CAROLINE FERNANDA DE AVILA

SPECIES AND TRICHOTHECENE GENOTYPES WITHIN *Fusarium incarnatum-equiseti* SPECIES COMPLEX INFECTING BRAZILIAN RICE

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2017

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
A958s
2017

Avila, Caroline Fernanda de, 1991-

Species and trichothecene genotypes within *Fusarium incarnatum-equiseti* species complex infecting Brazilian rice / Caroline Fernanda de Avila. – Viçosa, MG, 2017.

viii, 42f. : il. (algumas color.) ; 29 cm.

Inclui apêndices.
Orientador: Emerson Medeiros Del Ponte.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Referências bibliográficas: f.30-35.

1. *Fusarium incarnatum-equiseti. 2. Oryza sativa*. 3. Filogenia. 4. Micotoxinas. 5. Tricotecenos. I. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-graduação em Fitopatologia. II. Título.

CDD 22 ed. 579.5677

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APROVADA: 31 de julho de 2017.

Antonio Moretti

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Lucas Magalhães de Abreu

Gláucia Mara Moreira

Gláucia Mara Moreira
(Coorientadora)

Emerson Medeiros Del Ponte
(Orientador)

À minha família,
meu pai Jorge,
minha mãe Luzia,
meu irmão Victor,
e meu namorado José Lucas:
OFEREÇO E DEDICO.

AGRADECIMENTOS

Primeiramente agradeço a Deus, por mais esta oportunidade e pela proteção.

A minha mãe, Luzia e ao meu pai, Jorge, pelo exemplo de vida, esperança, fé e coragem. Muito obrigada pelo apoio, zelo, cuidado e por sempre acreditarem e confiarem em mim.

Ao meu irmão Victor pelo carinho, apoio e conselhos que serão levados para a vida.

Ao meu namorado José Lucas pelo amor, apoio e motivação em todos os momentos.

Ao meu orientador Prof. Emerson Medeiros Del Ponte, pela orientação, confiança, paciência e pelos ensinamentos passados a mim durante o desenvolvimento deste trabalho.

A minha coorientadora Dr. Gláucia Mara Moreira, pela amizade, confiança, companheirismo, conselhos e por sempre estar disposta a ajudar durante toda realização desse estudo.

Aos professores Lucas Magalhães de Abreu e Antonio Moretti, que se dispuseram a participar da banca examinadora, contribuindo de forma relevante ao trabalho aqui realizado.

Aos amigos do Laboratório de Epidemiologia pelo companheirismo e ótima convivência, vocês proporcionaram momentos inesquecíveis durante essa trajetória.

Aos colegas de mestrado pela amizade, em especial a Marcela e Yane, pelo apoio e amizade.

Aos amigos de Viçosa, especialmente a Nayara e Lívia pela amizade e apoio, tornando os momentos difíceis mais leves e calmos, e momentos de alegria em memoráveis.

Ao pessoal do Laboratório de Biologia de Populações de Fitopatógenos, pela paciência e atenção dada durante a utilização dos equipamentos e pela contribuição nas análises moleculares.

Aos funcionários do Departamento de Fitopatologia.

À Universidade Federal de Viçosa, ao Departamento de Fitopatologia e ao Programa de Pós-graduação em Fitopatologia por proporcionarem condições de realizar este trabalho.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo apoio financeiro.

A todos os professores que contribuíram para o meu aprendizado e formação profissional.

A todas as pessoas que direta e indiretamente deram apoio e incentivo, meus sinceros agradecimentos.

MUITO OBRIGADA!

BIOGRAFIA

CAROLINE FERNANDA DE AVILA, filha de Jorge Luiz de Avila e Luzia Elisa Del Pupo de Avila, nasceu em 03 de janeiro de 1991 em Jundiaí - SP.

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SUMÁRIO

ABSTRACT

AVILA, Caroline Fernanda de, M.Sc., Universidade Federal de Viçosa. July, 2017. **Species and trichothecene genotypes within Fusarium incarnatum-equiseti species complex infecting Brazilian rice.** Adviser: Emerson Medeiros Del Ponte. Co-adviser: Gláucia Mara Moreira.

Brazil is a major producer and consumer of rice. Fusarium species infect and colonize cereal grains, and some are able to produce harmful mycotoxins that can accumulate in the kernels. Species of Fusarium incarnatum-equiseti species complex (FIESC) are regularly detected in rice kernels and are known as producers of different mycotoxins. The present study aimed to identify the phylogenetic species, evaluate the potential for trichothecene production and morphological variation of the FIESC strains associated with Brazilian rice kernels. An initial set of 147 FIESC strains were isolated from rice kernels produced at all major rice-growing regions in the country. A subsample (67 isolates) representative of all regions was subjected do DNA extraction followed by amplification and sequencing of a portion of the translation elongation factor 1-α (EF-*1α*) gene. The DNA sequences alignment of FIESC isolates and reference strains were subjected to Bayesian inference, maximum parsimony and maximum likelihood. Microscopic characters were conducted with cultures grown on SNA at 25 ºC in 12 h photoperiod. Radial growth and colony pigmentation were evaluated on PDA at 25 ºC in complete darkness for 3 and 14 days, respectively. Toxigenic potential was assessed by the detection of terpene synthase (Tri5) gene by PCR. Phylogenetic analysis suggested the presence of 15 species, among them, eight corresponding to previously described species: FIESC 4 (F. lacertarum), 6, 16, 17, 20, 24, 26 and 29. Seven new phylogenetic species were proposed: FIESC 32−38. Among them, six were constituted by a single strain. Thirty-one Tri5$^+$ isolates were found in 46.2% of species, including the two most dominant species, FIESC 26 and 32, and were distributed randomly across geographic regions. Morphological traits were observed and measured, and differences among morphological characteristics allowed distinguishing the patterns found in this study. Our results provide a baseline data on morphology of FIESC species, as well as an improved understanding of the composition and toxigenic potential of species within this complex associated with Brazilian rice.

RESUMO

AVILA, Caroline Fernanda de, M.Sc., Universidade Federal de Viçosa, julho de 2017. **Espécies e genotipagem de tricoteceno no complexo de espécies Fusarium incarnatum-equiseti infectando arroz no Brasil.** Orientador: Emerson Medeiros Del Ponte. Coorientadora: Gláucia Mara Moreira.

O Brasil é um grande produtor e consumidor de arroz. Espécies de Fusarium infectam e colonizam grãos deste cereal, sendo muitas capazes de produzir micotoxinas prejudiciais que podem se acumular nos grãos. As espécies pertencentes ao complexo Fusarium incarnatum-equiseti (FIESC) são regularmente detectadas em arroz e são conhecidas como produtoras de diferentes micotoxinas. O presente estudo teve como objetivos identificar as espécies filogenéticas, avaliar o potencial para produção de tricotecenos e a variação morfológica de isolados de FIESC associados a grãos de arroz no Brasil. Um conjunto inicial de 147 isolados foram obtidos de grãos de arroz produzidos em todas as principais regiões produtoras de arroz do país. Uma subamostra (67 isolados) representativa de todas as regiões foi sujeita à extração de DNA seguida por amplificação e sequenciamento de uma porção do gene fator de elongação 1-α (EF-1a). O alinhamento das sequências de DNA dos isolados de FIESC e isolados de referência foi submetido aos métodos de inferência Bayesiana, máxima parcimônia e máxima verossimilhança. Características microscópicas foram realizadas com culturas cultivadas em SNA a 25 ºC em fotoperíodo de 12 h. O crescimento radial e a pigmentação das colônias foram avaliados em BDA a 25 ºC em ausência de luz por 3 e 14 dias, respectivamente. O potencial toxigênico foi avaliado pela detecção do gene terpeno sintase (Tri5) através de PCR. A análise filogenética sugeriu a presença de 15 espécies, entre elas, oito correspondentes às espécies descritas anteriormente: FIESC 4 (F. lacertarum), 6, 16, 17, 20, 24, 26 e 29. Foram propostas sete novas espécies filogenéticas: FIESC 32−38. Dentre estas, seis foram constituídas por um único isolado. Trinta e um isolados Tri5$^+$ foram encontrados em 46,2% das espécies, incluindo as duas espécies mais dominantes, FIESC 26 e 32, e foram distribuídos aleatoriamente nas regiões geográficas. Os traços morfológicos foram observados e medidos, e as diferenças entre as características morfológicas permitiram distinguir os padrões encontrados neste estudo. Nossos resultados fornecem dados de base sobre a morfologia das espécies de FIESC, bem como uma melhor compreensão da composição e do potencial toxigênico das espécies deste complexo associadas ao arroz no Brasil.

INTRODUCTION

Brazil is ranked among the top ten largest rice producers in the world, being the first outside Asia (FAO/USDA, 2016). The total country's production was estimated in 10.6 million tons in the 2015/2016 growing season, and is concentrated mainly in the southernmost states of Brazil. However, there are also important rice-producing areas in the central-western and northern regions in Brazil under irrigated (lowland) and rain fed (upland) conditions (CONAB, 2016).

Among the biotic stresses that affect the crop, fungal diseases that occur on leaves and panicles are the main concern to rice farmers due to the potential to indirectly reduce yield by damaging the leaves or directly by damaging the kernels (Ou, 1985). Moreover, several fungi may infect kernels during the pre- or post-harvest period and produce mycotoxins that can affect human and animal health. Mycotoxins such as those produced by Fusarium species are difficult to eliminate during food processing (Miller et al., 1991; Desjardins, 2006). Several Fusarium mycotoxins have been reported in association with rice seeds (Agarwal et al., 1989; Desjardins et al., 2000; Makun et al., 2011), but the most common are fumonisins and moniliformin produced by species of the Fusarium fujikuroi species complex (FFSC) (Desjardins et al., 1997; Leslie et al., 1992; Moretti et al., 1996; Desjardins et al., 2000), B-trichothecenes, such as nivalenol (NIV), deoxynivalenol (DON) and acetylated derivatives (15-ADON and 3-ADON), and zearalenone (ZEA). Trichothecenes and ZEA are commonly produced by members of the Fusarium graminearum species complex (FGSC) (Lee et al., 2009).

The ability of a fungal strain to produce a specific mycotoxin has been assessed indirectly using molecular assays or directly using chemical analysis of pure cultures on agar plates or natural substrates (host tissue) colonized by the fungus (Desjardins, 2008). The first is based on PCR amplification of genes that regulate toxin production. For example, the presence of Tri3, Tri5, Tri7, Tri8, Tri12 and Tri13 genes (B-trichothecene genotypes) have been used to predict the potential to produce DON and its acetylates or NIV (Ward et al., 2002; Proctor et al., 2009; Lee et al., 2001), while potential to produce ZEA can be predicted based on polyketide synthases (PKSs) genes, such as PKS3, PKS4, PKS14, PKS15 and PKS16 (Linnemannstons et al., 2002; Kim et al., 2005; Lysøe et al., 2006). Molecular identification of toxin genotypes should be confirmed by chemical analysis to ensure the reliability of the results and whether the former can be used with confidence in surveys (Desjardins, 2008). Various methods have been employed to detect and quantify trichothecene toxins, and the most commonly used nowadays are those based on chromatography (HPLC, LC-MS and GC-MS) or immunoassays kits, which are a more affordable option and reliable if the goal is qualitative (Lattanzio et al., 2009).

