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Keywords: Oryza sativa; Fusarium semitectum; trichothecenes; zearalenone

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Viçosa, 23 March 2019

Dr. L. Cocolin
Editor-In-Chief
International Journal of Food Microbiology

Dear Prof. Luca Cocolin

I am uploading a new version with the changes request after the technical check.

1) Style for the unit should be g/l and not gl-1

DONE. There were only three cases that were modified.

Yours sincerely,
pastedGraphic.png ¬

Emerson M. Del Ponte (Prof.)
On behalf of all authors

***Highlights (for review)**

- Nine species within the *Fusarium incarnatum-equiseti* species complex were found in Brazilian rice
- A new species, FIESC 37 was found to be second in prevalence, following FIESC 26
- Sixteen out of 46 strains produced mycotoxin in vitro
- FIESC 26 produced zearalenone (DON) and FIESC 37 produced DON and ZEAE
- The *incarnatum* morphotype was dominant compared to *equiseti* morphotype

Fusarium incarnatum-equiseti species complex associated with Brazilian rice: phylogeny, morphology and toxigenic potential

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Abstract

Fusarium incarnatum-equiseti species complex (FIESC) is commonly detected in rice kernels grown in Brazil, but knowledge of species limits and toxigenic potential is lacking. Seventy strains morphologically identified as FIESC-like, isolated from all rice-growing regions of Brazil, were subjected to sequencing of *EF-1α* gene. Among them, 18 selected strains were analyzed for their *RPB2* gene sequences. Nine phylogenetic species were identified, among which eight matched the previously reported FIESC 4 (*F. lacertarum*), 6, 16, 17, 20, 24, 26 and 29. One new phylogenetic species was identified, and named FIESC 37. Five strains formed new

singleton lineages. The most dominant species were FIESC 26 (22/70 strains) and FIESC 37 (21/70), the newly identified. The incarnatum morphotype was dominant (n = 10 phylogenetic species) over the equiseti (n = 4). Among forty-six selected strains representing all species, only 16 strains produced detectable levels of mycotoxins *in vitro*. FIESC 26 produced ZEA and FIESC 37 produced both ZEA and DON. ZEA was produced by nine isolates of three other species, among which few isolates produced trichothecenes: DON (5/46), NIV (3/46), 4-ANIV (2/46), 15-ADON (1/46) and 3-ADON (1/46). The T-2 and HT-2 mycotoxins were not detected. Our results contribute novel information on species limits and mycotoxin production within FIESC infecting cereals in the southern hemisphere and provide baseline data for further exploring morphological differences among the species.

Keywords: *Oryza sativa*, *Fusarium semitectum*, trichothecenes, zearalenone

1. Introduction

Brazil is ranked among the top ten largest rice-producing countries in the world and first outside Asia (FAO, 2018). The total country's production, averaging 12 million tons in recent seasons, is originated mainly (>80%) from the two southernmost states of Brazil: Rio Grande do Sul and Santa Catarina (FAO, 2018). Among the biotic stresses that affect rice crops, foliar and panicle fungal diseases are of significant concern to rice farmers due to losses to both grain yield and quality (Cartwright et al., 2018). Several fungi are capable of infecting and colonizing rice kernels during pre- or post-harvest periods, among which some produce mycotoxins of concern to human and animal health, including *Fusarium* spp. (Desjardins, 2006). Although a wide range of *Fusarium* mycotoxins has been reported in association with rice grains worldwide (Abbas et

al., 1998; Agarwal et al., 1989; Desjardins et al., 2000; Lee et al., 2009; Makun et al., 2011; Tanaka et al., 2007), the most commonly reported are fumonisins and moniliformin, produced by members of *Fusarium fujikuroi* species complex (FFSC; Leslie et al., 1992). Other mycotoxins include type B trichothecenes, such as nivalenol (NIV), deoxynivalenol (DON) and acetylated derivatives (15-ADON and 3-ADON), and zearalenone (ZEA) (Desjardins et al., 1997, 2000). The latter two mycotoxins are commonly produced by members of the *F. graminearum* species complex (FGSC) that infect rice (Gomes et al., 2015; Lee et al., 2009), but there are reports of trichothecene production by species referred to *F. incarnatum-equiseti* species complex (FIESC) (Goswami et al., 2008; Kosiak et al., 2005; Villani et al., 2016).

Literature data on the occurrence of *Fusarium* mycotoxins in Brazilian rice are scarce. Some reports showed that DON and ZEA were found as contaminants of parboiled rice sold at the markets (Dors et al., 2009) and natural and parboiled rice from rice experimental plots (Heidtmann-Bemvenuti et al., 2012). On the other hand, an extensive analysis of 230 samples of rice and byproducts showed that ZEA and DON occurred in a significant number of samples (45% and 8%, respectively) (Almeida et al., 2012). Recently, a survey reported ZEA in 60% of parboiled rice samples, being 36% with concentrations higher than the maximum permitted limit by the Brazilian regulation (Savi et al., 2018).

In order to protect consumers, regulatory agency in Brazil has promulgated maximum tolerated levels (MTLs) of mycotoxins for several cereal crops, including rice (ANVISA, 2011). The MTLs of two mycotoxins in rice were updated recently, to be effective in January 2017: DON in rice bran (1,250 µg/kg), ZEA in processed, raw and bran of rice (100, 400 and 600 µg/kg) (ANVISA, 2017). Therefore, it is imperative to increase surveillance and provide knowledge on the diversity of toxigenic *Fusarium* species and their toxigenic potential. Such

information is valuable to define targets in microbiological and mycotoxin surveys as well as future revision of MTLs. Our recent survey showed the presence of *Fusarium* mycotoxins and at least four species complexes associated with rice in Brazil, including FIESC (Moreira et al., unpublished). However, accurate identification of species has been made available only for FGSC strains (Gomes et al., 2015).

FIESC species are commonly regarded as saprophytes and opportunistic plant pathogens, but have also been found associated with human diseases of clinical environments (Jurado et al., 2005; O'Donnell et al., 2009). Species within FIESC have been found in wheat, barley, oat and maize kernels (Castellá and Cabañes, 2014; Marín et al., 2012; O'Donnell et al., 2018; Piacentini et al., 2019; Villani et al., 2016). In rice, FIESC has been detected in seeds produced in Asia, Europe and Africa (Amatulli et al., 2010; Castellá and Cabañes, 2014; Desjardins et al., 2000; Maheshwar and Janardhana, 2010; Makun et al., 2011; Marín et al., 2012). Strains of FIESC are capable of producing a range of mycotoxins, including fusarochromanone (FUSCHR), ZEA and trichothecenes (DON, NIV, T-2 toxin, 4-acetylnivalenol [4-ANIV], fusarenone X [FUS-X]), equisetin (EQ), diacetoxyscirpenol (DAS), 15-monoacetoxy-scirpentriol (MAS), neosolaniol (NEO), beauvericin (BEA), butenolide (BUT), fusaric acid (FA), 8-O-methylbostrycoidin (MBO) and moniliformin (MON) (Kosiak et al., 2005; Leslie and Summerell, 2006; Logrieco et al., 1998; O'Donnell et al., 2018; Villani et al., 2016).

