veneto (Northern Italy) - Verona	Durum wheat *Triticum durum*	Obelix	2018
P10a	Veneto (Northern Italy) - Verona	Durum wheat *Triticum durum*	Obelix	2018
P37a	Lombardy (Northern Italy) -Mantova	Durum wheat *Triticum durum*	Oliver	2018
P64d	Abruzzo (Central Italy) - L'Aquila	Durum wheat *Triticum durum*	Marco Aurelio	2018
P68a	Abruzzo (Central Italy) - L'Aquila	Durum wheat *Triticum durum*	Marco Aurelio	2018
P71f	Abruzzo (Central Italy) - L'Aquila	Durum wheat *Triticum durum*	Marco Aurelio	2018
P78c	Abruzzo (Central Italy) - Chieti	Durum wheat *Triticum durum*	Marco Aurelio	2018
P78d	Abruzzo (Central Italy) - Chieti	Durum wheat *Triticum durum*	Marco Aurelio	2018
P80a	Abruzzo (Central Italy) - Chieti	Durum wheat *Triticum durum*	Marco Aurelio	2018
P82b	Abruzzo (Central Italy) - Chieti	Durum wheat *Triticum durum*	Marco Aurelio	2018
P88a	Molise (Central Italy) Campobasso	Durum wheat *Triticum durum*	Marco Aurelio	2018
P89a	Molise (Central Italy) - Campobasso	Durum wheat *Triticum durum*	Marco Aurelio	2018
P91a	Molise (Central Italy) - Campobasso	Durum wheat *Triticum durum*	Marco Aurelio	2018
P92a	Molise (Central Italy) - Campobasso	Durum wheat *Triticum durum*	Marco Aurelio	2018
P96a	Campania (Southern Italy) - Benevento	Durum wheat *Triticum durum*	Aureo	2018