Surveys of rice grain have been conducted in many countries and the data show large variation in the toxins and associated Fusarium species, which include mainly those of the F. fujikuroi and F. graminearum species complexes. A pioneer study conducted during the 1990s showed the natural occurrence of fumonisins B1 and B2 in rice in the United States, which were associated with the occurrence of F. proliferatum (Abbas et al., 1998). As to trichothecenes, NIV have been found in rice in Korea (Lee et al., 2009). NIV, DON and Fus-X in Japan (Tanaka et al., 2007) and Fus-X, DON and NIV in Nepal (Desjardins et al. 2000). DON was considered by FAO-WHO as a common contaminant of rice (FAO, 2002).

In Brazil, a survey of mycotoxins in parboiled rice from markets showed the presence of aflatoxin B1 (AFB B1), ochratoxin (OTA), DON and ZEA (Dors et al., 2009). More recently, DON and ZEA mycotoxins have been found in natural and parboiled rice, as well in bran and husk fractions (Heidtmann-Bemvenuti et al., 2012). Apparently, higher levels of contamination with ZEA and DON are found in parboiled rice than in natural rice (111.7 and 8 μg kg^{-1}, respectively), which may be due to parboiling process allowing toxins to migrate from bark into the grain (Lazzari, 1997). The analysis of several mycotoxins in 230 samples of rice and products, from a year with reports of human health problems due to poor storage of rice, showed that two Fusarium mycotoxins, ZEA and DON, were present in 45.2 and 8.3% of the samples, respectively; the maximum level of DON was 244 μg kg^{-1} (Almeida et al., 2012).

A specific legislation to mitigate the mycotoxin risk to consumers in Brazil has been recently set by establishing maximum tolerated levels (MTLs) for a range of mycotoxins in cereals grains and derivatives (ANVISA, 2011). For rice, MTLs were defined for DON, fumonisins and ZEA, three Fusarium mycotoxins (ANVISA, 2011). Thus, there is an urgent need to increase surveillance and provide knowledge on the diversity of toxigenic Fusarium species and their toxigenic potential in order to improve detection methods and define potential targets in microbiological and mycotoxin surveys.

In Brazil, four FGSC members were identified associated with rice seeds in Rio Grande do Sul state, in the southern region. Fusarium asiaticum was predominant, followed by F. cortaderiae, F. graminearum and F. meridionale. In vitro mycotoxin production by strains belonging to the four species indicated F. asiaticum as producer of NIV and F. graminearum as producer of 15-ADON and 3-ADON (Gomes et al., 2015). Data about the occurrence of toxigenic species belonging to FFSC or other Fusarium complexes in Brazilian rice are scarce, but a recent study showed the presence of species from the Fusarium incarnatum-equiseti species complex (FIESC), although the identification was limited to morphological criteria (Gomes, 2014).

Species within FIESC are typical saprophytes and opportunistic pathogens associated with host plants, but also with humans, especially in immunocompromised patients causing superficial infections, digestive disorders, skin inflammations, tachycardia, haemorrhages and nervous system disorders (Jurado et al., 2005). FIESC species are commonly isolated from cereals, such as wheat, barley, oat and maize (Marín et al., 2012; Castellá & Cabañes, 2014; Villani et al., 2016). In rice, FIESC have been found in seeds produced in Asia, Europe and Africa (Castellá & Cabañes, 2014; Makun et al., 2011; Amatulli et al., 2010; Maheshwar & Janardhana, 2010; Desjardins et al., 2000, Stepień et al., 2012).

FIESC isolates have been shown to produce fusarochromanone (FUSCHR), ZEA and trichothecenes [DON, NIV, T-2 toxin and 4-acetylnivalenol (FUS-X)], equisetin (EQ), diacetoxy-scirpenol (DAS), 15-monoacetoxy-scirpentriol (MAS) and neosolaniol (NEO) (Leslie & Summerell, 2006; Kosiak et al., 2005, Villani et al., 2016). Fusarium equiseti strains isolated from different host/substrates (Allium cepa, Oryza sativa, Pinus root, Triticum sp., Zea mays, soil, among others) in Poland and Italy produced ZEA in rice cultures (1.34 ng/g to 34,000 ng/g) (Stepień et al., 2012). Over 80% of F. equiseti strains from cereals in Europe produced DON and 25% were able to produce NIV (Marín et al., 2012).

Knowledge of the variation in morphological traits within FIESC is very limited, only four species have a morphological description in the literature: F. equiseti, F. lacertarum, F. scirpi and F. semitectum, the last one was used in the literature, but through the study of the type collection revealed that this Latin binomial was misapplied (Leslie & Summerell, 2006; O'Donnell et al., 2009). A unique morphological character of the species belonging to this complex is a structure where meso- and microconidia are arranged in the aerial mycelium, often with “rabbit ears” appearance and is easily observed in situ through the microscope examination.

Based on literature description, Kosiak et al. (2005) identified two morphotypes varieties in the macroconidia of F. equiseti from Norwegian cereals: morphotype I, with short apical cells, and morphotype II, with long apical cells. To resolve large number of morphologically cryptic species, phylogenetic criteria is adopted and several genes have been used to determine species boundaries within Fusarium, under the concept of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor et al., 2000). Among them, the translation elongation factor 1-alpha (EF-*1α*) gene is highly informative at the species level, as non-orthologous copies have not been reported and it has shown sufficient polymorphism to separate Fusarium species, thus being it a good choice for single-locus identification tool and a genetic marker for Fusarium identification (Geiser et al., 2004). The EF-*1α* gene was the most informative locus to identify species within FIESC when compared to ITS+LSU 28S rRNA, the second largest subunit of RNA polymerase II (RPB2) and calmodulin (CAM) genes in a Fusarium study including FIESC (O'Donnell et al., 2009). That was the first phylogenetic study using multilocus DNA sequences data of FIESC which used clinical strains infecting animals and humans in the United States (O'Donnell et al., 2009). Species identification, based on GCPSR, resolved the isolates in twenty-eight phylogenetically distinct species nested in the complex. The Latin binomial nomenclature was applied to 3 of 28 species identified and for the rest the authors adopted haplotype nomenclature using Arabic numbers to species in order to facilitate accurate communication within scientific community. Two new species were later identified among isolates obtained from insects and named as FIESC 29 and FIESC 30 (O'Donnell et al., 2012).

To assess phylogenetic diversity of FIESC strains isolated from Spanish wheat, partial sequence of EF-*1α* gene was subjected to Maximum-parsimony and Bayesian Inference, which resolved FIESC strains into seven of the previously described species (Castellá & Cabañes, 2014). In a recent study with cereal-infecting strains of FIESC, a combined data set of partial sequences of CAM, RPB2, EF-*1α*, and β-tubulin (TUB2) genes from 69 strains (including three strains from rice) from Europe, North America and Turkey, assigned the FIESC isolates to five phylogenetic species, including a novel lineage designated as FIESC 31 (Villani et al., 2016). Thus, 31 phylogenetic species within FIESC have been described but only three of them received Latin binomials (sensu

O’Donnell): F. lacertarum (FIESC 4), F. scirpi (FIESC 9) and F. equiseti (FIESC 14) (O’Donnell et al., 2009).

The composition, spatial distribution, toxigenic potential, phylogeny and morphotypes within FIESC isolates associated with rice in Brazil, but also globally, are not well understood. We hypothesize that some of already described, or even new toxigenic species, may occur in Brazilian rice at frequencies that vary across the distinct rice-growing regions in the country. Therefore, the objectives of this study were to identify phylogenetic species, characterize the morphology, and assess the genetic ability to produce trichothecenes for a collection of FIESC-like strains obtained from rice in the main producing regions of Brazil.

MATERIALS AND METHODS

Sampled area, strains collection and isolation procedures

The collection of strains used in this study was obtained from samples of mature kernels or panicles collected in the major rice-producing regions of Brazil. The collection is composed of a historical sample (2011/12 season) of FIESC-like isolates (13 strains) obtained from mature kernels produced in Rio Grande do Sul (RS) state (Gomes, 2014) and two contemporary collections of isolates obtained from rice grown in 2014/15 and 2015/16 seasons. The isolates from the 2014/15 season and part of isolates from the 2015/16 season were obtained from samples of mature kernels produced at experimental field plots of RS, Goiás (GO), Mato Grosso (MT), Tocantins (TO), Maranhão (MA), Roraima (RR) and São Paulo (SP) states. The majority of 2015/16 strains were isolated from selected panicles collected randomly at commercial fields surveyed in the states of Santa Catarina (SC), RS and TO (Figure 1).

The strains were obtained from seven to ten-day old colonies grown from a sub-sample of 200 developing/mature kernels using a standard blotter test (25 ºC and 12/12 light/dark cycle), which were randomly selected from a composite sample of the field or seed lots. Mycelia and conidia were harvested from fungal colonies resembling Fusarium and grown on Synthetic Nutrient-poor Agar (SNA, Nirenberg, 1976) in order to assign them to species or species complex based on morphological traits, specially through the presence of "rabbit ears" appearance (Leslie & Summerell, 2006). One hundred and forty seven isolates were assigned to FIESC following these criteria and were available for this study (Table 1 Appendix, Figure 1).

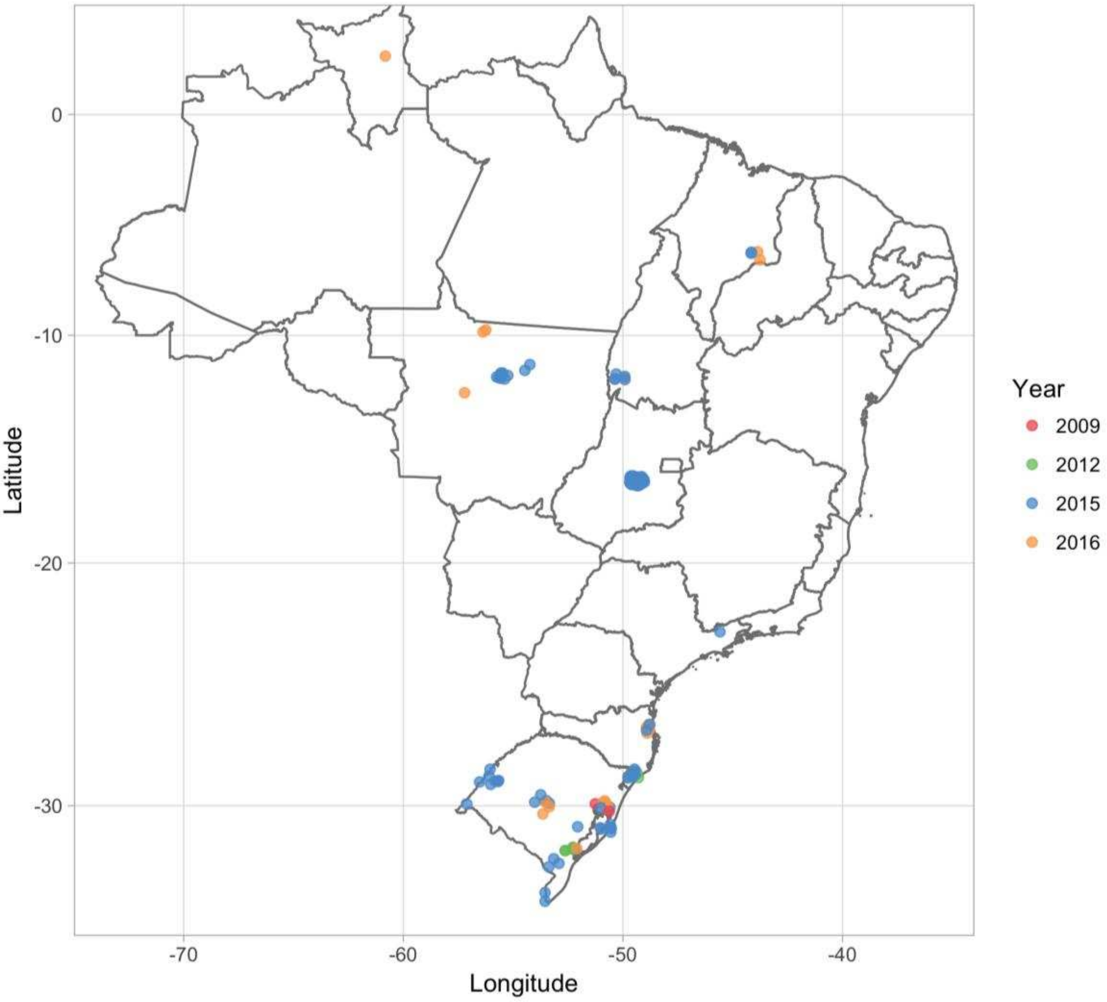


Figure 1. Approximate geographical origin of FIESC-like strains (n = 147) sampled from rice fields during four harvesting seasons.