Knowledge of the variation in morphological traits within FIESC is very limited and only four species are formally described: *F. equiseti*, *F. lacertarum*, *F. scirpi* and *F. semitectum*. Typification of *F. semitectum* and its supposed synonyms *F. pallidoroseum* and *F. incarnatum* is largely unclear and needs further investigations (Leslie and Summerell, 2006; O'Donnell et al., 2009). Most of the cryptic *Fusarium* species, which cannot be distinguished by morphological

93 markers, have been resolved by sequencing of key genes (Geiser et al., 2004; O'Donnell et al.,
94 2015). Within FIESC, the translation elongation factor 1-alpha (*EF-1α*) gene permitted the
95 separation of strains from clinical environments in North America into 28 species, among which
96 only 3 species have been formally described, while Arabic numbers were assigned to the rest of
97 the species to facilitate communication (O'Donnell et al., 2009). Further studies have reported
98 new species including FIESC 29 and FIESC 30 (insects) (O'Donnell et al., 2012); FIESC 31 and
99 FIESC 32 (plumbing drain water) (Short et al., 2011); FIESC 31 (cereal-borne) (Villani et al.,
100 2016), currently redesignated as FIESC 33 (Villani et al., in press); and FIESC 34, 35 and 36
101 (soybean, wheat and maize) (O'Donnell et al., 2018). Two studies using larger sampling from
102 cereals reported seven FIESC species in Spanish cereals (Castellá and Cabañes, 2014) and five
103 FIESC in cereals from North America, Europe and Turkey (Villani et al., 2016). Recently, one
104 strain isolated from barley (Piacentini et al., 2019) and another two from rice (Savi et al., 2018)
105 in Brazil grouped into FIESC clade. Another strain, identified as FIESC 21, was obtained from
106 barley in Uruguay (Garmendia et al., 2018). Nevertheless, to the best of our knowledge,
107 comprehensive information on the diversity and toxigenic potential of FIESC species infecting
108 cereals in South America is lacking.

109 Species composition and their spatial distribution, phylogenetic relationships, toxigenic
110 potential and morphology within FIESC associated with rice in Brazil and elsewhere are poorly
111 known. We hypothesized that some of the described species, or even underscribed ones, occur in
112 Brazilian rice at frequencies that vary across the distinct rice-growing regions in the country that
113 experience contrasting climate and rice-growing conditions. The objectives of this study were to
114 1) resolve FIESC from Brazilian rice into phylogenetic species, 2) characterize their morphology
115 and 3) assess their ability to produce trichothecenes and zearalenone.

2. Materials and Methods

2.1. Sampling, strains collection and isolation procedures

The strains used in this study were obtained from either mature grains or field-collected panicles obtained from the major rice-producing regions of Brazil during three different seasons. They were separated into three collections based on the season and time of sampling (pre- or post-harvest): a) historical sample (2012/13), constituted of 13 FIESC-like strains isolated from mature kernels produced in Rio Grande do Sul (RS) state; b) a contemporary sample (2014/15 and 2015/16 seasons) of 109 strains from field collections prior to harvest in RS, Goiás (GO), Mato Grosso (MT), Tocantins (TO), Maranhão (MA), Roraima (RR) and São Paulo (SP) states; c) a field strain collection of 29 strains from the 2015/16 season isolated from grains of rice panicles collected randomly, during harvest, at rice fields in the states of Santa Catarina (SC), RS and TO (Figure 1).

The isolates were obtained from seven to ten-days old colonies grown on developing/mature grains assayed in a standard blotter test (25 °C and 12/12 light/dark cycle). These were randomly selected from a composite sample from panicles or seed samples. Mycelia and conidia were harvested from fungal colonies resembling *Fusarium* and grown on synthetic nutrient-poor agar (SNA, Nirenberg, 1976). One hundred and fifty one isolates were assigned to FIESC based on their similarity to the colony color and micromorphological characteristics described by Leslie and Summerell (2006) for *F. equiseti* and *F. semitectum*, which represent the diagnostic morphotypes for members of this species complex. A subsample was selected based on geographic criteria in order to reduce sample size for conducting the molecular analyses. The isolates were single-spore cultured and preserved in microtubes at 4 ºC during storage.

139

140 *2.2. DNA extraction, PCR assays and sequencing*

141 The isolates were grown on potato dextrose agar (PDA, Acumedia, Neogen Corporation) for 7
142 days at 25 ºC. Fresh mycelia were harvest into 2 mL tubes and disrupted in TissueLyser (Qiagen,
143 Haan, Germany). The total genomic DNA was extracted using the Wizard® Genomic DNA
144 Purification Kit (Promega, Madison, USA) according to the manufacturer's protocol. Seventy
145 isolates were selected and used for the phylogenetic analyses as first step to identify the species
146 (Figure 1, Supplementary Table 1). Fragments of *EF-1α* gene were amplified using the primer
147 pair EF1 and EF2 (O'Donnell et al., 1998). Cycling conditions were: initial heating at 95 ºC for
148 90 s, following by 40 cycles (45 s at 95 ºC, 1 min at 52 ºC and 2 min at 72 ºC), final extension at
149 72 ºC for 5 min. A subsample of 18 representative isolates of each *EF-1α*-based identified
150 species was selected for phylogeny using a fragment of the second largest subunit of RNA
151 polymerase II (*RPB2*) (O'Donnell et al., 2008). All PCR runs used the GoTaq® Colorless Master
152 Mix (Promega, Madison, USA) in a final volume of 20 µL containing of 1 X PCR buffer, 1 µM
153 of primers, 1 µL of DMSO, 0.4 µL of BSA (10 mg/mL solution), and 50–100 ng of DNA in a
154 MG-96 MyGeneTM Thermal Cycler (Hangzhou LongGene Scientific Instruments Co., Ltd.).
155 PCR products were visualized in 1% agarose gel run in 1 X TBE buffer, under a UV light to
156 ensure the presence of single-band products. PCR products were purified with the enzymatic
157 mixture NucleoSAP (Cellco Biotec, São Carlos, Brazil). The fragments of amplified DNA were
158 sent for sequencing at Macrogen, Korea.

159

160 *2.3. Sequence alignment and phylogenetic analysis*

161 The DNA sequences were edited using SeqAssem (Hepperle, 2004) and compared with those

from GenBank using BLASTn search. Sequences of FIESC isolates were aligned with sequences of reference strains (O'Donnell et al., 2009, 2012, 2018; Short et al., 2011; Villani et al., 2016) using the MUSCLE algorithm (Edgar, 2004) implemented in the software MEGAX (Kumar et al., 2018). Alignments consisted of 198 parsimony-informative positions/713 bp for *EF-1α* and 116/867 bp for *RPB2*. The best-fitted model of nucleotide substitution for the phylogenetic analysis was estimated using jModelTest (Darriba et al., 2012). Bayesian Inference (BI) analysis was performed using MrBayes 3.2.6 (Ronquist et al., 2012) with GTR+G+I model for *EF-1α* and GTR+G for *RPB2*, 2,000,000 generations through two independent runs, sampled every 500 generations and burnin of 25% of initial trees. Phylogenetic trees were visualized using the FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Maximum-likelihood (ML) and Maximum-parsimony (MP) analyses were implemented in MEGAX software (Kumar et al., 2018) with 1,000 bootstrap replications. The models chosen for ML were GTR+G+I for *EF-1α* and GTR+G for *RPB2*. MP analysis was performed using 100 random additional sequences with tree bisection-reconnection (TBR) method of branch swapping. Phylogenetic analyses were performed for each gene partition and for the concatenated dataset with *Fusarium concolor* (NRRL 13459) used as outgroup. The generated sequences are deposited in the GenBank database under accession numbers MK298062–MK298131 (*EF-1α*) and MK298132–MK298149 (*RPB2*) (Supplementary Table 1).

2.4. Morphological characterization

A total of 39 strains representative of all identified phylogenetic species were selected for assessing morphological traits. A mycelial disk (5 mm diameter) was inoculated on the center of a plastic Petri dish containing PDA. The plates were incubated at 25 ºC in complete darkness.