P101a	Campania (Southern Italy) - Benevento	Durum wheat *Triticum durum*	Aureo	2018
P129a	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Anthalis	2018
P208a	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Anthalis	2018
P325b	Abruzzo (Central Italy) - Teramo	Durum wheat *Triticum durum*	Marco Aurelio	2018
P440a	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Antalis	2018
P449d	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Iride	2018
P454a	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Iride	2018
P492a	Abruzzo (Central Italy) - Teramo	Durum wheat *Triticum durum*	Marco Aurelio	2018
P493a	Abruzzo (Central Italy) - Teramo	Durum wheat *Triticum durum*	Marco Aurelio	2018
P507c	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Saragolla	2018
P508c	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Saragolla	2018
P509b	Apulia	Durum wheat	Saragolla	2018

	(Southern Italy) - Foggia	*Triticum durum*		
P509d	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Saragolla	2018
P510c	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Saragolla	2018
P514a	Apulia (Southern Italy) - Foggia	Durum wheat *Triticum durum*	Saragolla	2018
P2142b	Emilia Romagna (Northern Italy) - Parma	Durum wheat *Triticum durum*	Monastir	2018
P2146a	Emilia Romagna (Northern Italy) - Parma	Durum wheat *Triticum durum*	Monastir	2018
P2149a	Emilia Romagna (Northern Italy) - Parma	Durum wheat *Triticum durum*	Monastir	2018
P2163a	Emilia Romagna (Northern Italy) Reggio Emilia	Durum wheat *Triticum durum*	Levante	2018
P2164a	Emilia Romagna (Northern Italy) Reggio Emilia	Durum wheat *Triticum durum*	Levante	2018
P2165b	Emilia Romagna (Northern Italy) Reggio Emilia	Durum wheat *Triticum durum*	Levante	2018
P2168b	Emilia Romagna (Northern Italy) Reggio Emilia	Durum wheat *Triticum durum*	Levante	2018
P2170a	Emilia Romagna (Northern Italy) Reggio Emilia	Durum wheat *Triticum durum*	Levante	2018
P2170b	Emilia Romagna (Northern Italy) Reggio Emilia	Durum wheat *Triticum durum*	Levante	2018
P2214b	Emilia Romagna (Northern Italy) - Piacenza	Durum wheat *Triticum durum*	Athoris	2018
P2221b	Emilia Romagna (Northern Italy) - Carpi	Durum wheat *Triticum durum*	Tito Flavio	2018
P2262b	Lombardy (Northern Italy) - Mantova	Durum wheat *Triticum durum*	PR22D66	2018
P2270	Lombardy (Northern Italy) - Ostiglia	Durum wheat *Triticum durum*	Pigreco	2018
P2281a	Lombardy (Northern Italy) - Ostiglia	Durum wheat *Triticum durum*	Pigreco	2018
