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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 Brazilian rice, but knowledge of the species limits and their toxigenic potential is lacking. Seventy strains morphologically identified as FIESC-like, isolated from the major rice-growing regions of Brazil, were subjected to sequencing of *EF-1α* gene. Among them, 18 strains were selected and 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 (*F. pernambucanum*), 20 (*F. caatingaense*), 24, 26 and 29. One new phylogenetic species was identified, and named FIESC 38. Five strains formed new singleton lineages. The most dominant species were FIESC 26 (22/70 strains) and FIESC 38 (21/70), the newly identified species. The *incarnatum* morphotype was dominant (10 phylogenetic species) over the *equiseti* (4 species). Among 46 strains selected to represent all species, only 16 strains produced detectable levels of mycotoxins *in vitro*. FIESC 26 produced ZEA and FIESC 38 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 cereal-infecting FIESC in the southern hemisphere and provide baseline data for further exploring morphological differences among the species.

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, including mycotoxigenic species of concern to human and animal health, such as *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 reported in rice 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). Trichothecenes and zearalenone are commonly produced by members of the *F. graminearum* species complex (FGSC), that also infect rice, (Gomes et al., 2015; Lee et al., 2009), but *F. incarnatum-equiseti* species complex (FIESC) are also capable of producing these mycotoxins (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 and reports showed that DON and ZEA were found contaminating natural and parboiled rice from rice experimental

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plots (da Silva et al., 2019; Heidtmann-Bemvenuti et al., 2012). The analysis of 230 samples of rice and byproducts showed the presence of ZEA and DON occurred in a significant number of samples (45% and 8%, respectively) (Almeida et al., 2012). A recent survey reported ZEA in 60% of parboiled rice samples, being 36% with concentrations higher than the maximum tolerated levels (MTLs) by the Brazilian regulation (Savi et al., 2018).

In order to protect consumers, regulatory agency in Brazil has promulgated MTLs for mycotoxins in several cereal crops, including rice (ANVISA, 2011). The MTLs of two mycotoxins in rice were updated recently, effective from January 2017: DON in rice bran (1250 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], diacetoxyscirpenol [DAS], 15-monoacetoxy-scirpentriol [MAS], neosolaniol [NEO]), equisetin (EQ), 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 has allowed so far to distinguish among four species: *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 markers, have been resolved by sequencing of key genes (Geiser et al., 2004; O'Donnell et al., 2015). Within FIESC, the translation elongation factor 1-alpha (*EF-1α*) gene allowed the separation of strains from clinical environments in North America into 28 species, among which only three species have been formally described, while Arabic numbers were assigned to the rest of the species to facilitate communication (O'Donnell et al., 2009). Recently, FIESC 17 and FIESC 20 were formally described as *F. pernambucanum* and *F. caatingaense*, respectively, based on morphological and molecular phylogenetic markers and the fertility of sexual crosses (Santos et al., 2019). Other studies have reported new species including FIESC 29 and FIESC 30 (insects) (O'Donnell et al., 2012); FIESC 31 and FIESC 32 (plumbing drain water) (Short et al., 2011); FIESC 31 (cereal-borne) (Villani et al., 2016), currently redesignated as FIESC 33 (Villani et al., 2019); FIESC 34, 35 and 36 (soybean, wheat and maize) (O'Donnell et al., 2018); and FIESC 37 (soybean) (Hartman et al., 2019). Maryani et al. (2018) reported three and described other three new FIESC species associated with diseased bananas in Indonesia (*F. kotabaruense*, *F. tanahbumbuense* and *F. sulawense*), but the authors did not take into consideration the updates made in the numbering system adopted for distinct clades since the work of O'Donnell et al. (2009), resulting in conflicts with the numbering system adopted to designate FIESC. Two studies using a relatively large sample from cereals reported seven FIESC species in Spanish cereals (Castellá and Cabañes, 2014) and five FIESC in cereals from North America, Europe and Turkey (Villani et al., 2016). Recently, one strain isolated from barley (Piacentini et al., 2019) and another two from rice (Savi et al., 2018) in Brazil grouped into FIESC clade. Another strain, identified as FIESC 21, was obtained from barley in Uruguay (Garmendia et al., 2018). Nevertheless, to the best of our knowledge, comprehensive information on the diversity and toxigenic potential of FIESC species infecting cereals in South America is lacking.