DNA extraction, PCR assays and sequencing

A subsample of the rice strains was selected following geographic criteria in order to reduce sample size for conducting the molecular analyses. Sixty-seven isolates were selected and used for the phylogenetic analyses as first step of the work (Table 1 Appendix, Figure 1 Appendix).

For this subset, single spore cultures were obtained, preserved in microtubes and stored at 4 ºC. At different times, isolates were grown on potato dextrose agar (PDA, Acumedia, Neogen Corporation) for 7 days at 25 ºC. Fresh mycelia were harvest 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. 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 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.). 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 ºC for 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 72 ºC for 5 min. 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 do Brasil, São Carlos, Brazil). The fragments of amplified DNA were sent for sequencing by a commercial service.

Sequence alignment and phylogenetic analysis

The DNA sequences of EF-*1α* 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; Villani et al., 2016) using the MUSCLE algorithm (Edgar, 2004) implemented in the software MEGA6 (Tamura et al., 2013). 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 (Ronquist et al., 2012) with GTR+I+G model, 1,500,000 generations through two independent runs, sampled every 500 generations and burnin-in of 25% of initial trees. Only posterior probability values ≥95% were considered for this analysis. Phylogenetic tree was visualized using the FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and edited using InkScape 0.9.1 (www.inkscape.org). Maximum-likelihood (ML) and Maximum-parsimony (MP) analyses were implemented in MEGA6 software (Tamura et al., 2013) with 1,000 bootstrap replications. The model chosen for ML was GTR+I+G. MP analysis was performed using 100 random additional sequences with tree bisection-reconnection (TBR) method of branch swapping. Only bootstrap values ≥70% were considered for these analyses. Fusarium concolor (NRRL 13459) was used as outgroup.

Trichothecene genotype analyses

The genetic ability of strains to produce B-trichothecene mycotoxins was assessed by PCR targeting the terpene synthase (Tri5) gene that codes for a key enzyme within the biosynthetic pathway of trichothecene mycotoxins, which resulted in the classification as Tri5^{+} or Tri5^{-} (Ward et al., 2002; Proctor et al., 2009). PCR conditions were the same as described in Villani et al. (2016). Amplification products were resolved on 1 % agarose gels and the sized scored in comparison to a 100-bp DNA size ladder (Promega, Madison, USA). Fusarium fujikuroi (CML 3140) and Fusarium cortaderiae (10Tr193) were used as negative and positive controls, respectively.

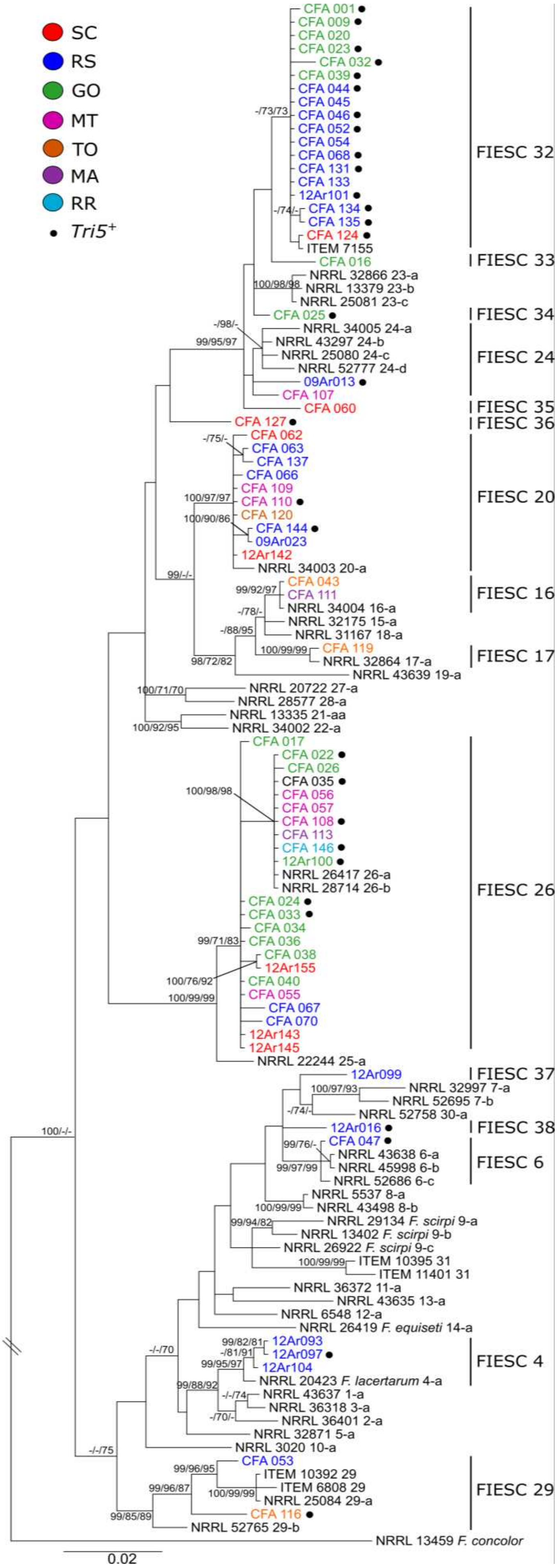
Morphological characterization

For the morphological assessment, 46 strains representative of the full set of phylogenetic species were select. 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. The mycelial growth (mm) was assessed after 3 days, by measuring the 2 right-angled colonies diameter using a pachymeter, and the colony pigmentation was evaluated after 14 days (Leslie & Summerell, 2006). Microscopic examination was assessed using 7 to 14 day-old colonies grown on plastic Petri dishes containing SNA with pieces of carnation leaves incubated at 25 ºC and photoperiod (Leslie & Summerell, 2006). Morphological traits were evaluated and photographed, including 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.

RESULTS

FIESC species assignment

The amplification of EF-*1α* gene yielded a fragment of ≈700 bp. The resulting phylogenetic trees were highly topologically concordant and the phylogeny inferred by BI analysis is shown in Figure 2. The phylogenetic analyses suggested the presence of 15 species, among them, eight grouped with the following previously described species: FIESC 4 (F. lacertarum), 6, 16, 17, 20, 24, 26 and 29. All these species formed monophyletic groups with high statistical support (i.e., 72-100%), with exception of lineage 24. FIESC 26 formed a subclade containing the reference sequences.


SC
RS
GO
MT
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MA
RR
• Tri5+
CFA 001 •
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NRRL 13379 23-b
NRRL 25081 23-c
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CFA 025 •
FIESC 34
NRRL 34005 24-a
NRRL 43297 24-b
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NRRL 52777 24-d
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NRRL 34003 20-a
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100/90/86
FIESC 20
99/-/-
CFA 043
CFA 111
NRRL 34004 16-a
NRRL 32175 15-a
NRRL 31167 18-a
99/92/97
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FIESC 16
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100/99/99
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FIESC 17
NRRL 43639 19-a
NRRL 20722 27-a
NRRL 28577 28-a
100/71/70
NRRL 13335 21-aa
NRRL 34002 22-a
100/92/95
CFA 017
CFA 022 •
CFA 026
CFA 035 •
CFA 056
CFA 057
CFA 108 •
CFA 113
CFA 146 •
12Ar100 •
NRRL 26417 26-a
NRRL 28714 26-b
100/98/98
CFA 024 •
CFA 033 •
CFA 034
CFA 036
CFA 038
12Ar155
CFA 040
CFA 055
CFA 067
CFA 070
12Ar143
12Ar145
99/71/83
100/76/92
100/99/99
FIESC 26
NRRL 22244 25-a
12Ar099
FIESC 37
NRRL 32997 7-a
NRRL 52695 7-b
NRRL 52758 30-a
100/97/93
-/74/-
12Ar016 •
FIESC 38
CFA 047 •
NRRL 43638 6-a
NRRL 45998 6-b
NRRL 52686 6-c
99/76/-
99/97/99
FIESC 6
100/-/-
NRRL 5537 8-a
NRRL 43498 8-b
100/99/99
NRRL 29134 F. scirpi 9-a
NRRL 13402 F. scirpi 9-b
NRRL 26922 F. scirpi 9-c
99/94/82
ITEM 10395 31
ITEM 11401 31
100/99/99
NRRL 36372 11-a
NRRL 43635 13-a
NRRL 6548 12-a
NRRL 26419 F. equiseti 14-a
12Ar093
12Ar097 •
12Ar104
NRRL 20423 F. lacertarum 4-a
99/82/81
-/81/91
99/95/97
-/-/70
FIESC 4
NRRL 43637 1-a
NRRL 36318 3-a
NRRL 36401 2-a
99/88/92
-/-/74
-/70/-
NRRL 32871 5-a
NRRL 3020 10-a
-/-/75
CFA 053
ITEM 10392 29
ITEM 6808 29
NRRL 25084 29-a
CFA 116 •
NRRL 52765 29-b
99/96/95
99/96/87
100/99/99
99/85/89
FIESC 29
NRRL 13459 F. concolor
0.02

Figure 2. Phylogenetic tree inferred from partial EF-1α gene sequences from members of the Fusarium incarnatum-equiseti species complex from Brazilian rice using Bayesiana Inference (BI). Posterior probability ≥95% (BI) and bootstrap values ≥70% (MP and ML) are in the internodes, respectively. Fusarium concolor (NRRL 13459) was used as outgroup. Colors represent the origin of isolates of this study. Reference sequences were from O'Donnell et al. (2009, 2012) and Villani et al. (2016). (●) presence of Tri5 gene. Abbreviation: NRRL (The ARS Culture Collection, Peoria, Illinois, USA), ITEM (Institute of Sciences of Food Production, Bari, Italy). Brazilian States (GO: Goiás, TO: Tocantins, RS: Rio Grande do Sul, MT: Mato Grosso, SC: Santa Catarina, MA: Maranhão, SP: São Paulo, RR: Roraima).