P2283a	Lombardy (Northern Italy) - Ostiglia	Durum wheat *Triticum durum*	Pigreco	2018
P2285a	Lombardy (Northern Italy) - Ostiglia	Durum wheat *Triticum durum*	Pigreco	2018
P2289a	Lombardy (Northern Italy) - Ostiglia	Durum wheat *Triticum durum*	Pigreco	2018
P2307	Lombardy (Northern Italy) - Mantova	Durum wheat *Triticum durum*	Levante	2018
P2308a	Lombardy (Northern Italy) - Mantova	Durum wheat *Triticum durum*	Levante	2018
P2309a	Lombardy (Northern Italy) - Mantova	Durum wheat - *Triticum durum*	Levante	2018
R83	Iran	Foam	NA	2018
R273	Iran	Sediment	NA	2018
R972	Iran	Submerged decaying stem or root	NA	2018
R1389	Iran	Sediment	NA	2018
R1409	Iran	Water	NA	2018
NRRL 34036 = UTHSC 01-1965*****	CO, USA	Ethmoid aspirate	NA	Unknown
CBS 143609 = OrSaAg3*****	Orumieh-Salmas, Iran	*Agaricus bisporus*	NA	2016
NRRL 36147 = CBS 109232*****	Unkown	Human bronchial secretion	Unknown	Unknown
NRRL 25481 = CBS 393.93 = BBA 64485 (neotype)*****	Germany	Winter wheat	Diplomat	1984
NRRL 25128 = ARSEF 1331*****	Poland	*Hymenoptera ichneumonidae*	NA	Unknown
NRRL 52726 = ARSEF 8299*****	Turkey	Unknown	Unknown	Unknown

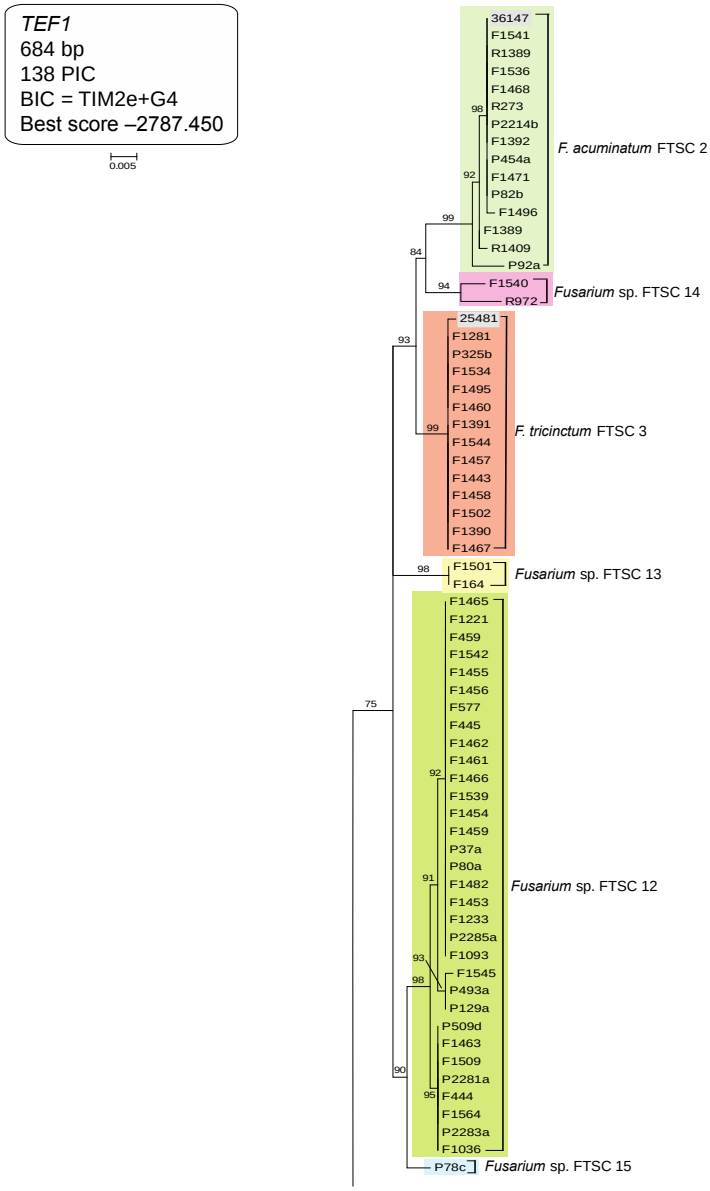
NRRL 52933 = ARSEF 8648*****	Turkey	Unknown	Unknown	Unknown