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

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 (Fig. 1).

The isolates were obtained from seven to ten day-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 °C during storage.

2.2. DNA extraction, PCR assays and sequencing

The isolates were grown on potato dextrose agar (PDA, Acumedia, Neogen Corporation) for 7 days at 25 °C. Fresh mycelia were harvested into 2 mL tubes and disrupted in TissueLyser (Qiagen, Haan, Germany). The total genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's protocol. Seventy isolates were selected and used for the phylogenetic analyses as first step to identify the species (Fig. 1, Supplementary Table 1). Fragments of *EF-1α* gene were amplified using the primer pair EF1 and EF2 (O'Donnell et al., 1998). Cycling conditions were: initial heating at 95 °C for 90 s, following by 40 cycles (45 s at 95 °C, 1 min at 52 °C and 2 min at 72 °C), final extension at 72 °C for

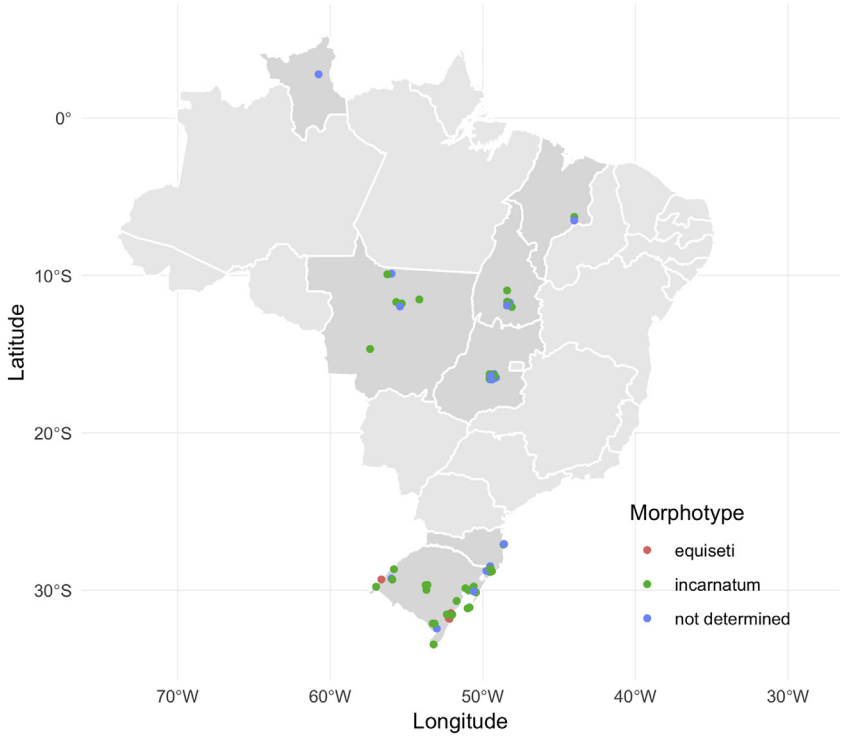

Fig. 1. Approximate geographical origin of FIESC-like strains (n = 151) sampled from rice fields during four harvesting seasons. Seventy isolates were identified to species level and grouped in two morphotypes.

5 min. A subsample of 18 representative isolates of each *EF-1α*-based identified species was selected for phylogeny using a fragment of the second largest subunit of RNA polymerase II (*RPB2*) (O'Donnell et al., 2008). All PCR runs used the GoTaq® Colorless Master Mix (Promega, Madison, USA) in a final volume of 20 µL containing of 1 X PCR buffer, 1 µM of primers, 1 µL of pure DMSO, 0.4 µL of BSA (10 mg mL^{-1} solution), and 50–100 ng of DNA in a MG-96 MyGeneTM Thermal Cycler (Hangzhou LongGene Scientific Instruments Co., Ltd.). PCR products were visualized in 1% agarose gel run in 1 X TBE buffer, under a UV light to ensure the presence of single-band products. PCR products were purified with the enzymatic mixture NucleoSAP (Cellco Biotec, São Carlos, Brazil). The fragments of amplified DNA were sent for sequencing at Macrogen, Korea.