A large monophyletic group, represented by 19 strains, was statically supported by MP and ML analysis (73% bootstrap, respectively), which suggests a new phylogenetic species, named here as FIESC 32. In addition, six other individual strains (CFA016, CFA025, CFA060, CFA127, 12Ar099, 12Ar016) did not cluster with any reference sequences and represents a putatively phylogenetically distinct new FIESC species constituted by a single strain, named FIESC 33 to 38. Overall, 60% of the rice isolates (40/67) examined here were assigned to two species: FIESC 26 and one of the new species (FIESC 32) (Figure 2). These species were found in the two most sampled states, with FIESC 26 dominating in GO and FIESC 32 in RS state. Species 4, 6 and 29 were recovered from kernels produced in RS state and isolates of FIESC 26 and 20 were found at various locations (Figure 3-a).

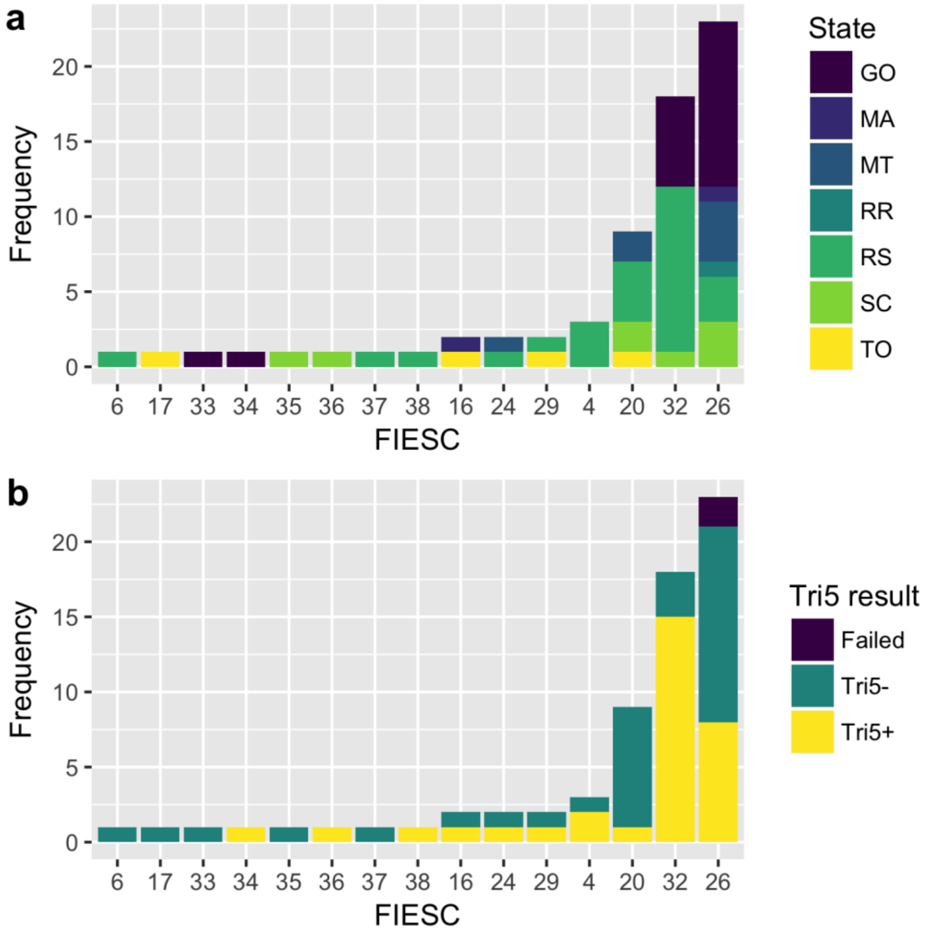

Figure 3. Frequency of species. **a)** State of origin for all identified linages, and **b)** Presence/absence of Tri5 gene among FIESC species. Brazilian States (GO: Goiás, TO: Tocantins, RS: Rio Grande do Sul, MT: Mato Grosso, SC: Santa Catarina, MA: Maranhão, SP: São Paulo, RR: Roraima).

Terpene synthase (Tri5) gene analysis

There were 31 Tri5^{+} out of 67 isolates, and both Tri5^{+} and Tri5^{-} were found in the two most dominant species: FIESC 32 (15 Tri5^{+} strains) and FIESC 26 (seven Tri5^{+} strains). Six other Tri5^{+} strains were found in four previously described species (FIESC 20, 24, 4 and 29) and the last three strains were found in the newly described species (FIESC 34, 36 and 38). These Tri5^{+} isolates appeared to be randomly distributed across the species (Figure 3-b, Table 1 Appendix).

Morphological characterization

The mean diameter of the mycelial radial growth ranged from 39 to 52 mm across species (Figure 4-a, Table 2 Appendix) after three days old on PDA at 25 °C in the dark. The faster growth was found for FIESC 33 (57 mm) and the slowest for FIESC 35 (35 mm) isolates. White to brown mycelia were produced by all species, with two exceptions, FIESC 6 and 20, which were light-brown or cream (Figures 6-e and 9-h, respectively). The reverse varied from beige or cream (e.g. Figures 5-i and 6-f) to orange to light brown (e.g. Figures 10-e and 11-g), but there was predominance of cream colour with subtle variations to light brown (e.g. Figures 9-g and 11-i). Color of reverse colony from FIESC 24 and 26 were orange and brown, respectively (Figures 10-e and 11-g). When present, in 13% of the species, sporodochia was orange in color (Figure 5-c, 10-h and 12-d-e) and was usually observed near to the carnation leaves on SNA. Chlamydospores were found in FIESC 4, 6, 16 and 26, in chains (Figures 5-e and 6-d) or solitary (7-b and 11-d; Table 2 Appendix). Macroconidia were formed in mono- and polyphialides (e.g. Figures 20-d-e, 13-e and 17-d).

Conidia shape and size varied among the species. Macroconidia were observed in all species, whereas mesoconidia and microconidia were produced by 13 and 8 species, respectively (Table 3 Appendix). Mean macroconidia length varied from 25 to 34 µm for all, but for F. lacertarum conidia length was approximately 49 µm (Figure 4-b). The mean width ranged from 3 to 4 µm across the species, with F. lacertarum (FIESC 4), contrarily to length, showing the lowest width (Figure 4-b).

Three morphologically distinct patterns were observed for macroconidia shape. 1) The most predominant (8 out of 15 species) was falcate, with variation in apical and basal cells: hooked apical cell and foot-shaped cell (FIESC 6, 17, 24, 33) (Figures 6-a, 8-b, 10-a, 14-b, respectively); curved apical cell and foot-shaped cell (FIESC 16, 32, 38) (Figures 7-a, 13-a-b and 19-a); hooked apical cell and distinctly notched basal cell (FIESC 26)

(Figure 11-d-e); 2) another shape appeared with a dorsiventral curvature, tapering and elongate apical cell with prominent basal foot-shape cell (F. lacertarum, FIESC 37) (Figures 5-a, 18-a); and 3) nearly straight macroconidia, with blunt apical cell and distinctly notched basal cell (FIESC 34, 35) (Figures 15-a, 16-a) or slightly curved apical cell and barely notched basal cell (FIESC 20, 36) (Figures 9-a, 17-a).

Mesoconidia were not produced only by two species (FIESC 16, 17). The shapes observed for this conidium type were falcate (e.g. Figure 12-b), curved (e.g. Figure 6-b), or straight (e.g. Figure 17-b). Microconidia were produced by eight phylogenetic species (Table 3 Appendix), and were reniform (Figures 6-c and 12-c), obovoid (Figure 7-a) or fusiform (Figures 9-c, 11-d-e, 13-c, 16-d and 17-c).

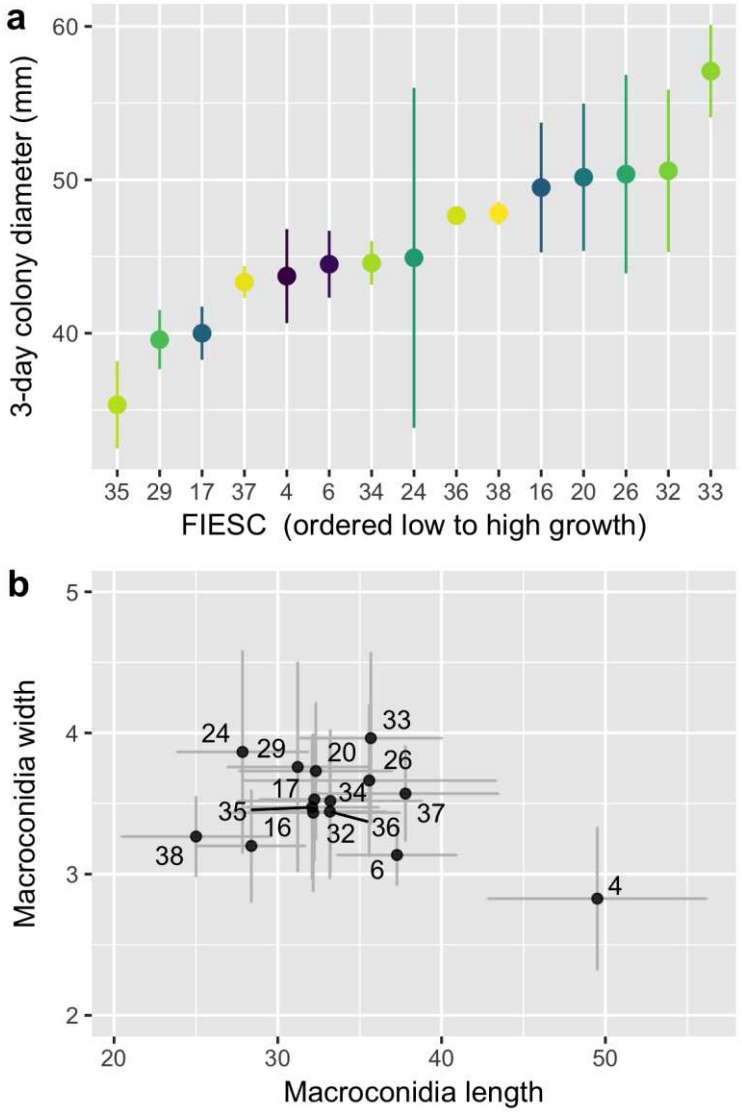

Figure 4. Morphology measurements. **(a)** Colony diameter of 15 species after 3-days of growth on PDA under 25 ºC (mm), and (**b)** Variation in macroconidia length (µm) and width (µm) across the identified species. The error bar represents the standard deviation.