CBS 143611 = OrSaAg3*****	Orumieh-Salmas, Iran	*Agaricus bisporus*	NA	2016
NRRL 45999 = UTHSC 06-3449*****	CA, USA	Human scalp	Unknown	Unknown
CBS 143231 = JW14004*****	Netherlands	Soil	NA	2017
NRRL 52722 = ARSEF 6401*****	Turkey	*Eurygaster* sp.	NA	1999
NRRL 52772 = ARSEF 5560*****	Norway	*Galleria mellonella* larva	NA	Unknown
NRRL 52720 = ARSEF 6410*****	Turkey	*Eurygaster* sp.	NA	1999
MRC 2532*****	Japan	Soybean	Unknown	Unknown
NRRL 20692*****	Ethiopia	*Cynodon dactylon*	Unknown	Unknown
NRRL 20693*****	Netherlands	*Claviceps purpurea* on *Lolium perenne*	NA	Unknown
NRRL 36452 = CBS 831.85 = BBA 64346*****	Australia	Soil	NA	Unknown

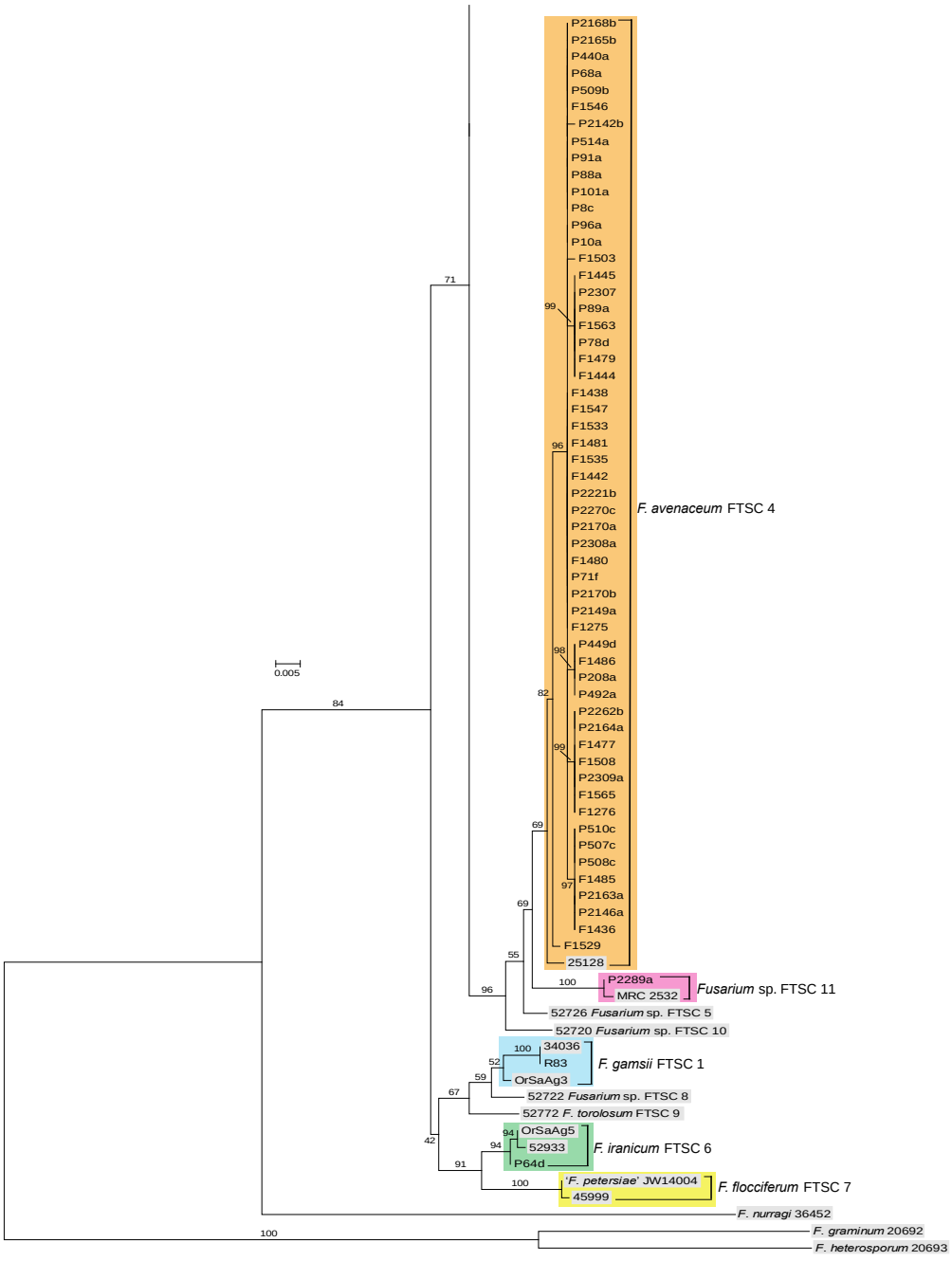
[a] F, P and R strains belong to a collection maintained at the Department of Agricultural and Food Science, University of Bologna, Bologna, Italy; ARSEF, ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, New York, USA; BBA, Biologische Bundesanstalt für Land-und Forstwirtschaft, Institute für Mikrobiologie, Berlin, Germany; CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; MRC, former South African Medical Research Council Collection, currently housed at the Agricultural Research Council, Pretoria, South Africa; NRRL, ARS Culture Collection, Peoria, Illinois, USA; UTHSC, University of Texas Health Sciences Center, San Antonio, TX, USA. Strains followed by an Asterix were used as a reference (See Fig. 2).