2.3. Sequence alignment and phylogenetic analysis

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 (Hartman et al., 2019; O'Donnell et al., 2009, 2012, 2018; Santos et al., 2019; 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 196 parsimony-informative positions/705 bp for *EF-1α* and 119/867 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 SYM + 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 1000 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 °C in complete darkness. The mycelial growth (mm) was assessed after 3 days, by measuring the two right-angled colonies diameter using a pachometer, and the colony pigmentation was evaluated after 14 days (Leslie and Summerell, 2006). Microscopic examination was performed on 7 to 14 day-old colonies grown on plastic Petri dishes containing SNA with sterilized pieces of carnation leaves incubated at 25 °C and 12 h photoperiod (Leslie and Summerell, 2006). The following morphological traits were measured and photographed: presence/absence of sporodochia and chlamydospores and type of conidiogenous cells; conidia type, shape, septation and size (30 to 50 conidia per isolate, measured digitally using Digimizer v4.6.1 software). The morphometric data were summarized as frequency, minimum, maximum and mean values.

2.5. Isolates and in vitro culturing for mycotoxin analysis

A subsample of 46 strains representative of all phylogenetic clades was selected for the evaluation of *in vitro* production of trichothecenes type A (T-2 and HT-2), type B (DON, 3-ADON, 15-ADON, NIV and 4-ANIV) and ZEA. Autoclaved rice grains (30 g grains/13 mL water) were inoculated with mycelial plugs (5 mm) of colonies of each isolate and incubated for 21 days in the dark at 25 °C. Non-inoculated rice grains served as control. Portions of 1 g of grinded rice culture were extracted with 5 mL of acetonitrile/water (84,16, *v*/v) with 1% of acetic acid by

orbital shaking for 2 h. After filtration through filter paper (Whatman n. 4), 100 µL were diluted with 900 µL ultrapure water. The residue was filtered using RC through 0.20 µm regenerated cellulose filter. The mycotoxins were quantified by comparing peak areas with calibration curves obtained with standard solutions. The detection limits were 0.1 µg/g for DON, NIV, 4-ANIV and ZEA, 1 µg/g for 3-ADON and 15-ADON, 0.05 µg/g for T-2 and HT-2. The analyses were performed according to each mycotoxin as described below.

2.5.1. Determination of DON, NIV and acetylates

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

2.5.2. Determination of T-2 and HT-2

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

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 (*F. pernambucanum*), FIESC 20 (*F. caatingaense*), FIESC 24, FIESC 26 and FIESC 29, and a new species named FIESC 38, which formed a large monophyletic group (20 strains) with statistical support (MP = 98%, ML = 97% and BI = 1 PP) (Fig. 2). All but FIESC 24 species formed strongly supported monophyletic groups (*i.e.*, 82–100%). 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 (Fig. 2).

In general, the combined dataset (*EF-1α* and *RPB2*) confirmed the monophyly of species inferred by the *EF-1α* tree (Fig. 3), but only two incongruences were detected. FIESC 24 formed a well-supported clade