185 The mycelial growth (mm) was assessed after 3 days, by measuring the two right-angled
186 colonies diameter using a pachometer, and the colony pigmentation was evaluated after 14 days
187 (Leslie and Summerell, 2006). Microscopic examination was performed on 7 to 14 day-old
188 colonies grown on plastic Petri dishes containing SNA with pieces of carnation leaves incubated
189 at 25 ºC and 12 h photoperiod (Leslie and Summerell, 2006). The following morphological traits
190 were measured and photographed: presence/absence of sporodochia and chlamydospores and
191 type of conidiogenous cells; conidia type, shape, septation and size (30 to 50 conidia per isolate,
192 measured digitally using Digimizer v4.6.1 software). The morphometric data were summarized
193 as frequency, minimum, maximum and mean values.
194
195 *2.5. Isolates and in vitro culturing for mycotoxin analysis*
196 A subsample of 46 strains representative of all phylogenetic clades was selected for the
197 evaluation of *in vitro* production of trichothecenes type A (T-2 and HT-2), type B (DON, 3-
198 ADON, 15-ADON, NIV and 4-ANIV) and ZEA. Autoclaved rice grains (30 g) were inoculated
199 with mycelial plugs (5 mm) of colonies of each isolate and incubated for 21 days in the dark at
200 25 ºC. Non-inoculated rice grains served as control. Portions of 1 g of grinded rice culture were
201 extracted with 5 mL of acetonitrile/water (84:16, v/v) with 1 % of acetic acid by orbital shaking
202 for 2 h. After filtration through filter paper (Whatman n. 4), 100 µL were diluted with 900 µL
203 ultrapure water. The residue was filtered using RC through 0.20 µm regenerated cellulose filter.
204 The mycotoxins were quantified by comparing peak areas with a calibration curves obtained
205 with standard solutions. The detection limits were 0.1 µg/g for DON, NIV, 4-ANIV and ZEA, 1
206 µg/g for 3-ADON and 15-ADON, 0.05 µg/g for T-2 and HT-2. The analyses were performed
207 according to each mycotoxin as described below.

208

209 *2.5.1. Determination of DON, NIV and acetylates*

210 Fifty µL of extract was injected into an Agilent 1260 Series HPLC (Agilent Technology, Santa
211 Clara, CA, USA) coupled to a diode array (DAD) detector; detections were set to 220 nm. The
212 analytical column was a Synergi Hydro-RP 80A (150 × 3 mm, 4 µm, Phenomenex) preceded by
213 a SecurityGuard™ cartridge (4 × 3 mm, Phenomenex), the column heater set at 40 °C. The
214 mobile phase consisted of a binary gradient was applied as follows: the initial composition was
215 92% of (A) water and 8% of (B) acetonitrile, the concentration of solvent B was linearly
216 increased to 9% in 35 min and kept constant for 27 min. The flow rate of the mobile phase was
217 0.5 mL/min. Under these analytical conditions the retention times of target toxins were NIV 4.1
218 min, DON 8.2 min, 4-ANIV 20.2 min, 3-ADON 50.8 min and 15-ADON 53.2 min.

219

220 *2.5.2. Determination of T-2 and HT-2*

221 Ten µL of extract were injected into an Agilent UHPLC apparatus (Agilent Technology, Santa
222 Clara, CA, USA). Data acquisition and instrument control were performed by LC Openlab
223 software (Agilent). The column used was a ZORBAX Eclipse Plus C18 (50 mm × 2.1 mm i.d.,
224 1.8 µm). T-2 and HT-2 were detected by DAD detector. The mobile phase consisted of a binary
225 gradient was applied as follows: the initial composition of the mobile phase 70% of (A)
226 water/30% of (B) acetonitrile was kept constant for 1.5 min, solvent B was linearly increased to
227 35 % in 0.5 min, and kept constant for 2 min. The flow rate of the mobile phase was 0.5 mL/min.
228 The column temperature was 50 °C the detector was set at 202 nm wavelength. Retention times
229 were 1.97 min for HT-2 and 4.9 min for T-2.

230

2.5.3. Determination of ZEA

A volume of 100 µL was injected in the HPLC system (Agilent 1260 Series) with a full loop injection system. The analytical column was a Luna PFP (150 × 4.60 mm, 3 µm) (Phenomenex) preceded by SecurityGuard™ cartridges PFP (4 × 3 mm, Phenomenex). The column was thermostated at 30 °C. The mobile phase consisted of a mixture of water/acetonitrile/methanol (46:46:8, v/v/v) eluted at a flow rate of 1 mL/min. The fluorometric detector was set at wavelengths, ex= 274 nm, em= 440 nm. Retention time was 7.8 min.

3. Results

3.1. Phylogenetic analysis

The *EF-1α* trees were topologically concordant and the phylogeny inferred by MP criterion allowed to identify nine species including eight previously reported species: FIESC 4 (*F. lacertarum*), FIESC 6, FIESC 16, FIESC 17, FIESC 20, FIESC 24, FIESC 26 and FIESC 29, and a new species named FIESC 37, which formed a large monophyletic group (20 strains) with statistical support (MP = 97%, ML = 98% and BI = 1 PP). All but FIESC 24 species formed strongly supported monophyletic groups (i.e., 79–100%). In addition, four strains (16Ar037, 16Ar043, 12Ar099, 12Ar016) formed singleton lineages in the phylogenies and could represent additional phylogenetic FIESC species, provisionally named *Fusarium* sp. 1 to 4, respectively.

In general, the combined dataset (*EF-1α* and *RPB2*) confirmed the monophyly of species inferred by the *EF-1α* tree (Figure 3), but only two incongruencies were detected. FIESC 24 formed a well-supported clade in the combined tree, but it did not contain 15Ar083 strain, which was grouped (unsupported) with FIESC 24 in the *EF-1α* tree (Figure 3). We thus decided to assign 15Ar083 to a singleton lineage (*Fusarium* sp. 5). The ungrouped 15Ar025 strain in the

EF-1α tree, grouped with the FIESC 37 in the combined tree (Figure 3).

3.2. FIESC prevalence and distribution

Overall, three species dominated the collection and accounted for 77% (54/70) of the strains. These were, in this order, FIESC 26 (22/70), the newly identified species FIESC 37 (21/70), and FIESC 20 (11/70). Two species were represented by three strains (FIESC 16 and *F. lacertarum*), one by two strains (FIESC 29) and the other three previously reported species and the new singleton lineages by only one strain. Of the two most represented species, FIESC 26 was found in all but TO state, while FIESC 37 was found in RS state and also at the experimental field in GO state. Of the five singleton lineages, four were found in south of Brazil (Supplementary Table 1).

3.3. Morphological characterization

Two morphological aggregates (morphotypes) were found in our 39-strain sample representative of all species/lineages. The most common morphotype was the incarnatum found in seven species and three singleton lineages. The least represented was the equiseti morphotype, which was found in *F. lacertarum* (FIESC 4), FIESC 6 and *Fusarium* sp. 3 and 4.

A distinct feature of the equiseti morphotype is the presence of a prominent foot-shaped basal cell and elongated apical cell of macroconidia (not present in incarnatum morphotype) and abundant chlamydospores produced in chains. The four species with this morphotype produced macroconidia with a dorsiventral curvature on aerial mycelium and sporodochia, usually 5-septate (Figure 4 A). Chlamydospores were produced in chains, smooth to rough walled (Figure 4 F). Smaller conidia without an elongated apical cell were also observed (Figure 4 B–C). FIESC

6 and *Fusarium* sp. 5 produced microconidia, 0 to 1-septate (Figure D–E), typical of *F. scirpi* (Leslie and Summerell 2006). This equiseti morphotype corresponds to the clade of FIESC 1 to 14, 30, 31, 34 and 35 (Figure 2).

Species belonging to the incarnatum morphotype produced straight to slightly curved macroconidia on sporodochia, without distinctive foot shaped basal cells. The species also produced abundant mesoconidia, while chlamydospores were produced by some strains and were generally solitary and not abundant (Figure 4 G–N). All strains with incarnatum morphology are distributed among the FIESC species 15 to 29, 32, 36 and 37 in the phylogenetic tree (Figure 2).

Most species (8/10) of the incarnatum morphotype showed nearly straight or relatively slender macroconidia with straight ventral surface and curved dorsal surface varying shapes of apical and basal cells. FIESC 29 and *Fusarium* sp. 1 macroconidia were nearly straight, with curved apical cell and distinctly notched basal cell (Figure 4 G). FIESC 17, 20, 26 and *Fusarium* sp. 2 macroconidia were straight to falcate (Figure 4 H). FIESC 16 and 24 macroconidia were ventral nearly straight and dorsal curved, with variation to falcate observed for FIESC 24. Other two species, FIESC 37 and *Fusarium* sp. 5, have falcate macroconidia (Figure 4 I) with apical and basal cells, respectively, hooked and foot shaped. Macroconidia were usually 5-septate. Mesoconidia were formed from polyphialides in aerial mycelia (Figure 4 N), appearing “rabbit ears” when observed under the microscope (Figure 4 M). The shapes observed for this type of conidia were fusoid to falcate or curved, usually 3-septate (Figure 4 J). Microconidia were produced by eight phylogenetic species (Figure 4 K), and were obovoid, fusiform or slightly curved, 0 to 1-septate, and generally difficult to find. Chlamydospores were observed only in FIESC 16 and 26, solitary and smooth walled (Figure 4 L).