[b] See Fig. 1 for map showing regions where pathogen surveys were conducted.

[c] NA = not applicable.

Supplementary Figure S1: Maximum likelihood tree based on *TEF1* sequences (684 bp alignment) of 123 analyzed FTSC isolates collected across Italy and Iran in relation to FTSC reference sequences (isolates highlighted in grey). Analysis was based on TIM2e + G4 model of molecular evolution. Bootstrap values (% based on 5000 pseudoreplications) are shown on branches.




P2168b
P2165b
P440a
P68a
P509b
F1546
P2142b
P514a
P91a
P88a
P101a
P8c
P96a
P10a
F1503
F1445
P2307
P89a
F1563
P78d
F1479
F1444
F1438
F1547
F1533
F1481
F1535
F1442
P2221b
P2270c
P2170a
P2308a
F1480
P71f
P2170b
P2149a
F1275
P449d
F1486
P208a
P492a
P2262b
P2164a
F1477
F1508
P2309a
F1565
F1276
P510c
P507c
P508c
F1485
P2163a
P2146a
F1436
F1529
25128
F. avenaceum FTSC 4
0.005
P2289a
MRC 2532
Fusarium sp. FTSC 11
52726 Fusarium sp. FTSC 5
52720 Fusarium sp. FTSC 10
34036
R83
OrSaAg3
F. gamsii FTSC 1
52722 Fusarium sp. FTSC 8
52772 F. torolosum FTSC 9
OrSaAg5
52933
P64d
F. iranicum FTSC 6
'F. petersiae' JW14004
45999
F. flocciferum FTSC 7