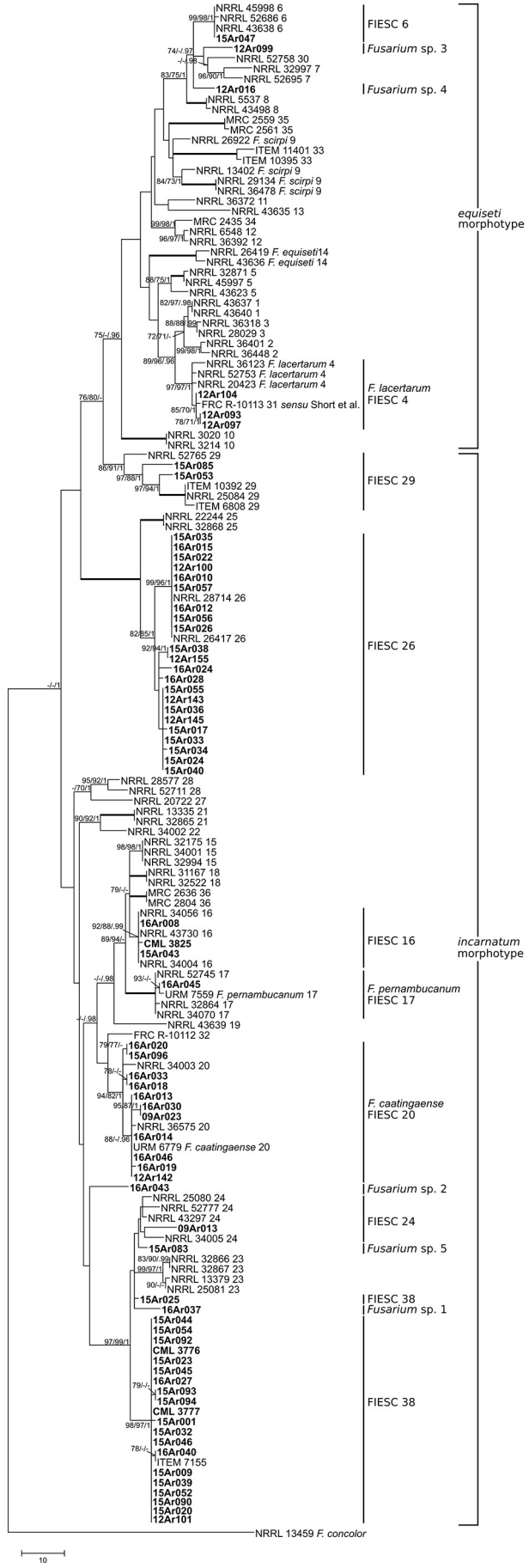

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Fig. 2. Phylogenetic tree inferred from partial *EF-1α* gene sequences from members of the *Fusarium incarnatum-equiseti* species complex from Brazilian rice using maximum parsimony method (MP). Bootstrap values ≥70% (MP and maximum likelihood) and 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), Santos et al. (2019), 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), URM (Universidade do Recife Micologia, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil).

in the combined tree, but it did not include 15Ar083 strain, which was grouped (unsupported) with FIESC 24 in the *EF-1α* tree. 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 38 in the combined tree (Fig. 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 38 (21/70), and *F. caatingaense* (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 38 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).