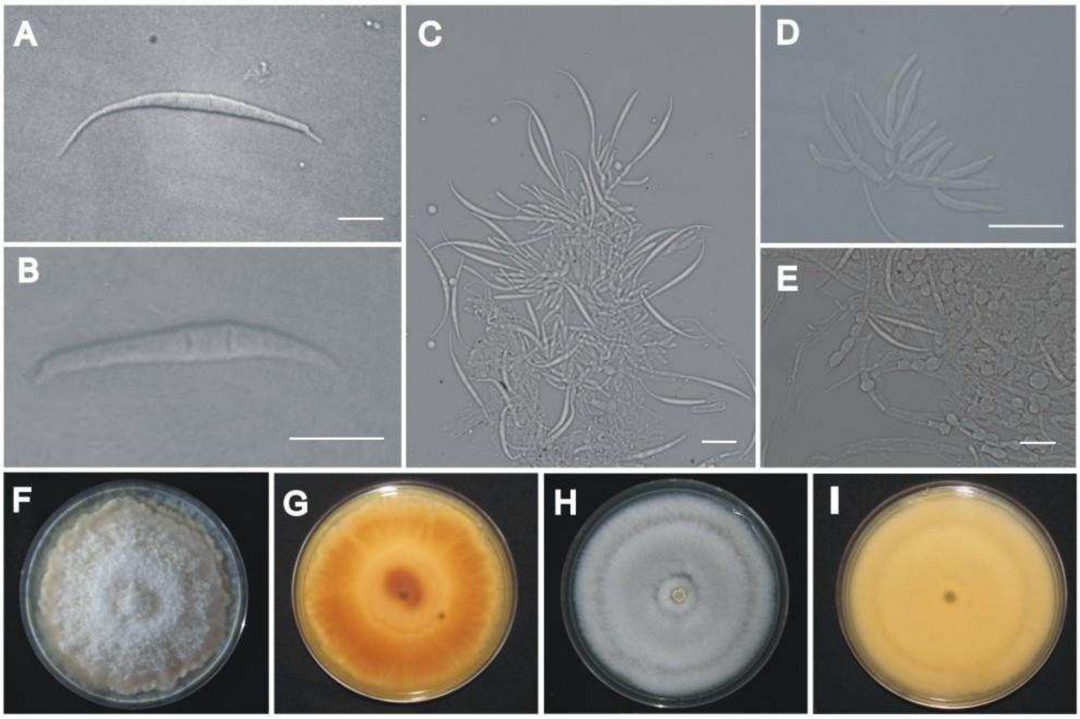

Figure 5. Fusarium lacertarum (FIESC 4). **A:** macroconidia with dorsiventral curvature; **B:** falcate mesoconidia; **C–D:** sporodochia; **E:** chlamydospores in chains; **F–I:** colonies on PDA. Surface brown (F) or white (H); reverse brown (G) or cream (I). Scale bars: 10 µm.

Figure 6. FIESC 6. **A:** falcate macroconidia; **B:** curved mesoconidia; **C:** reniform microconidia; **D:** chlamydospores in chains; **E–F:** colonies on PDA. Surface (E) and reverse (F) light-brown. Scale bars: 20 µm, C - 10 µm.

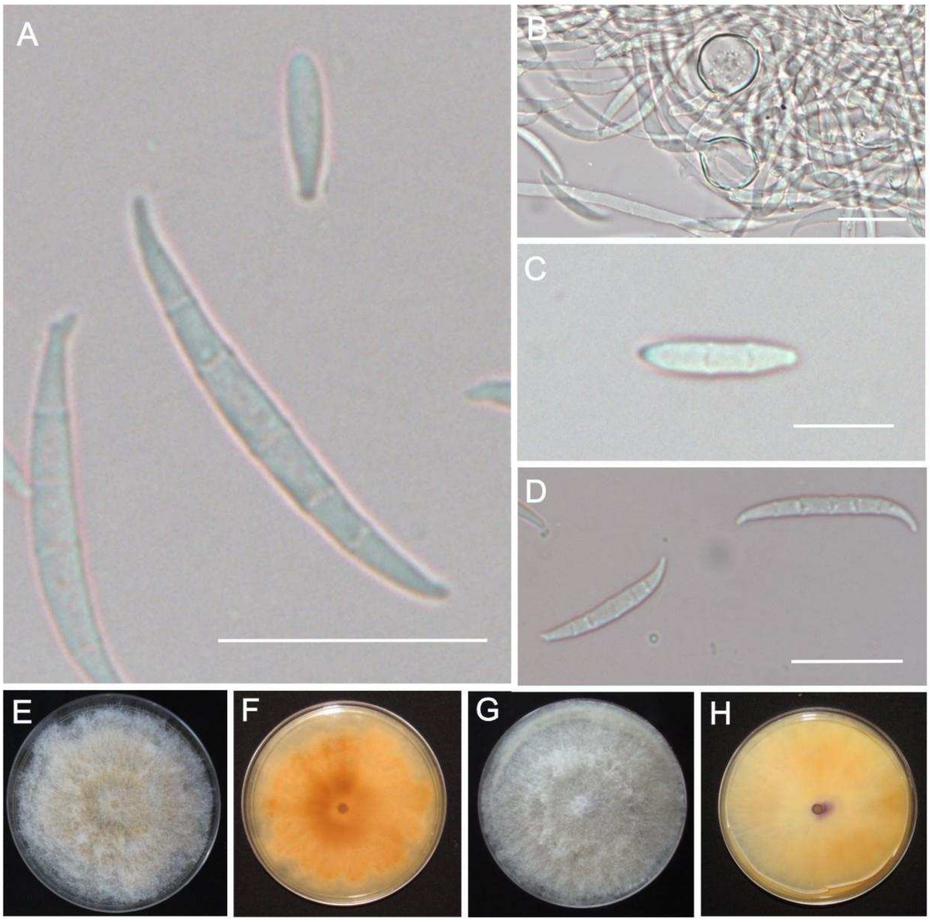

Figure 7. FIESC 16. **A and D:** falcate macroconidia and obovoid microconidia (A); **B:** solitary chlamydospores; **C:** obovoid microconidia. **E–H:** colonies on PDA. Surface white (E) and (G); reverse light-brown (F) and cream (H). Scale bars: 20 µm, C - 10 µm.

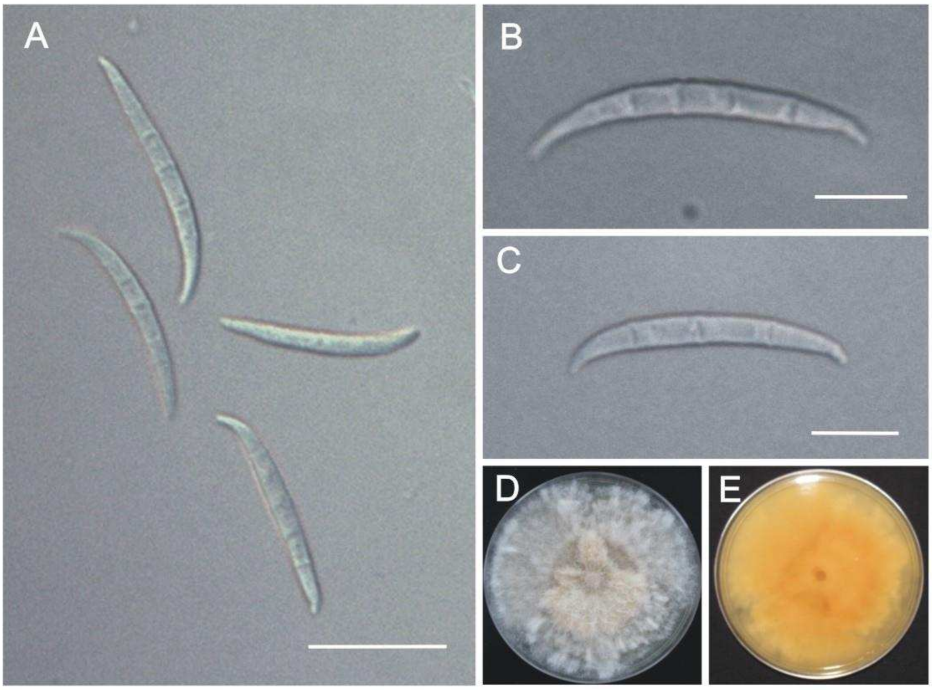

Figure 8. FIESC 17. **A–C:** falcate macroconidia; **D–E:** colonies on PDA. Surface white (D); reverse brownish orange (E). Scale bars: 20 µm.

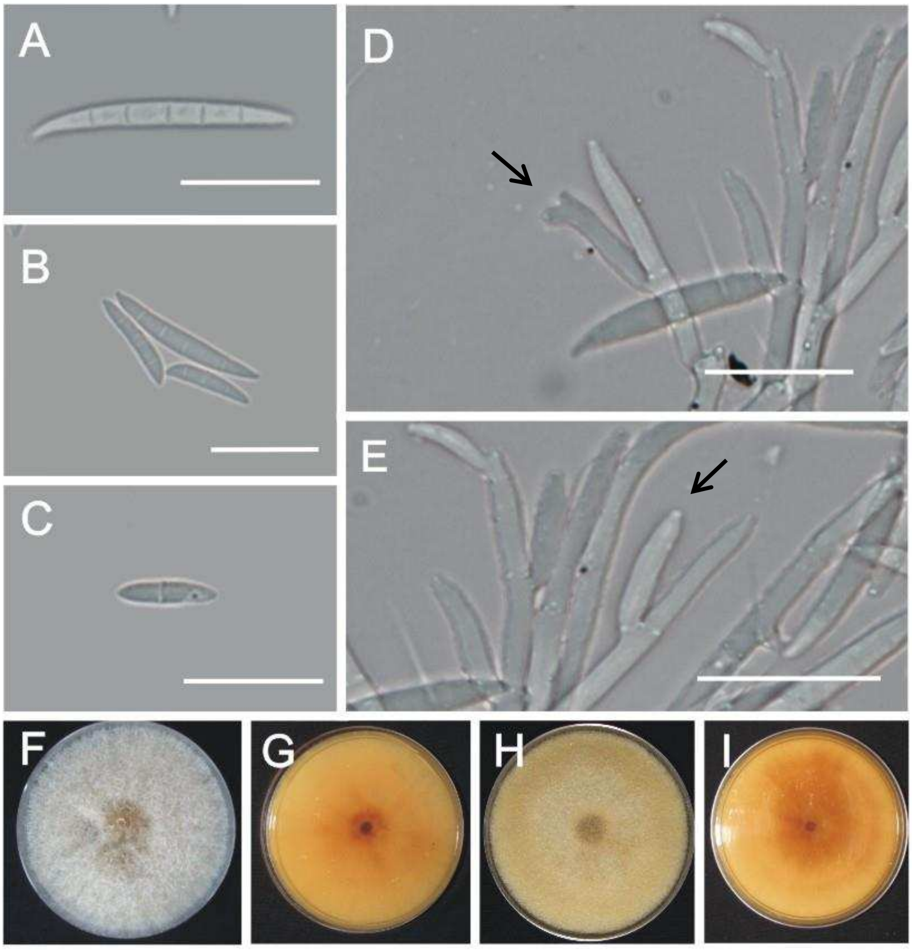

Figure 9. FIESC 20. **A:** nearly straight macroconidia; **B:** falcate mesoconidia; **C:** fusiform microconidia; **D:** polyphialide indicated by arrow; **E:** monophialide indicated by arrow; **F–I:** colonies on PDA. Surface white (F) or cream (H) and reverse brownish orange (G, I). Scale bars: 20 µm.