The growth rate and color of the colony was not structured by the morphotypes. The mean diameter of the mycelial radial growth ranged from 35 to 55 mm across the species. The fastest-growing strain was a FIESC 24 (57 mm) and the slowest-growing ones were *Fusarium* sp. 1 and 5 (38 and 36 mm, respectively). White, cream or light brown mycelia were observed among the strains (Figure 4 Q–S). The reverse side of the colony varied from cream to orange to light brown, but there was a predominance of cream color with subtle variations to light brown. FIESC 24 and 26 produced orange and brown colors in the reverse of colonies, respectively. The full set of quantitative data on morphology of each species can be found in the supplemental material (Supplementary Figures 1 and 2, Supplementary Table 2).

3.4. Mycotoxin production

Only 34.8% (16/46) of the isolates, belonging to seven of the nine species, produced detectable levels of mycotoxins (Supplementary Table 1). ZEA was produced by nine isolates (FIESC 26, 26 and 37), followed by DON produced by five isolates (FIESC 16, 37 and *Fusarium* sp. 4) and NIV by three isolates (FIESC 16 and 20), among which two also produced 4-ANIV (FIESC 20). One strain (15Ar047, FIESC 6) that produced DON, also produced 3-ADON and 15-ADON. The strain 16Ar008 (FIESC 16) produced both DON and NIV. The mycotoxins T-2 and HT-2 were not detected.

4. Discussion

This is the first study to elucidate phylogeny, morphology and toxigenic potential for a relatively large collection of FIESC strains obtained from the major rice-producing regions in Brazil. Phylogeny suggested the occurrence of four species with several representative strains, including

323 a putatively novel phylogenetic species (FIESC 37), which was one of the dominant species, and
324 the presence of five singleton lineages that may represent putatively new phylogenetic species.
325 Studies on the diversity of FIESC in cereals are incipient. After the seminal paper by
326 O'Donnell et al. (2009), where 28 phylogenetic species were described in a collection of
327 medically important FIESC strains, only three studies identified phylogenetically FIESC species
328 among a collection of isolates recovered from agricultural crops, mainly cereals such as maize,
329 barley, oat, wheat and rice (Castellá and Cabañes, 2014; O'Donnell et al., 2018; Villani et al.,
330 2016). Although the singleton lineages need to be further confirmed as new species, the genetic
331 diversity found in our survey of FIESC isolates from rice is extremely large and confirm
332 previous reports on FIESC that showed the high biological variability existing within this species
333 complex isolated from multiple cereal hosts. Prior to our study, 15 FIESC species have been
334 identified in cereals: FIESC 1, FIESC 5, FIESC 10, FIESC 14, FIESC 24 and FIESC 28 (wheat,
335 Spain) (Castellá and Cabañes, 2014); FIESC 12 (wheat, Germany), FIESC 23 (rice, India) and
336 FIESC 25 (rice, China) (O'Donnell et al., 2009); FIESC 29 (maize and wheat, Italy) and FIESC
337 31 (maize, oat and wheat from Netherlands, Canada and Italy, respectively) (Villani et al., 2016);
338 FIESC 1 (barley, India), FIESC 4 and FIESC 25 (rice, India), FIESC 7 (wheat, India), FIESC 14
339 (barley, Germany and USA), FIESC 25 (sorghum, India), FIESC 36 (maize and wheat, India)
340 (O'Donnell et al., 2018), FIESC 21 (barley, Uruguay) (Garmendia et al., 2018). Five species
341 were known from rice, two of them reported by O'Donnell et al. (2009) (FIESC 23 and 25), two
342 species reported by Villani et al. (2016) (FIESC 5 and 29) and two by O'Donnell et al. (2018)
343 (FIESC 4 and 25).
344 Among the eight species identified in our study, three have been previously reported
345 from winter cereals (FIESC 23, 24 and 29), while five species are reported here for the first time

for a cereal crop. Villani et al. (2016) identified five cereal-infecting strains from Europe and Canada (maize, barley, oat, rice and wheat), while Castellá and Cabañes (2014) identified seven species associated with wheat in Spain. The latter suggested that lineage composition in Spanish wheat was likely shaped by climate given to geographical structure: FIESC 14 isolates were found in Castilla-La Mancha region, which has cold winters and dry climate; and FIESC 24, 25 and 29 were found in Catalonia, where winters are mostly mild and summers with moderate temperatures. The hypothesis of climate influence has been further speculated. For instance, FIESC 29 reported by Villani et al. (2016) was isolated from warm climate regions, Italy and Mexico, similar to Castellá and Cabañes (2014), who reported the same species in Spain.

Contrastingly, one FIESC 29 isolate (15Ar053) found in our study originated in the subtropics of southern Brazil (Arroio Grande, RS), and the other strain (15Ar085) from the tropics in northern Brazil (Formoso do Araguaia, TO), two regions of contrasting climates. Differently from winter wheat, regions in Brazil where rice is cultivated should not vary considerably for the weather conditions, during the rice-growing seasons. Nevertheless, the two most dominant species of our survey appeared to be structured by region: FIESC 26 was dominant in Goiás state (11/22) and FIESC 37 was dominant in RS state (11/21). The former species was more frequent in the tropical Cerrado region, such MT and GO, and up north of Brazil, MA and RR states, together with FIESC 16 and FIESC 17. The species with equiseti morphotype were restricted to southernmost rice regions of Brazil, such as FIESC 4, 6, *Fusarium* sp. 3 and 4 found in RS state, but the number of isolates were small. Therefore, it is difficult to draw any hypothesis on the effect of climate on spatial distribution. Additional studies with larger sample size are needed to confirm whether climate is an important driver of FIESC species distribution in Brazilian rice.

In this study we included the gene sequences of one isolate (ITEM 7155) from a previous study, for which the phylogeny was not well resolved becaused the strain had been grouped close to FIESC 23 and 24 (Villani et al., 2016). We found that the ITEM 7155 strain grouped with our newly discovered FIESC 37. Two strains (15Ar053 and 15Ar085), together with a sequence of reference strain NRRL 52765, may represent three distinct phylogenetic species, since they diverged from FIESC 29 (Figures 2 and 3). The strain FRC R-10113 was identified by Short et al. (2011) as a new phylogenetic species (FIESC 31), however, the phylogenies including more sequences indicate that this strain belongs to *F. lacertarum* species (Figures 2 and 3).

Latin binomials have been applied to only three of the now thirty-three FIESC species, which may be due to the high levels of cryptic speciation and the morphological homoplasy that lead to underestimates of species diversity within this complex based on morphological taxonomy. In the absence of morphological concordance, molecular phylogeny improves the accuracy of the identification of isolates within FIESC. Our study is the first to provide detailed information on the variability in morphology traits across FIESC species. We observed two morphotypes of which varied in macroconidia shape, type of apical and basal cell, as well as shape of mesoconidia and presence/absence and shape of microconidia.

Three traits were observed in radial growth, four species (FIESC 16, 20, 26, 37) had similar growth rate (48–52 mm) while other four species (FIESC 4, 6, *Fusarium* sp. 2, 3, 4) ranged between 44–49 mm. The last group comprises species with equiseti morphotype, except *Fusarium* sp. 2. FIESC 17 and 29 had the same growth rate. Two exceptions were observed, FIESC 24 grew faster and *Fusarium* sp. 1 and 5 slower than the others. The most commonly species in the present study, FIESC 26 and 37 were among the fastest-growing strains, which may contribute to higher fitness and further dominance among the sampling areas, a hypothesis

392 that needs to be further explored.