F. nurragi 36452
F. graminum 20692
F. heterosporum 20693
71
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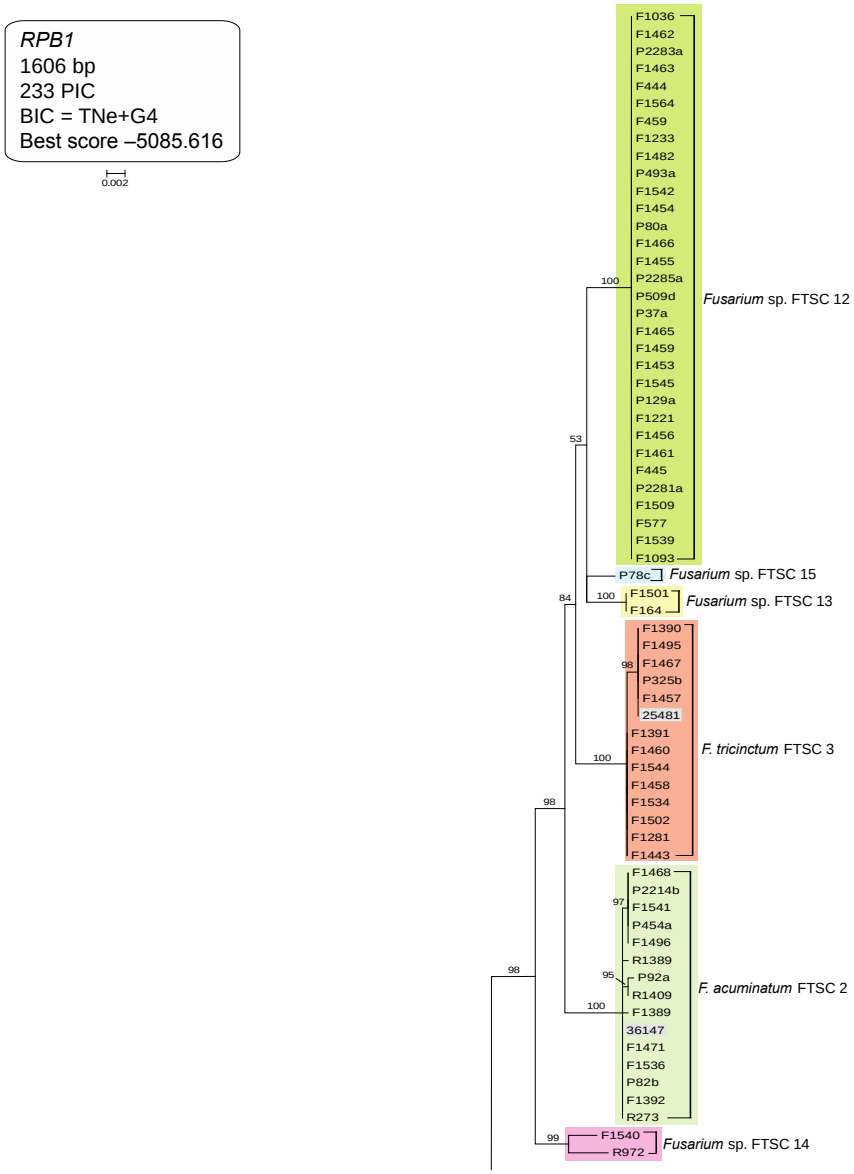
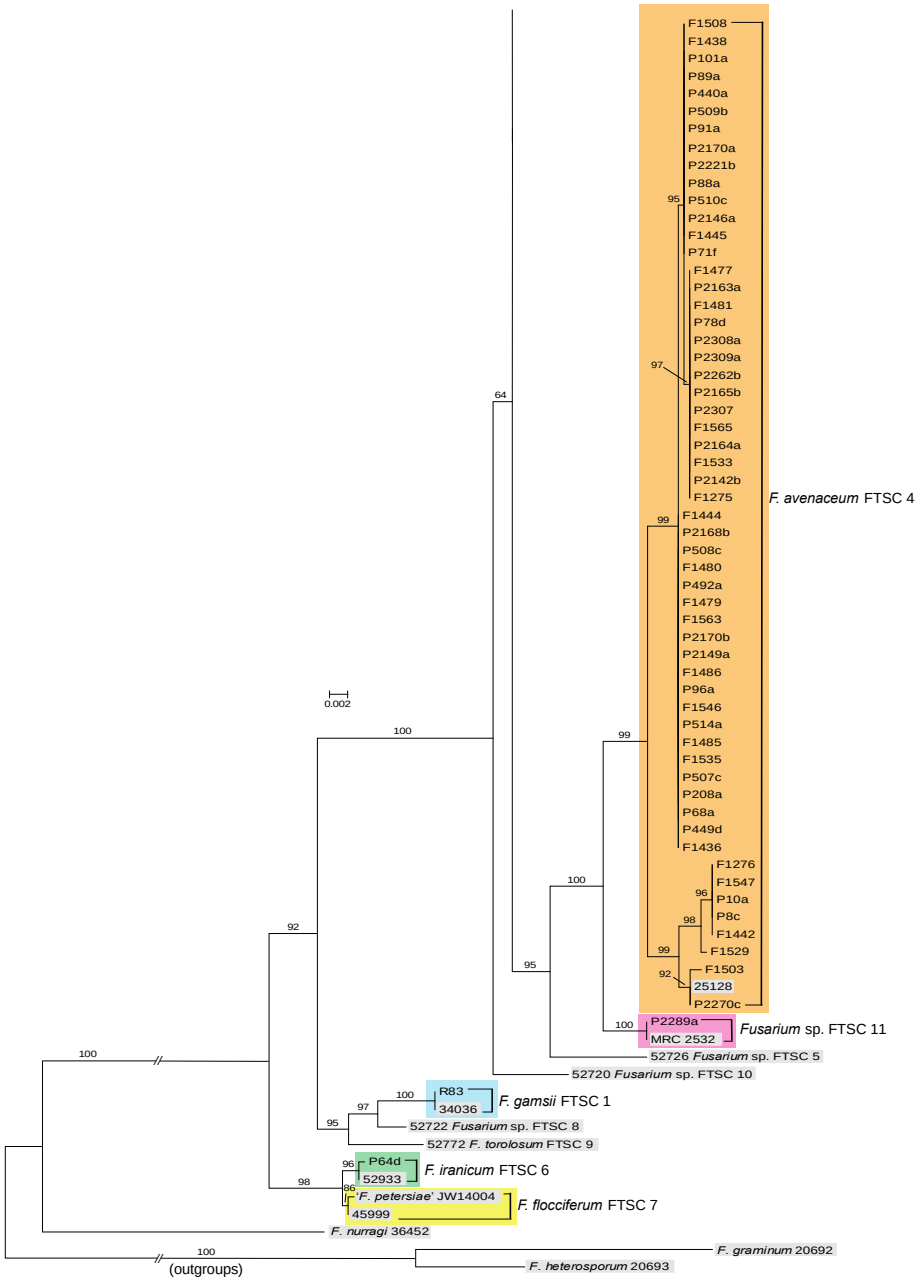
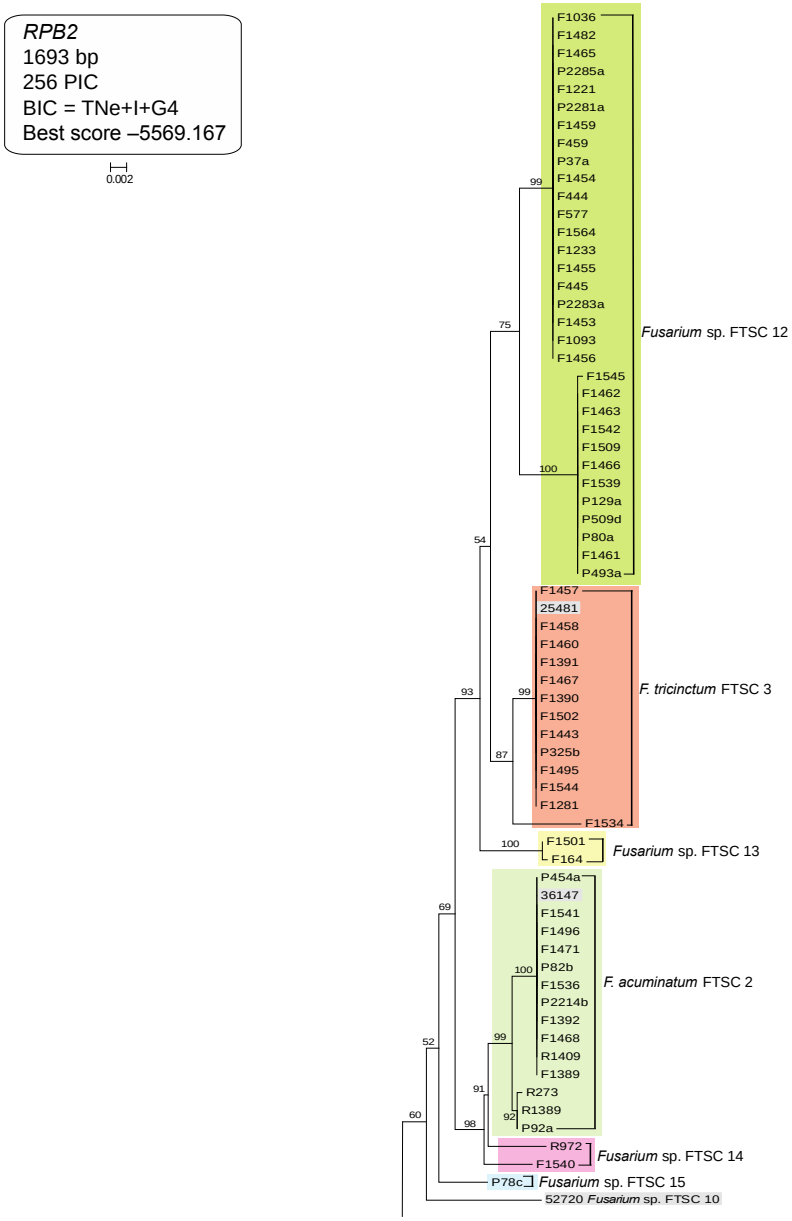
84
100

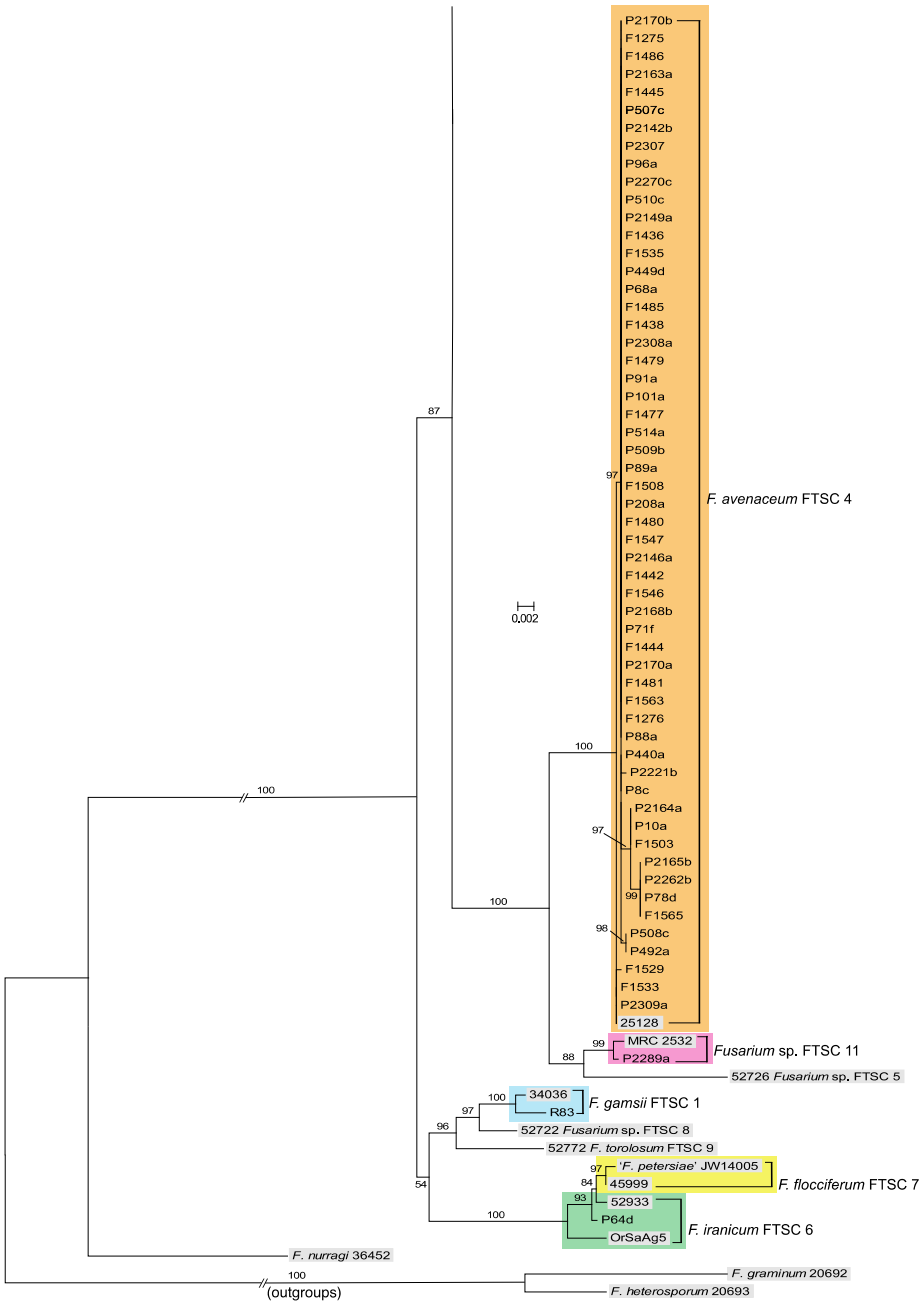
Supplementary Figure S2: Maximum likelihood tree based on *RPB1* sequences (1606 bp alignment) of 123 analyzed FTSC isolates collected across Italy (n= 117) and Iran (n= 6) in relation to FTSC reference sequences (isolates highlighted in grey). Analysis was based on TNe + G4 model of molecular evolution. Bootstrap values (% based on 5000 pseudoreplications) are shown on branches.




F1508
F1438
P101a
P89a
P440a
P509b
P91a
P2170a
P2221b
P88a
P510c
P2146a
F1445
P71f
F1477
P2163a
F1481
P78d
P2308a
P2309a
P2262b
P2165b
P2307
F1565
P2164a
F1533
P2142b
F1275
F. avenaceum FTSC 4
F1444
P2168b
P508c
F1480
P492a
F1479
F1563
P2170b
P2149a
F1486
P96a
F1546
P514a
F1485
F1535
P507c
P208a
P68a
P449d
F1436
F1276
F1547
P10a
P8c
F1442
F1529
F1503
25128
P2270c
P2289a
MRC 2532
Fusarium sp. FTSC 11
52726 Fusarium sp. FTSC 5
52720 Fusarium sp. FTSC 10
R83
34036
F. gamsii FTSC 1
52722 Fusarium sp. FTSC 8
52772 F. torolosum FTSC 9
P64d
52933
F. iranicum FTSC 6