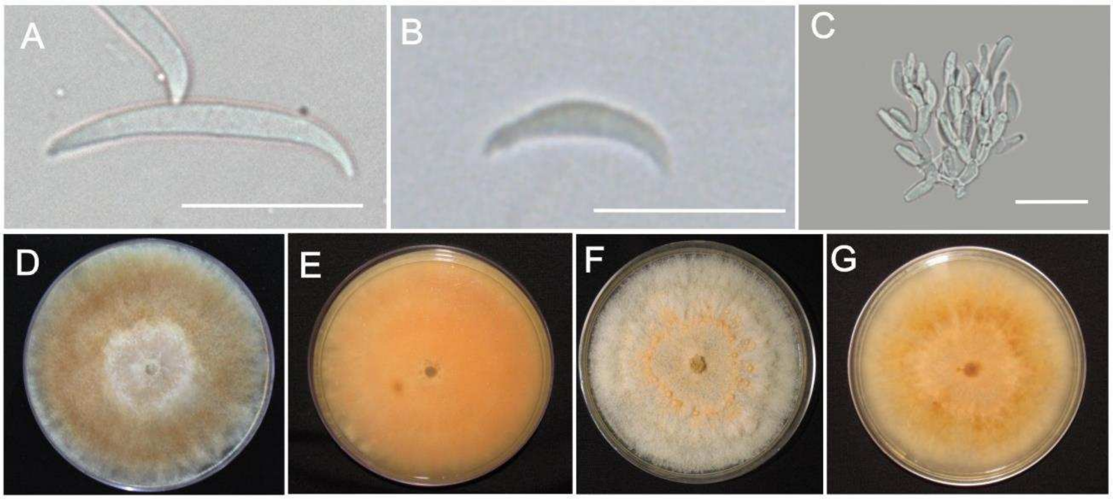

Figure 10. FIESC 24. **A:** falcate macroconidia; **B:** curved mesoconidia; **C:** sporodochia; **D–G:** colonies on PDA. Surface light-brown (D) or white (F) and reverse orange (E) or brownish orange (F). Scale bars: 10 µm.

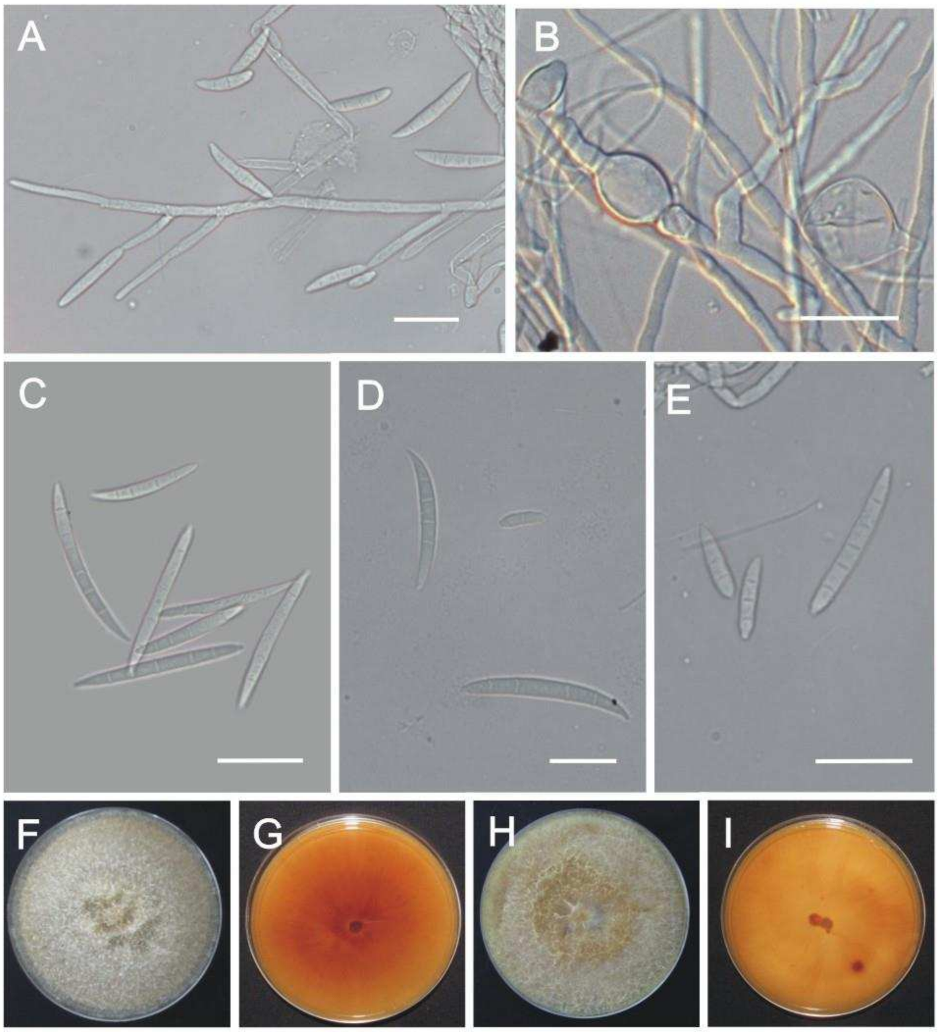

Figure 11. FIESC 26. **A, C, D:** falcate macroconidia; **B:** solitary chlamydospore; **E:** fusiform microconidia **F–I:** colonies on PDA. Surface white (F) and light-brown (H); reverse brownish orange to brown (G, I). Scale bars: 20 µm.

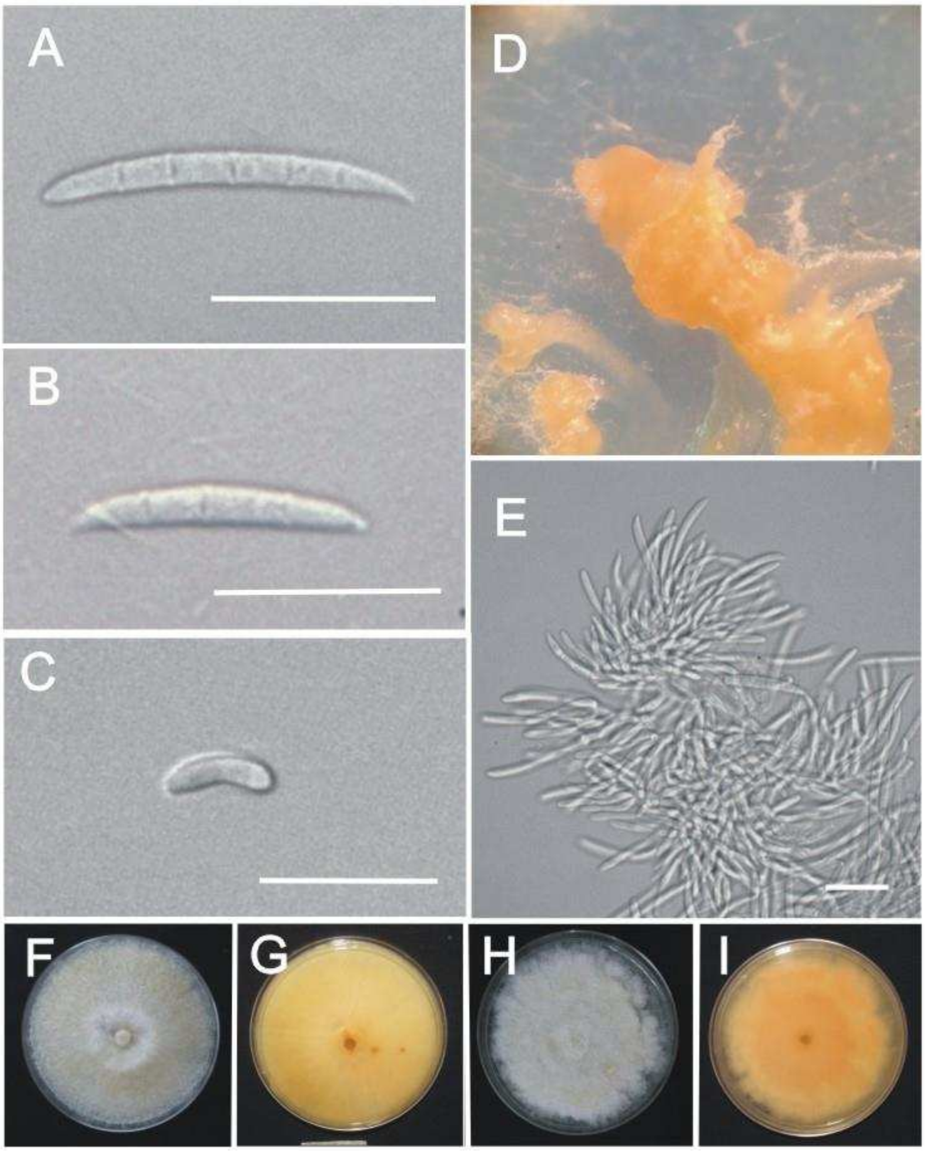

Figure 12. FIESC 29. **A–B:** nearly straight macroconidia; **C:** reniform microconidia; **D:** orange sporodochia; **E:** sporodochia; **F:** colonies on PDA. Surface white (F, H) and reverse cream (G) or orange (I). Scale bars: 20 µm.

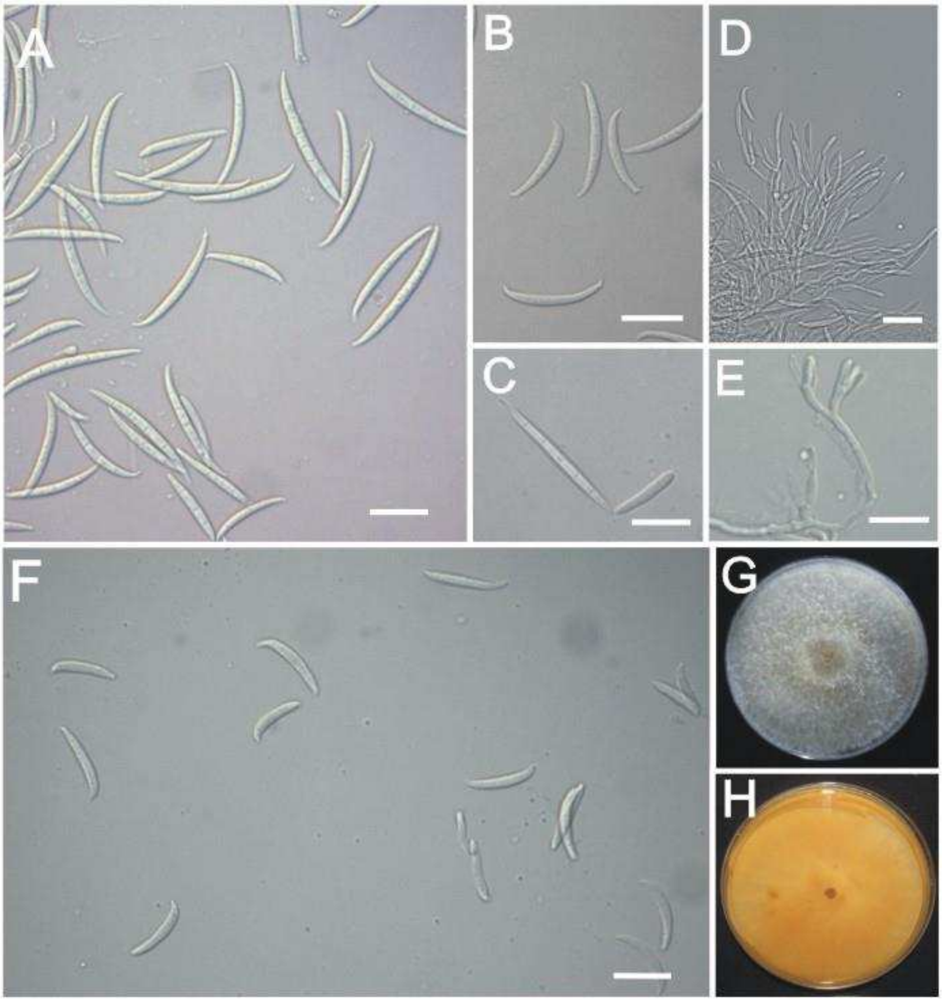

Figure 13. FIESC 32. **A, B, F:** falcate macroconidia; **C:** macroconidia and fusiform microconidia; **D:** sporodochia; **E:** polyphialides; **G–H:** colonies on PDA. Surface white (G); reverse orange (H). Scale bars: 20 µm.