393 FIESC isolates are able to produce B-trichothecenes (DON and NIV) due to the presence
394 of *Tri5* gene within trichothecene biosynthetic loci (Proctor et al., 2009) and other mycotoxins
395 such as fusarochromanone, beauvericin and zearalenone as reported in the literature (Kosiak et
396 al., 2005; Leslie and Summerell, 2006; O'Donnell et al., 2018, Villani et al., 2016). Recently,
397 80% of FIESC strains from Spanish cereals produced DON and 25% produced NIV (Marín et
398 al., 2012). Contrastingly, FIESC isolates from Norwegian cereals produced higher quantities of
399 type A trichothecenes but neither detectable levels of DON nor DON derivatives, yet significant
400 amounts of NIV and FUS was produced (Kosiak et al., 2005). In this study, 35% of the strains
401 produced different B-trichothecenes mycotoxins or ZEA, which were distributed randomly
402 across the species and geographic regions. This suggests that FIESC not only may be an
403 important contributor to mycotoxin levels detected in Brazilian rice (Almeida et al., 2012;
404 Moreira et al., unpublished; Savi et al., 2018), but also that in FIESC isolated from rice there is a
405 wide variability in mycotoxin production. Thus our data confirms the conclusions of a recent
406 report by Villani et al. (in press), that analyzed the genomes of 13 FIESC members and showed
407 that the production of secondary metabolites, included the mycotoxins, could be affected by the
408 different distribution of functional and related gene clusters. Finally, our data on mycotoxin
409 production by FIESC members, together with previous papers on same topic, strongly suggest
410 that a wider, global survey, must be performed on FIESC originated from different sources, in
411 order to relate the occurrence of mycotoxin gene clusters and the phenotypic production of
412 mycotoxins *in vitro*, and therefore better assess the risk associated to FIESC occurrence in agro-
413 food important crops.

414

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Legends

Figure 1. Approximate geographical origin of FIESC-like strains (n = 151) sampled from rice fields during four harvesting seasons.

Figure 2. Phylogenetic tree inferred from partial *EF-1α* gene sequences from members of the *Fusarium incarnatum-equiseti* species complex from Brazilian rice using maximum parsimony method (MP). Bootstrap values ≥70% (MP and maximum likelihood) and posterior probability ≥95% (Bayesian inference) are in the internodes, respectively. Bold branches refer to nodes with ≥99% bootstrap values and a posterior probability value of ≥0.99. *Fusarium concolor* (NRRL 13459) was used as outgroup. Reference sequences were from O'Donnell et al. (2009, 2012, 2018), Short et al. (2011) and Villani et al. (2016). Abbreviation: NRRL (The ARS Culture Collection, Peoria, Illinois, USA), ITEM (Agro-Food Microbial Fungi Culture Collection, Bari, Italy), MRC (Agricultural Research Council, Pretoria, South Africa), FRC (Fusarium Research Center, Pennsylvania, USA).

Figure 3. Phylogenetic tree inferred from partial *EF-1α* and *RPB2* genes sequences from

members of the *Fusarium incarnatum-equiseti* species complex from Brazilian rice using maximum parsimony method (MP). Bootstrap values ≥70% (MP and maximum likelihood) and posterior probability ≥95% (Bayesian inference) are in the internodes, respectively. Bold branches refer to nodes with ≥99% bootstrap values and a posterior probability value of ≥0.99. *Fusarium concolor* (NRRL 13459) was used as outgroup. Reference sequences were from O'Donnell et al. (2009, 2012), Short et al. (2011) and Villani et al. (2016). Abbreviation: NRRL (The ARS Culture Collection, Peoria, Illinois, USA), ITEM (Agro-Food Microbial Fungi Culture Collection, Bari, Italy), FRC (Fusarium Research Center, Pennsylvania, USA).

Figure 4. General morphology of *Fusarium incarnatum-equiseti* species complex. A–F Micromorphology of species resembling *F. equiseti* description. Macroconidia with dorsiventral curvature (A, *F. lacertarum*). Smaller conidia (B, *F. lacertarum*; C, *Fusarium* sp. 4). Microconidia of FIESC 6 (D) and *Fusarium* sp. 4 (E). Chlamydospores in chains smooth to rough walled (F, FIESC 6). G–N Micromorphology of species resembling *Fusarium semitectum* description. Nearly straight (G, *Fusarium* sp. 1), straight to falcate (H, FIESC 20) and falcate macroconidia (I, FIESC 37). Mesoconidia (J, *Fusarium* sp. 2). Microconidia (K, *Fusarium* sp. 2). Solitary chlamydospore smooth walled (L, FIESC 26). Mesoconidia formed in aerial mycelium with "rabbit ears" appearance (M, FIESC 37). Mono- and polyphialides from aerial mycelium (N, FIESC 20). Sporodochia *in situ* (O, FIESC 29) and on SNA (P, FIESC 26). Colonies on PDA 14 days old incubated at 25 °C in the dark. (Q, FIESC 37; R, FIESC 26; S, FIESC 6). Scale bars: 20 µm.

Supplementary Table 1. Strains of *Fusarium incarnatum-equiseti* species complex isolated from

631 rice in Brazil identified by molecular phylogeny and the mycotoxins produced by strains grown
632 on rice grains.
633
634 Supplementary Table 2. Morphological characteristics of phylogenetic species within *Fusarium*
635 *incarnatum-equiseti* species complex used in this study.
636
637 Supplementary Figure 1. Morphology of FIESC species. FIESC 4 (*Fusarium lacertarum*), 6, 16,
638 17, 20, 24, 26, 29 (A–H, respectively). Colonies on PDA 14 days old incubated at 25 °C in the
639 dark. Verse (A1–H1) and reverse (A2–H2). Macroconidia (A3–H3, A4, D4). Smaller conidia
640 without elongate apical cell and prominent foot-shape basal cell (A5, B4–B5). Macro- and
641 mesoconidia (G4). Macro- and microconidia (C4, F6, G5). Mesoconidia (C5–C6, D5, E4, F4–F5,
642 H4). Microconidia (B6, E5–E6, H5–H6).
643
644 Supplementary Figure 2. Morphology of FIESC species. FIESC 37 and *Fusarium* sp. 1 to 5 (A–
645 F, respectively). Colonies on PDA 14 days old incubated at 25 °C in the dark. Verse (A1–F1)
646 and reverse (A2–F2). Macroconidia (A3–F3, D4, F4). Smaller conidia without elongate apical
647 cell and prominent foot-shape basal cell (D5, E4). Macro- and microconidia (A5). Macro-, meso-
648 and microconidia (B5). Mesoconidia (A4–C4, F5). Microconidia (C5, E5–E6).
649

Table 1. Strains of *Fusarium incarnatum-equiseti* species complex isolated from rice in Brazil identified by mol