'F. petersiae' JW14004
45999
F. flocciferum FTSC 7
F. nurragi 36452
F. graminum 20692
F. heterosporum 20693
(outgroups)
0.002

Supplementary Figure S3: Maximum likelihood tree based on *RPB2* sequences (1693 bp alignment) of 123 analyzed FTSC isolates collected across Italy and Iran in relation to FTSC reference sequences (isolates highlighted in grey). Analysis was based on TNe + I + G4 model of molecular evolution. Bootstrap values (% based on 5000 pseudoreplications) are shown on branches.




P2170b
F1275
F1486
P2163a
F1445
P507c
P2142b
P2307
P96a
P2270c
P510c
P2149a
F1436
F1535
P449d
P68a
F1485
F1438
P2308a
F1479
P91a
P101a
F1477
P514a
P509b
P89a
F1508
P208a
F1480
F1547
P2146a
F1442
F1546
P2168b
P71f
F1444
P2170a
F1481
F1563
F1276
P88a
P440a
P2221b
P8c
P2164a
P10a
F1503
P2165b
P2262b
P78d
F1565
P508c
P492a
F1529
F1533
P2309a
25128
F. avenaceum FTSC 4
0.002
MRC 2532
P2289a
Fusarium sp. FTSC 11
52726 Fusarium sp. FTSC 5
34036
R83
F. gamsii FTSC 1
52722 Fusarium sp. FTSC 8
52772 F. torolosum FTSC 9
'F. petersiae' JW14005
45999
F. flocciferum FTSC 7
52933
P64d
OrSaAg5
F. iranicum FTSC 6
F. nurragi 36452
F. graminum 20692
F. heterosporum 20693
(outgroups)
87
100
100
100
97
99
98
99
88
100
97
96
54
97
84
93
100
100
100