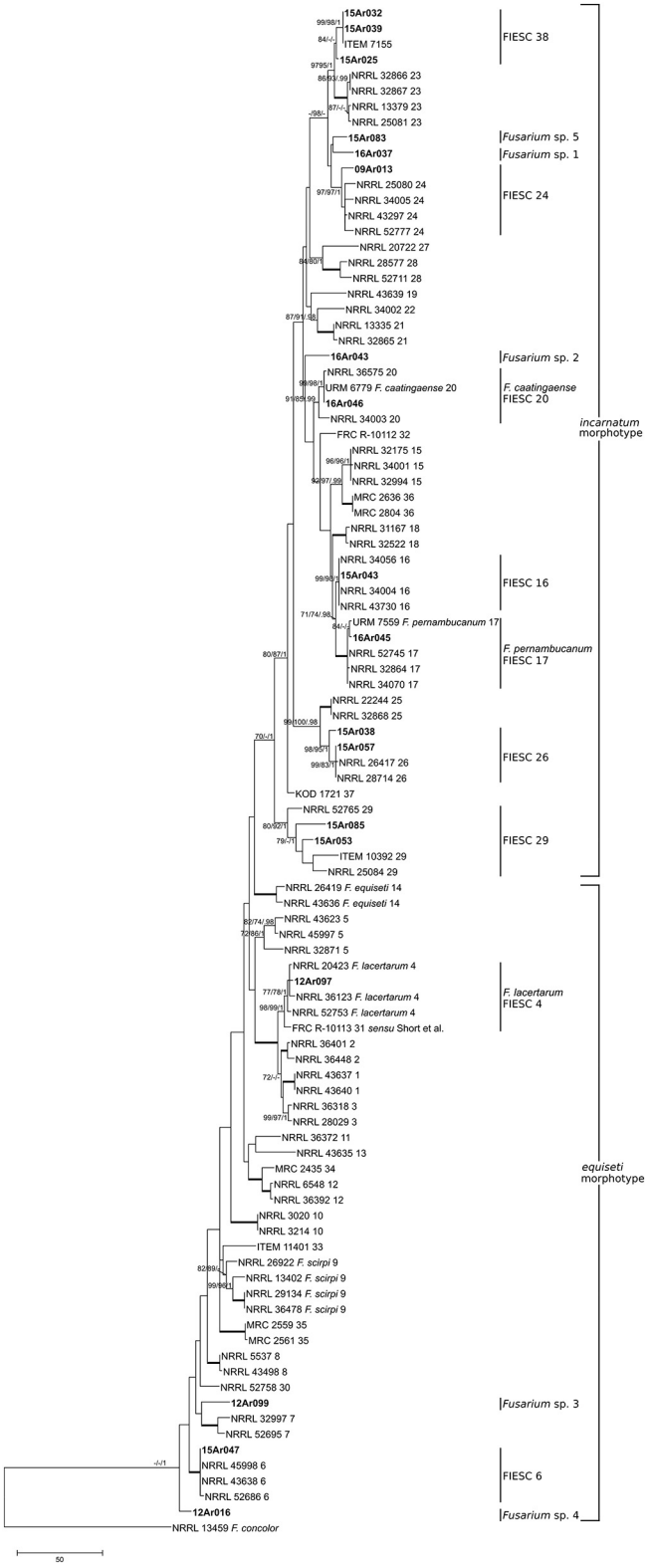
3.3. Morphological characterization

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 (Fig. 4 A). Chlamydospores were produced in chains, smooth to rough walled (Fig. 4 F). Smaller conidia without an elongated apical cell were also observed (Fig. 4 B–C). FIESC 6 and *Fusarium* sp. 4 produced microconidia, 0 to 1-septate (Fig. 4 D–E), typical of *F. scirpi* (Leslie and Summerell, 2006). This *equiseti* morphotype corresponds to the clade of FIESC 1 to 14, 30, 33, 34 and 35 (Fig. 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 (Fig. 4 G–N). All strains with *incarnatum* morphology are distributed among the FIESC species 15 to 29, 32, 36 to 38 in the phylogenetic tree (Fig. 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 (Fig. 4 G). *Fusarium pernambucanum* (FIESC 17), *F. caatingaense* (FIESC 20), FIESC 26 and



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Fusarium sp. 2 macroconidia were straight to falcate (Fig. 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 38 and *Fusarium* sp. 5, have falcate macroconidia (Fig. 4 I) with apical and basal cells, respectively, hooked and foot shaped. Macroconidia were usually 5-septate. Mesoconidia were formed from

Fig. 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 Hartman et al. (2019), O'Donnell et al. (2009, 2012, 2018), Santos et al. (2019), 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), URM (Universidade do Recife Micologia, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil).

polyphialides in aerial mycelia (Fig. 4 N), appearing "rabbit ears" when observed under the microscope (Fig. 4 M). The shapes observed for this type of conidia were fusoid to falcate or curved, usually 3-septate (Fig. 4 J). Microconidia were produced by eight phylogenetic species (Fig. 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 (Fig. 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 (Fig. 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 Figs. 1 and 2, Supplementary Table 2).