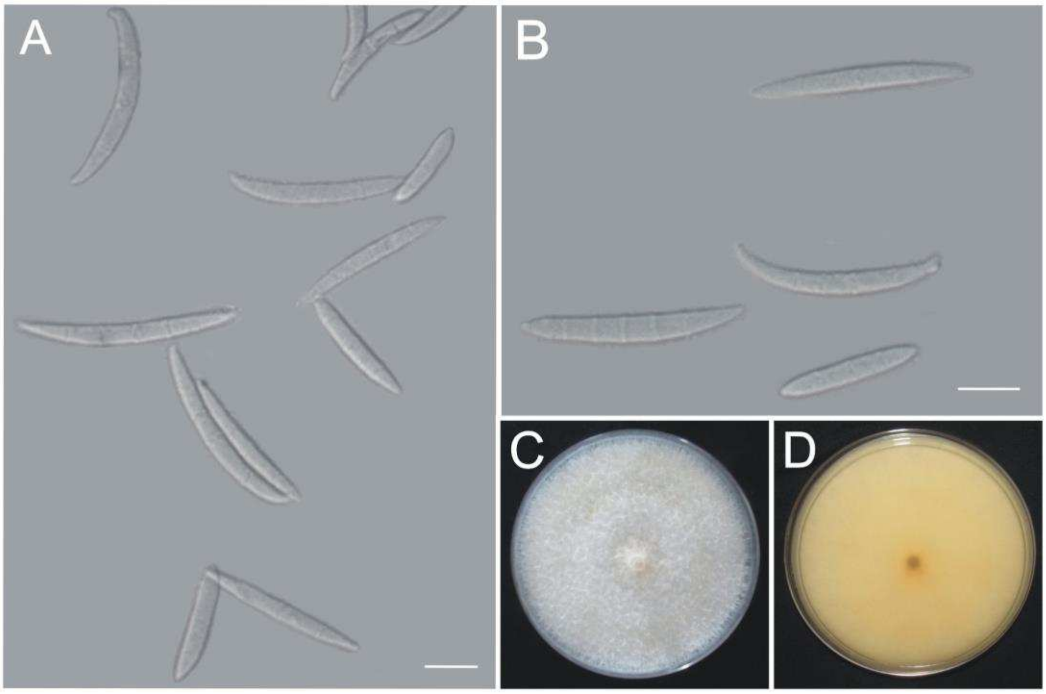

Figure 14. FIESC 33. **A–B:** falcate macroconidia and straight mesoconidia. **C–D:** colonies on PDA. Surface white (C); reverse cream (E). Scale bars: 10 µm.

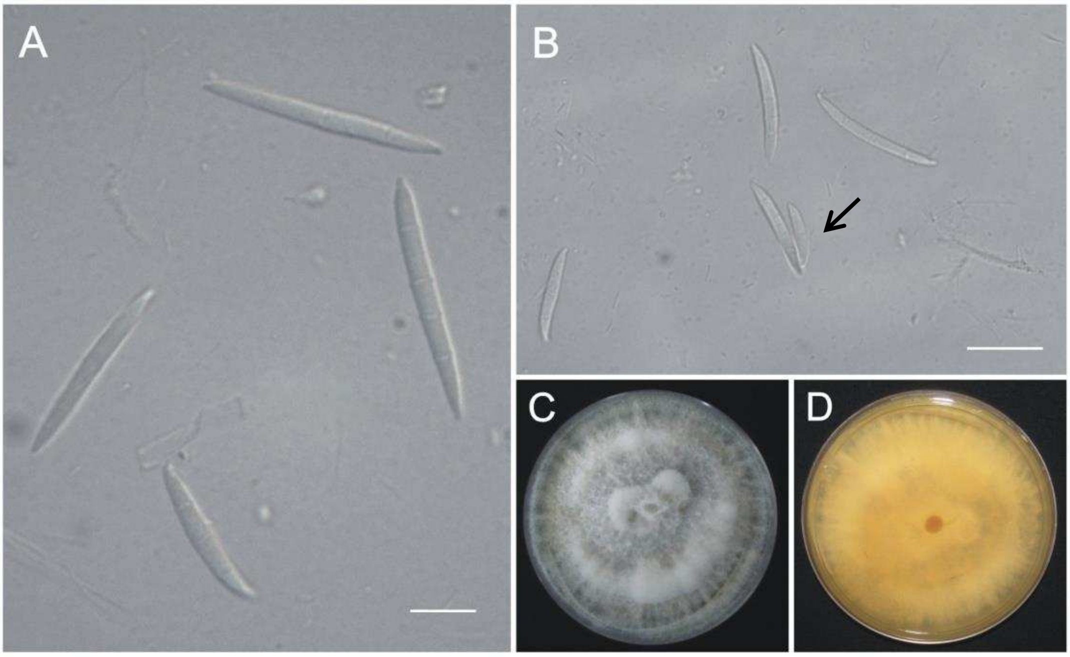

Figure 15. FIESC 34. **A:** nearly straight macroconidia; **B:** falcate mesoconidia indicated by arrow; **C–D:** colonies on PDA. Surface white (C); reverse cream (D). Scale bars: 20 µm.

Figure 16. FIESC 35. **A–B:** nearly straight macroconidia; **C:** straight mesoconidia; **D:** fusiform microconidia; **E–F:** colonies on PDA. Surface white (E); reverse cream (F). Scale bars: A - 20 µm, B–D - 10 µm.

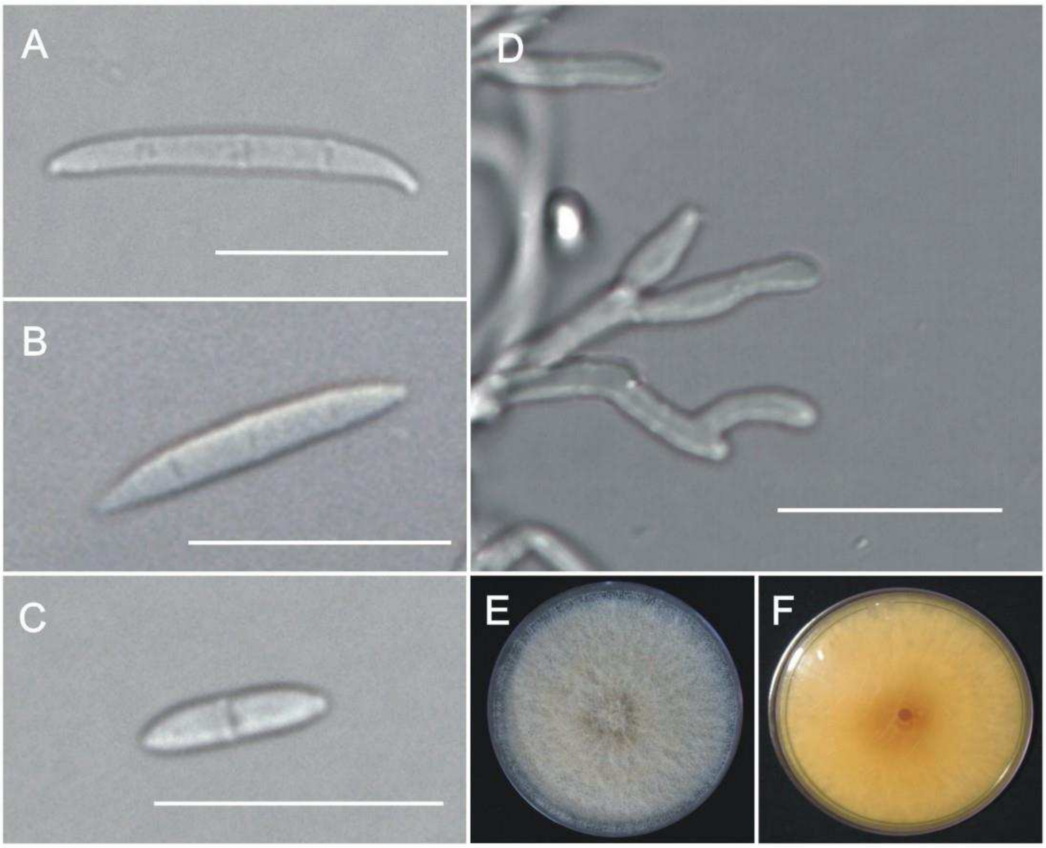

Figure 17. FIESC 36. **A:** nearly straight macroconidia; **B:** straight mesoconidia; **C:** fusiform microconidia; **D:** monophialides; **E–F:** colonies on PDA. Surface white (E); reverse light-brown (F). Scale bars: A, D - 10 µm, B, C - 20 µm.

Figure 18. FIESC 37. **A–B:** macroconidia with dorsiventral curvature; **C:** curved mesoconidia; **D:** sporodochia. Scale bars: 20 µm.

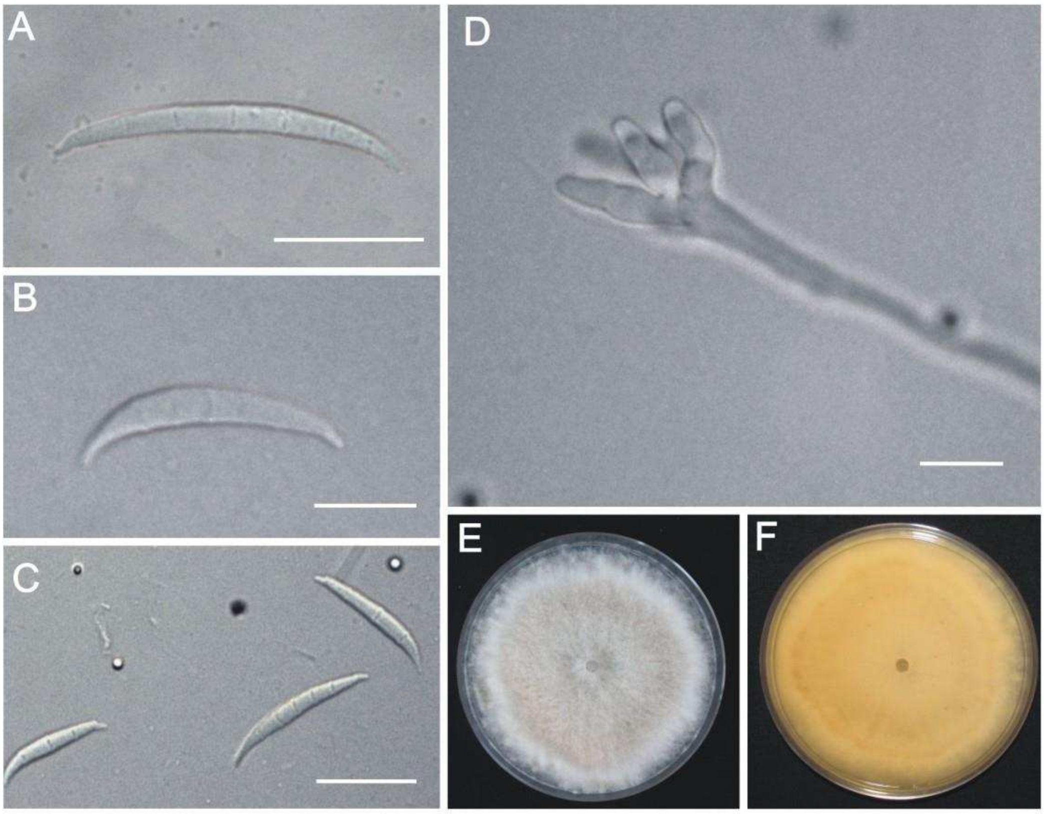

Figure 19. FIESC 38. **A:** falcate macroconidia; **B–C:** curved mesoconidia; **D:** monophialides; **E–F:** colonies on PDA. Surface white (E) and reverse cream (F). Scale bars: A, C - 20 µm, B - 10 µm.