Phylogenetic species	Isolate	ITEM code [a]	Location [b]	Year	GenBank access
					EF-1α
F. lacertarum (FIESC 4)	12Ar093	17662	Pelotas, RS	2012	MK298062
F. lacertarum (FIESC 4)	12Ar097	17663	Pelotas, RS	2012	MK298063
F. lacertarum (FIESC 4)	12Ar104	17667	Pelotas, RS	2012	MK298064
FIESC 6	15Ar047	17680	Itaqui, RS	2015	MK298065
FIESC 16	15Ar043		Formoso do Araguaia, TO	2015	MK298068
FIESC 16	16Ar008	17695	Paraibano, MA	2016	MK298069
FIESC 16	CML 3825	17649	Formoso do Araguaia, TO	2015	MK298070
FIESC 17	16Ar045	17697	Lagoa da Confusão, TO	2016	MK298071
FIESC 20	09Ar023		Palmares do Sul, RS	2009	MK298081
FIESC 20	12Ar142	17668	Nova Veneza, SC	2012	MK298082
FIESC 20	16Ar033		Itajaí, SC	2016	MK298072
FIESC 20	16Ar020	17686	Glorinha, RS	2016	MK298073
FIESC 20	16Ar018	17687	Glorinha, RS	2016	MK298074
FIESC 20	16Ar019	17688	Glorinha, RS	2016	MK298075
FIESC 20	16Ar013		Alta Floresta, MT	2016	MK298076
FIESC 20	16Ar014	17694	Alta Floresta, MT	2016	MK298077
FIESC 20	16Ar046	17698	Formoso do Araguaia, TO	2016	MK298078
FIESC 20	15Ar096	17704	Arroio Grande, RS	2015	MK298079
FIESC 20	16Ar030	17705	Pelotas, RS	2016	MK298080
FIESC 24	09Ar013	17654	Cachoeirinha, RS	2009	MK298104
FIESC 26	12Ar100	17665	Brazabrantes, GO	2012	MK298128
FIESC 26	12Ar155		Nova Veneza, SC	2012	MK298129
FIESC 26	15Ar017		Santo Antônio do Goiás, GO	2015	MK298108
FIESC 26	15Ar022		Santo Antônio do Goiás, GO	2015	MK298109
FIESC 26	15Ar024		Santo Antônio do Goiás, GO	2015	MK298110
FIESC 26	15Ar026	17673	Brazabrantes, GO	2015	MK298111
FIESC 26	15Ar033	17674	Brazabrantes, GO	2015	MK298112
FIESC 26	15Ar034	17675	Brazabrantes, GO	2015	MK298113
FIESC 26	15Ar035		Brazabrantes, GO	2015	MK298114
FIESC 26	15Ar036	17676	Brazabrantes, GO	2015	MK298115
FIESC 26	15Ar038		Brazabrantes, GO	2015	MK298116
FIESC 26	15Ar040	17677	Brazabrantes, GO	2015	MK298117
FIESC 26	15Ar055	17683	Sinop, MT	2015	MK298118
FIESC 26	15Ar056	17684	Sinop, MT	2015	MK298119
FIESC 26	15Ar057		Sinop, MT	2015	MK298120
FIESC 26	16Ar024	17689	Santa Maria, RS	2016	MK298121
FIESC 26	16Ar028	17691	São Sepé, RS	2016	MK298122
FIESC 26	16Ar012	17693	Tangará da Serra, MT	2016	MK298123
FIESC 26	16Ar010		Paraibano, MA	2016	MK298124
FIESC 26	16Ar015		Boa Vista, RR	2016	MK298125
FIESC 26	12Ar143	17669	Nova Veneza, SC	2012	MK298126

Table 2. Morphological characteristics of phylogenetic species within *Fusarium incarnatum-equiseti* species c

Phylogenetic species	Morphotype	Colony - verse / reverse color
F. lacertarum (FIESC 4)	equiseti	White / cream – light-brown
FIESC 6	equiseti	White – light-brown / light-brown
FIESC 16	incarnatum	White – light-brown / cream – light-brown
FIESC 17	incarnatum	White / brownish orange
FIESC 20	incarnatum	White – light-brown / cream – light-brown
FIESC 24	incarnatum	light-brown / orange
FIESC 26	incarnatum	White – light-brown / cream – brown
FIESC 29	incarnatum	White – cream / cream – light orange
FIESC 37	incarnatum	White – light-orange / light orange, cream – light-brown
Fusarium sp. 1	incarnatum	Light-brown / light-orange
Fusarium sp. 2	incarnatum	Light-brown / light-brown
Fusarium sp. 3	equiseti	White / cream
Fusarium sp. 4	equiseti	Light-brown / cream
Fusarium sp. 5	incarnatum	White / brownish orange

Figure 1

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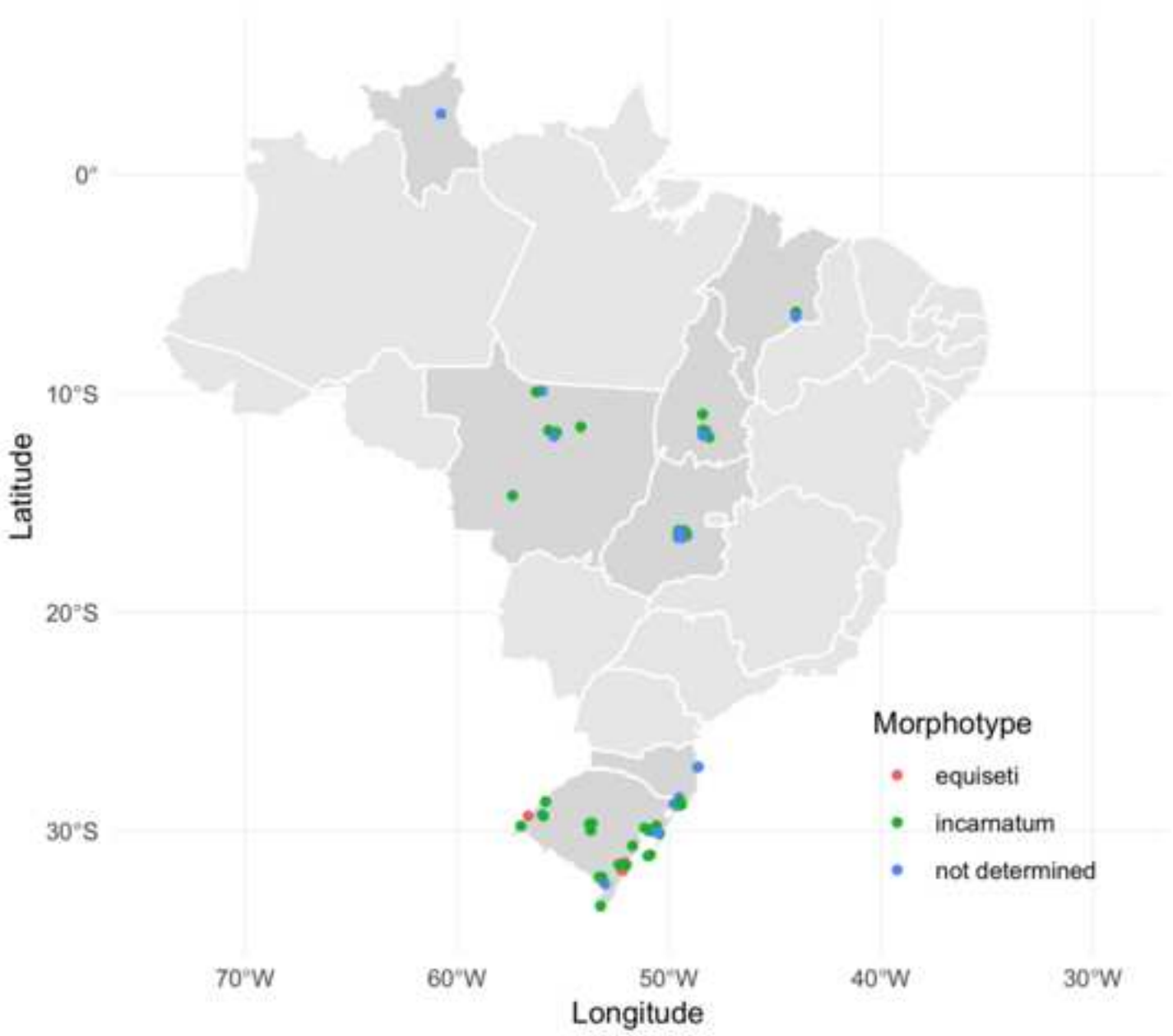