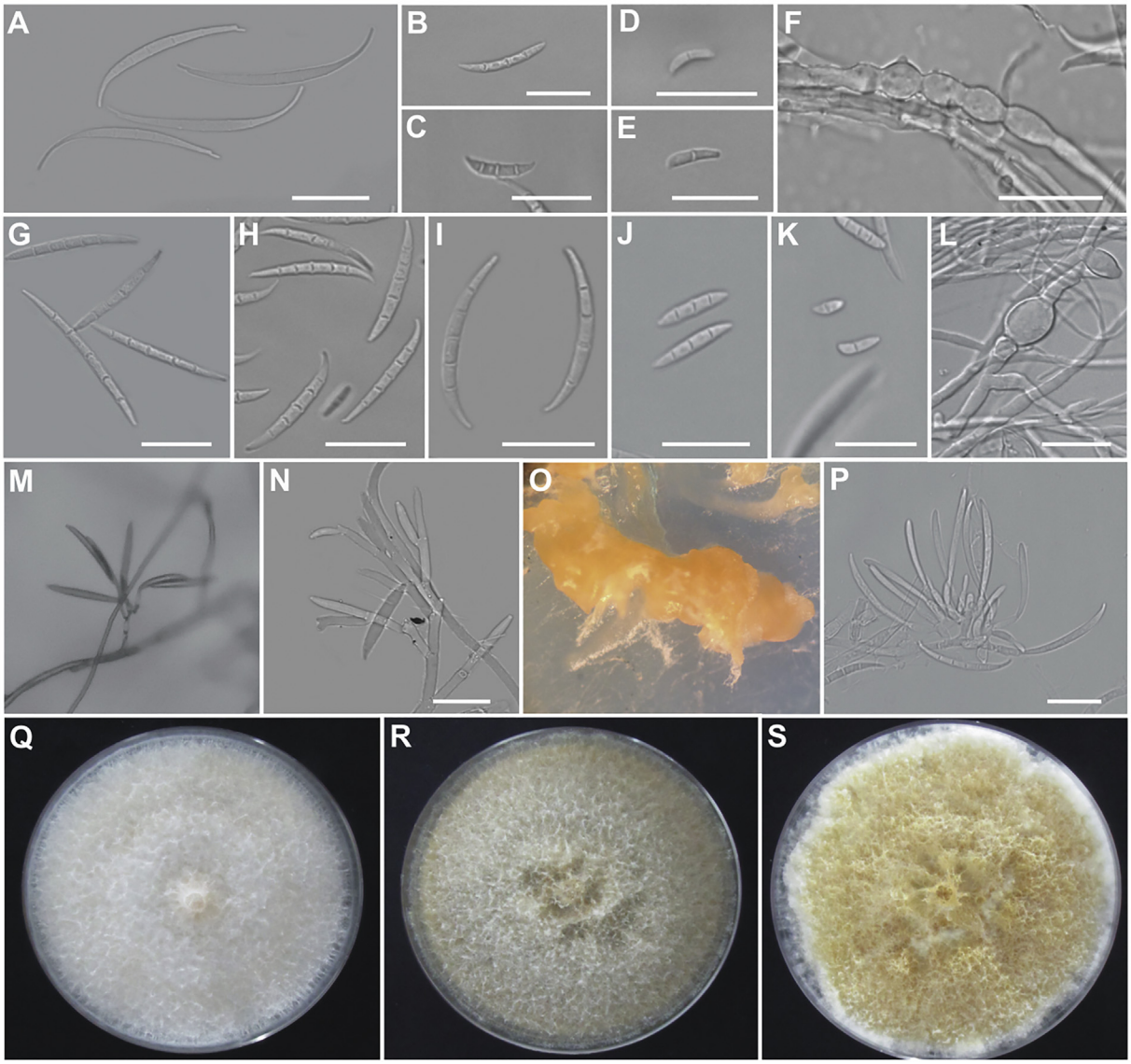

Fig. 4. General morphology of *Fusarium incarnatum-equiseti* species complex. A–F Micromorphology of species resembling *F. equiseti* description. Macroconidia with dorsiventral curvature (A, *F. lacertarum*). Smaller conidia (B, *F. lacertarum*; C, *Fusarium* sp. 4). Microconidia of FIESC 6 (D) and *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, *F. caatingaense*) and falcate macroconidia (I, FIESC 38). 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 38). Mono- and polyphialides from aerial mycelium (N, *F. caatingaense*). 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 38; R, FIESC 26; S, FIESC 6). Scale bars: 20 μm.

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 38), followed by DON produced by five isolates (FIESC 16, 38 and *Fusarium* sp. 4) and NIV by three isolates (FIESC 16 and *F. caatingaense*), among which two also produced 4-ANIV (*F. caatingaense*). One DON-producing strain (15Ar047, FIESC 6) 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 important rice-producing regions in Brazil. Phylogeny suggested the occurrence of four species with several representative strains, including a putatively novel phylogenetic species (FIESC 38), which was one of the dominant species, and the presence of five singleton lineages that may represent putatively new phylogenetic species.