Figure 2

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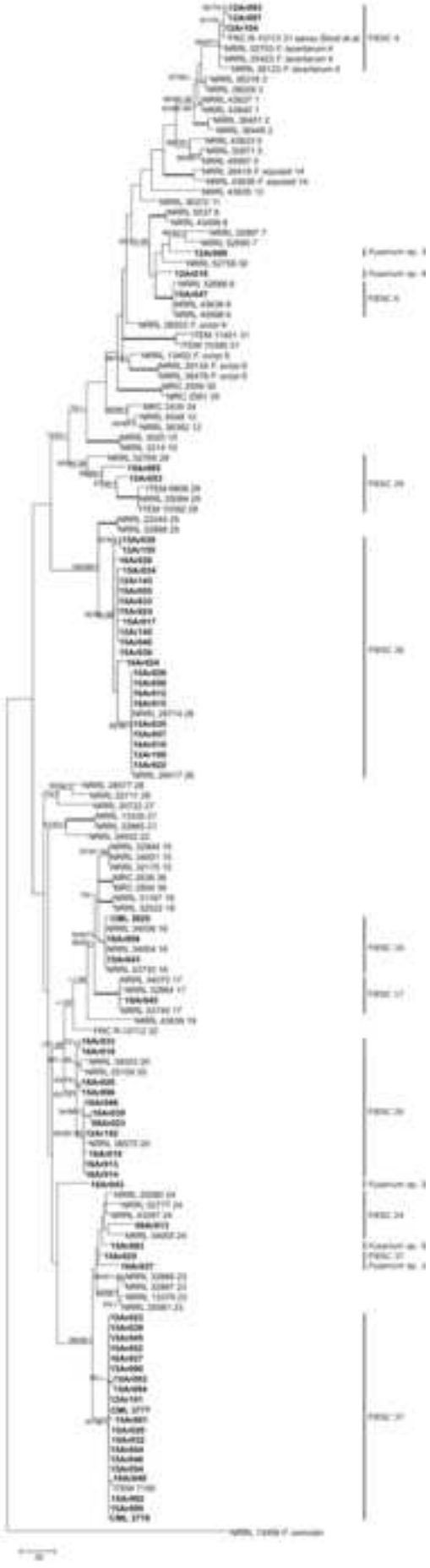

Figure 3

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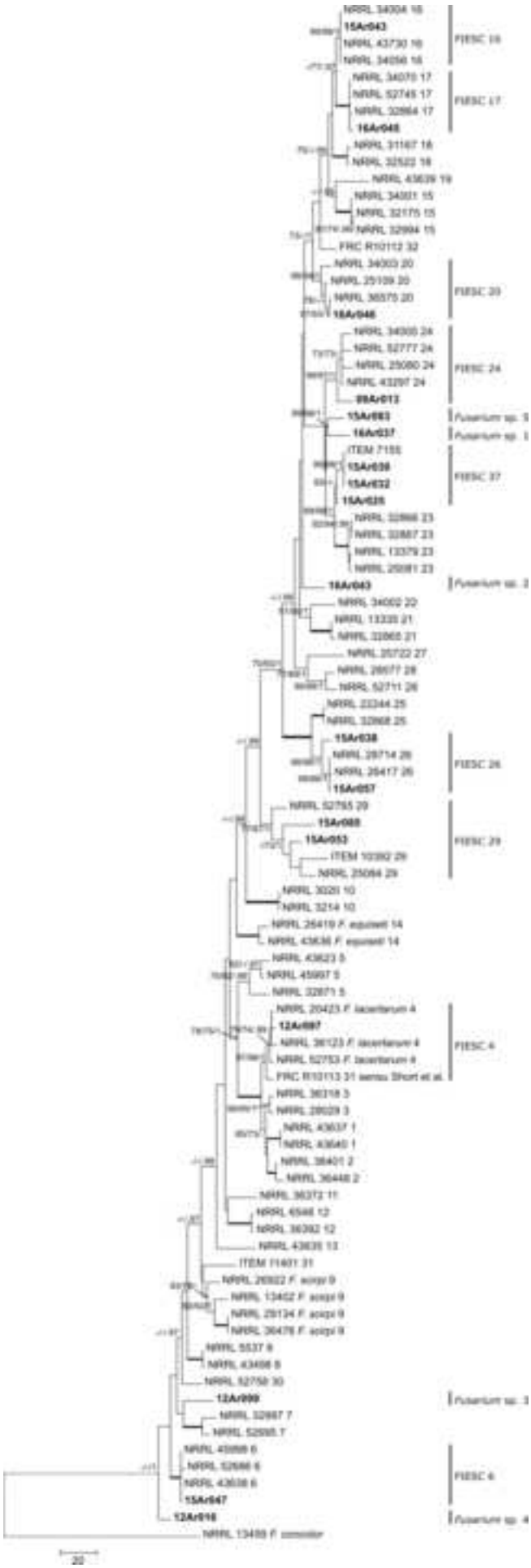

Figure 4

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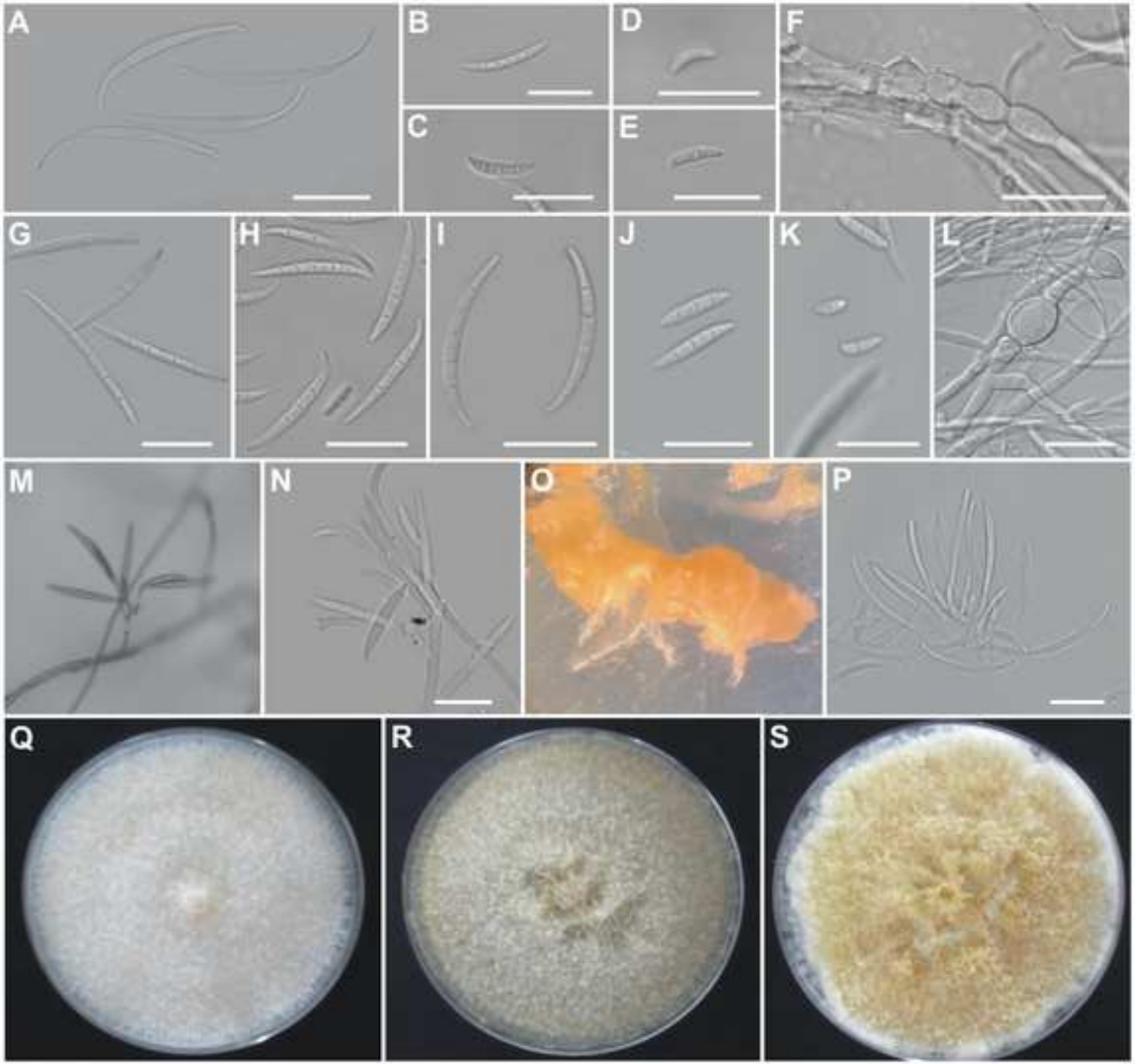

Supplementary Figure 1

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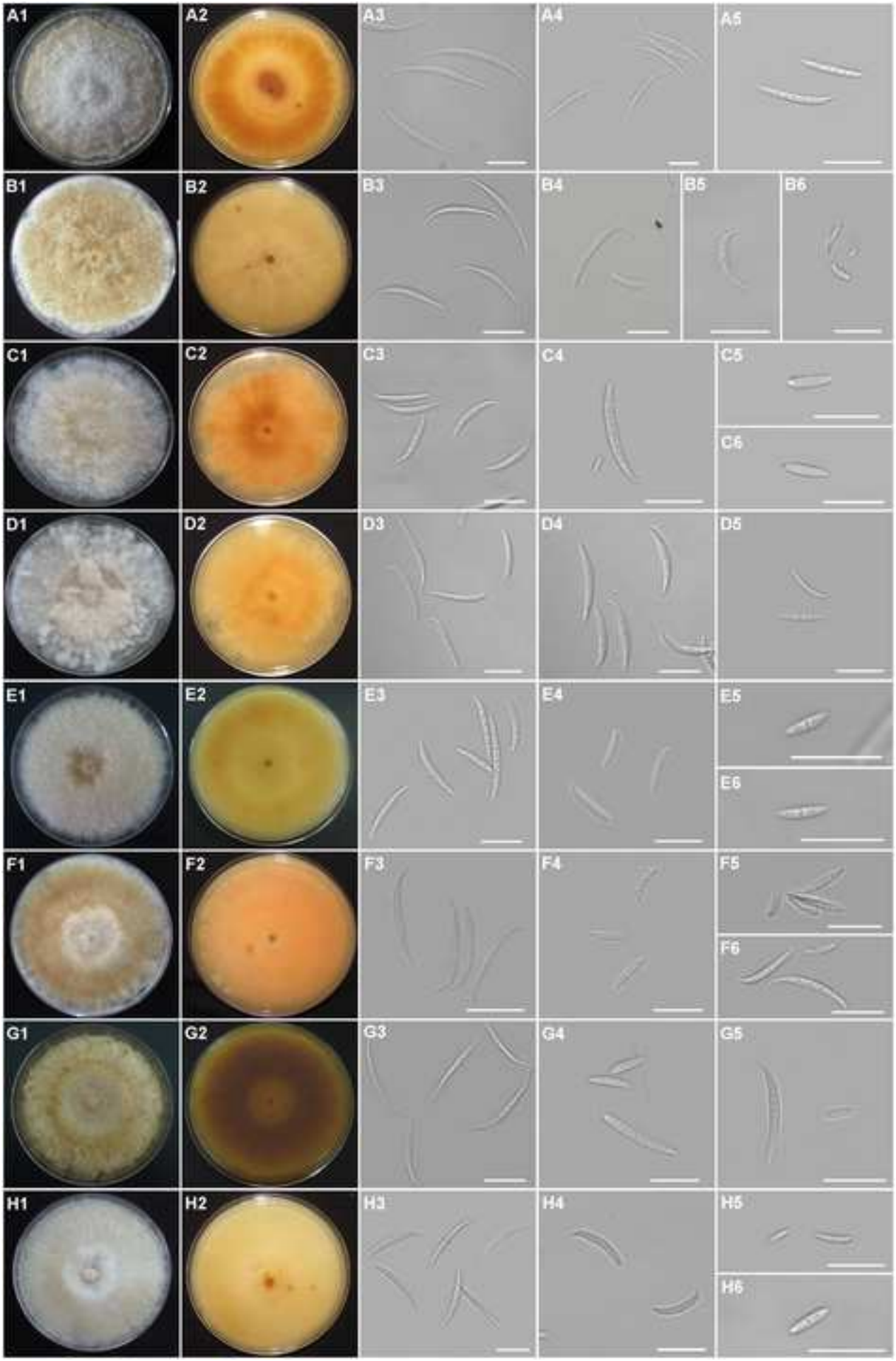

Supplementary figure 2

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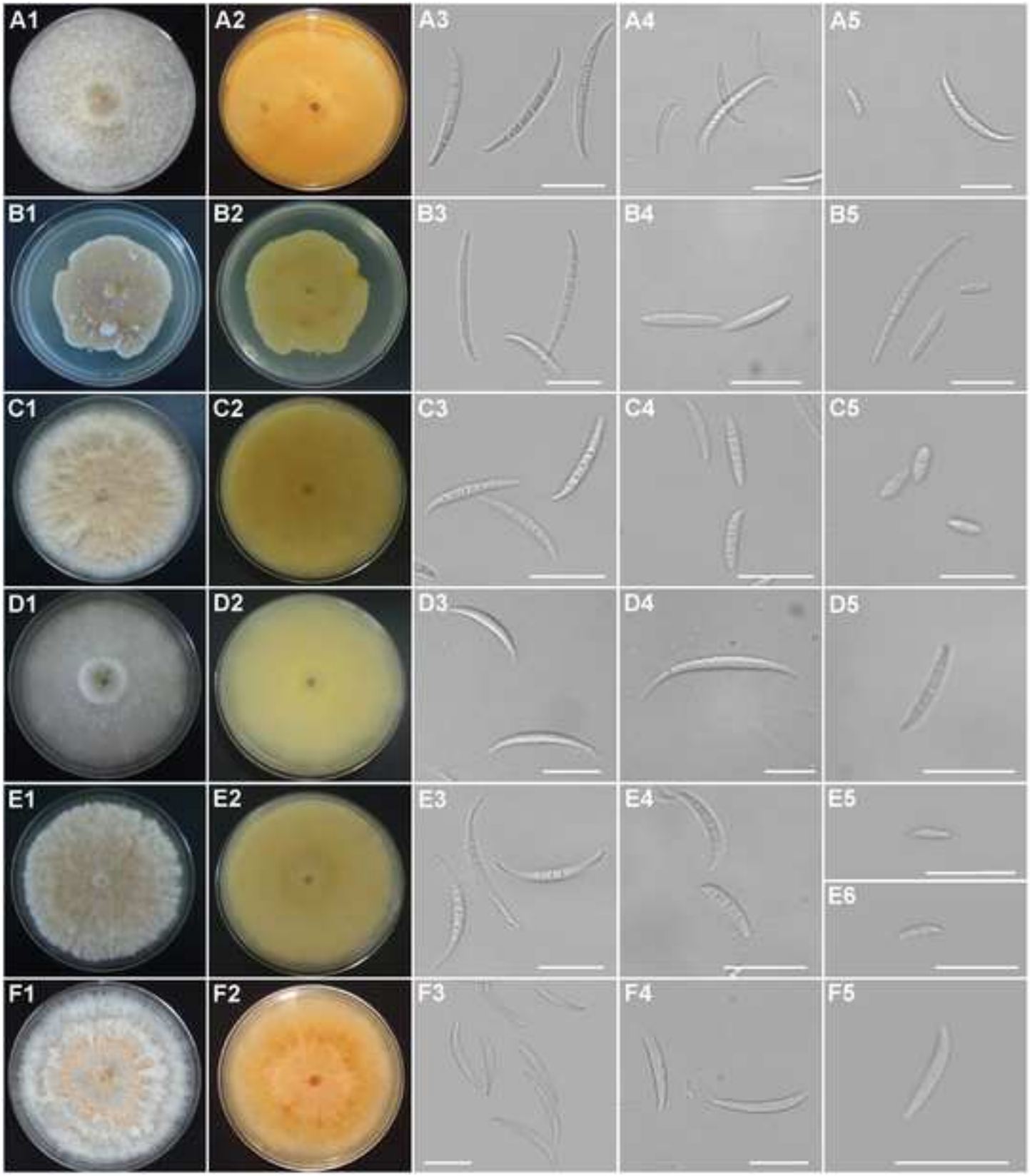

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