Studies on the diversity of FIESC in cereals are incipient. After the seminal paper by O'Donnell et al. (2009), who described 28 phylogenetic species in a collection of medically important FIESC strains, only three studies identified phylogenetic species among a collection of FIESC isolates recovered from agricultural crops, mainly cereals such as maize, barley, oat, wheat and rice (Castellá and Cabañes, 2014; O'Donnell et al., 2018; Villani et al., 2016). Although the singleton lineages need to be further confirmed as new species, the genetic diversity found in our survey of FIESC isolates from rice is extremely large and confirm previous reports on FIESC that showed the high biological variability existing within this species complex isolated from multiple cereal hosts. Prior to our study, 15 FIESC species have been identified in cereals: FIESC 1, FIESC 5, FIESC 10, FIESC 14, FIESC 24 and FIESC 28 (wheat, Spain) (Castellá and Cabañes, 2014); FIESC 12 (wheat, Germany), FIESC 23 (rice, India) and FIESC 25 (rice, China) (O'Donnell et al., 2009); FIESC 29 (maize and wheat, Italy) and FIESC 33 (maize, oat and wheat from Netherlands, Canada and Italy, respectively) (Villani et al., 2016); FIESC 1 (barley, India), FIESC 4 and FIESC 25 (rice, India), FIESC 7 (wheat, India), FIESC 14 (barley, Germany and USA), FIESC 25 (sorghum, India), FIESC 36 (maize and wheat, India) (O'Donnell et al., 2018), FIESC 21 (barley, Uruguay) (Garmendia et al., 2018). Five species have been known to infect rice, two of them reported by O'Donnell et al. (2009) (FIESC 23 and 25), two species reported by Villani et al. (2016) (FIESC 5 and 29) and two by O'Donnell et al. (2018) (FIESC 4 and 25).

Among the eight species identified in our study, three have been previously reported from winter cereals (FIESC 23, 24 and 29), while five species are reported here for the first time in a cereal crop. Villani et al. (2016) identified five cereal-infecting species 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 study 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 dramatically in the summer 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 38 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 *F. pernambucanum*. Species of the *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 is 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 our study we included the gene sequences of one isolate (ITEM 7155) from a previous study, for which the phylogeny was not well resolved because 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 38. 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 (Figs. 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 (Figs. 2 and 3).

Latin binomials have been applied to only eight of the now forty-two designated 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 (*F. caatingaense*, FIESC 16, 26, 38) had similar growth rate (48–52 mm) while for other four species (FIESC 4, 6, *Fusarium* sp. 2, 3, 4) growth rate ranged between 44 and 49 mm. The last group comprises species with *equiseti* morphotype, except *Fusarium* sp. 2. *Fusarium pernambucanum* and FIESC 29, which grew at similar rate. Two exceptions were observed, FIESC 24 grew faster and *Fusarium* sp. 1 and 5 slower than the others. The most common species in the present study, FIESC 26 and 38, were among the fastest-growing strains, which may contribute to enhanced fitness and further dominance among the sampling areas, a hypothesis that needs to be further explored.

FIESC isolates are able to produce B-trichothecenes (DON and NIV) due to the presence of *Tri5* gene within trichothecene biosynthetic loci (Proctor et al., 2009) and other mycotoxins such as fusarochromanone, BEA and ZEA as reported in the literature (Kosiak et al., 2005; Leslie and Summerell, 2006; O'Donnell et al., 2018; Villani et al., 2016). Recently, 80% of FIESC strains from Spanish cereals produced DON and 25% produced NIV (Marín et al., 2012). Contrastingly, FIESC isolates from Norwegian cereals produced higher quantities of type A trichothecenes but neither detectable levels of DON nor DON derivatives, yet significant amounts of NIV and FUS was produced (Kosiak et al., 2005). In this study, 35% of the strains produced different B-trichothecenes mycotoxins or ZEA, which were distributed randomly across the species and geographic regions. This suggests that FIESC not only may be an important contributor to mycotoxin levels detected in Brazilian rice (Almeida et al., 2012; Moreira et al., unpublished; Savi et al., 2018), but also that in FIESC isolated from rice there is a wide variability in mycotoxin production. Our data agree with a recent analysis of genomes for 13 FIESC member suggesting that the production of secondary metabolites, including mycotoxins, could be affected by the different distribution of functional and related gene clusters

(Villani et al., 2019). Collectively, ours and previous data on mycotoxin production by FIESC members suggest that a wider, global survey should be conducted on FIESC to understand how this trait relates with the presence of mycotoxin gene clusters in order to better assess the risk associated to FIESC occurrence in agro-food important crops.

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