DISCUSSION

This is the first global study that attempted to identify phylogenetic species, assess the trichothecene potential and variation in morphology for a relatively large collection of FIESC strains obtained from the major-producing region of rice in the Americas. A major finding is the proposition of seven putatively novel species within this complex, among 15 species found in Brazilian rice, after Villani et al. (2016). Thus far, 11 FIESC species have been previously identified in cereals: 1, 5, 10, 14, 24 and 28 (wheat, Spain) (Castellá & 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 31 (maize, oat and wheat from Netherlands, Canada and Italy, respectively) (Villani et al., 2016). In rice, besides the two species reported by O'Donnell et al. (2009) (FIESC 23 and 25), two species, FIESC 5 and 29, were reported by Villani et al. (2016), totalling four species before our study. Therefore, our data contribute the most comprehensive knowledge on the diversity of FIESC in rice globally.

Studies on the diversity of FIESC in cereals are incipient and the data are scarce. After the seminal paper by O'Donnell et al. (2009), who proposed 28 phylogenetic species for a collection of medically important FIESC strains, only two studies accurately identified FIESC species in a collection of isolates recovered from agricultural crops, mainly cereals such as maize, barley, oat, wheat and rice (Castellá & Cabañes, 2014; Villani et al., 2016). The diversity of species found in our study is larger than that reported in those studies where strains from multiple cereal crop were analysed.

Among the eight previously described species identified in our study, only two were reported in winter cereals (FIESC 24 and 29) and the other six are for the first time reported for a cereal crop. Villani et al. (2016) identified five cereal-infecting (maize, barley, oat, rice and wheat) strains from Europe and Canada, while Castellá and Cabañes (2014) identified seven species associated with wheat in Spain. The latter suggested that lineage composition in Spanish wheat was shaped by climate because species were structured by geography: all 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. Other researchers also hypothesized that FIESC distribution is affected by climate. For example, FIESC 29 strains reported by Villani et al. (2016) were isolated from warm climate regions, Italy and Mexico, similar to Castellá and Cabañes (2014), who reported the same lineage found in Spain. Contrastingly, the FIESC 29 isolate (CFA 053) found in our study originated in the subtropics of southern Brazil (Arroio Grande, RS), and the other strain (CFA 116) from tropics in northern Brazil (Formoso do Araguaia, TO). Differently from winter wheat, rice is a typical summer crop in Brazil and so weather conditions during the seasons for rice among regions in Brazil are more similar within the growing season. The two most represented species appeared to be structured by region: FIESC 26 was dominant in Goiás state (11/22) and FIESC 32 was dominant in RS state (12/19). The former lineage was more frequent in tropical climate of the Cerrado region, such MT and GO, and up north of Brazil, MA and RR states. FIESC 16 and 17 strains were also found in these regions. Some species were restricted to southernmost rice regions of Brazil, such as FIESC 4, 6, 37 and 38 found in RS state, but they were much less represented and thus it is difficult to draw hypothesis on the effect of climate on spatial distribution (Figure 3-a). Additional studies with larger sample size are needed to hypothesize if climate drives lineage distribution in Brazilian rice.

The FIESC species were identified in this study using a single locus, EF-*1α*, which was considered the most informative because it yielded results which were consistent with the multi-locus analysis of combined four-locus phylogeny (EF-*1α*, rDNA, RPB2 and CAM) of a FIESC collection of strains from clinical environments (O'Donnell et al., 2009). It would be instructive to further sequence other genes (e.g. RPB2) for a sample of rice strains representative of each species and all of the seven putative novel species (FIESC 32–38) in order to confirm our hypothesis or obtain greater resolution.

Among the seven proposed new species, six were constituted by a single strain (FIESC 33–38), which did not cluster with any known species and were distant apart from the others, further supporting our hypothesis. We included the EF-*1α* gene sequence of one isolate (ITEM 7155) from a previous study (Villani et al., 2016), whose phylogeny was not well resolved because the strain grouped close to FIESC 23 and 24. We found that the ITEM 7155 belongs to the newly discovered FIESC 32. FIESC 24 had no statistical support. Two strains (CFA 053 and CFA 116), plus a sequence of reference strain NRRL 52765, may represent three distinct phylogenetic species, since they grouped divergently from FIESC 29 (Figure 2).

FIESC isolates can 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, beauvericin and zearalenone as reported in the literature (Kosiak et al., 2005; Leslie & Summerell, 2006). Recently, 80% of F. equiseti strains from Spanish cereals produced DON and 25% produced NIV (Marín et al., 2012). Contrastingly, F. equiseti isolated from Norwegian cereals produced higher quantities of type A trichothecenes but no detectable levels of DON nor DON derivatives, yet significant amounts of NIV and FUS was produced (Kosiak et al., 2005).

In our study, 46.2% (31/67) isolates were Tri5^{+}, which was distributed randomly across the species and geographic regions (Figure 2). This suggests that FIESC may be an important contributor to the trichothecene levels detected in Brazilian rice (Almeida et al., 2012; Moreira, unpublished). In vitro toxin production studies are needed to check whether the molecular identification is accurate and which of the trichothecenes (DON or NIV) are produced, so that the molecular assays can be used as a surrogate variable to predict the chemotype (Desjardins, 2008).

Latin binomials have been applied to only three of the thirty-one FIESC species (O’Donnell et al., 2009), which may be due to the high levels of cryptic speciation and the morphological homoplasy, that lead to underestimates 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 the FIESC species. Some differences between them allowed distinguishing the morphological patterns, such as shape of apical and basal cell, as well as shaping of mesoconidia and presence or absenting of microconidia and its shape. We observed three morphologically distinct patterns on macroconidia shape. Most of species produced falcate macroconidia, hooked apical cell and foot-shaped cell. Among FIESC species with this morphological pattern, the shape of apical cell of macroconidia (hooked or curved), as well as its size and, when present, shape of meso- and microconidia are morphological traits that can be used to identify them.

FIESC 4 and 37 were the only that produced dorsiventral conidia (Figures 5 and 18), with similar growth rate (44 mm) and colony pigmentation. However, FIESC 4 macroconidia was much longer (Figure 4-b), thus this character can be used to differentiate them. These two species have similar morphology to F. equiseti described by Leslie and Summerell (2006). Another morphological pattern was observed in five species (FIESC 20, 29, 34, 35 and 36), which produced nearly straight or relatively slender macroconidia with a curved dorsal surface and a straighter ventral surface, the apical cell was slightly curved and basal cell foot shaped. These characters are very similar to those described for F. semitectum by Leslie and Summerell (2006).

FIESC 16 and 24 produced shorter (Figure 4-b), falcate macroconidia with hooked apical cell and foot cell. However, mesoconidia was absent and obovoid microconidia were observed in FIESC 16 (Figure 7), whereas FIESC 24 produced falcate mesoconidia and microconidia was absent (Figure 10, Table 3 Appendix).

Two traits were observed in radial growth, four species had similar growth rate (48–52 mm) while other four strains ranged between 44–49 mm. However, two exceptions were observed, FIESC 33 grew faster and FIESC 35 slower than the others. The growth rate and color of the colony are characteristics that did not differentiate the strains, contrary to the morphology of the conidia. The most commonly species in the present study, FIESC 26 and 32, had radial growth ≈52 mm, which may explain their predominance in the sampling areas (Figure 4-a).

Results of the present study provide baseline data on the morphology of each rice-FIESC lineage. Despite the predominance on macroconidia shape, FIESC species in the present study were quite distinct when all morphological characteristics were considered. All species seem to be distinct morphologically and phylogenetically. Thus further work may be conducted to confirm the findings and define the set of criteria (key) that can be used to differentiate FIESC species.

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APPENDIX

Table 1. Information for the full set of 147 strains of Fusarium incarnatum-equiseti species complex isolated from rice in Brazil, with indication of the species, whenever identified (shaded), or non-identified (NI) due to not being selected for identification.

Phylogenetic species	Study code	Origin[a]	Crop	Tri5[b]
32	CFA 001	Santo Antônio do Goiás, GO	2014/2015	+
NI	CFA 002	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 003	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 005	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 006	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 007	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 008	Santo Antônio do Goiás, GO	2014/2015	
32	CFA 009	Santo Antônio do Goiás, GO	2014/2015	+
NI	CFA 010	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 011	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 012	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 013	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 014	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 015	Santo Antônio do Goiás, GO	2014/2015	
33	CFA 016	Santo Antônio do Goiás, GO	2014/2015	-
26	CFA 017	Santo Antônio do Goiás, GO	2014/2015	-
NI	CFA 018	Santo Antônio do Goiás, GO	2014/2015	
NI	CFA 019	Santo Antônio do Goiás, GO	2014/2015	
32	CFA 020	Santo Antônio do Goiás, GO	2014/2015	-
NI	CFA 021	Santo Antônio do Goiás, GO	2014/2015	
26	CFA 022	Santo Antônio do Goiás, GO	2014/2015	+
32	CFA 023	Santo Antônio do Goiás, GO	2014/2015	+
26	CFA 024	Santo Antônio do Goiás, GO	2014/2015	+
34	CFA 025	Santo Antônio do Goiás, GO	2014/2015	+
26	CFA 026	Brazabrantes, GO	2014/2015	-
NI	CFA 027	Brazabrantes, GO	2014/2015	
NI	CFA 028	Brazabrantes, GO	2014/2015	
NI	CFA 029	Brazabrantes, GO	2014/2015	
NI	CFA 030	Brazabrantes, GO	2014/2015	
NI	CFA 031	Brazabrantes, GO	2014/2015	
32	CFA 032	Brazabrantes, GO	2014/2015	+
26	CFA 033	Brazabrantes, GO	2014/2015	+
26	CFA 034	Brazabrantes, GO	2014/2015	-
26	CFA 035	Brazabrantes, GO	2014/2015	+
26	CFA 036	Brazabrantes, GO	2014/2015	-
NI	CFA 037	Brazabrantes, GO	2014/2015	

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Table 1 – Continued

Phylogenetic species	Study code	Origin	Crop	Tri5
26	CFA 038	Brazabrantes, GO	2014/2015	-
32	CFA 039	Brazabrantes, GO	2014/2015	+
26	CFA 040	Brazabrantes, GO	2014/2015	-
NI	CFA 041	Brazabrantes, GO	2014/2015	
NI	CFA 042	Brazabrantes, GO	2014/2015	
16	CFA 043	Formoso do Araguaia, TO	2015/2016	+
32	CFA 044	Maçambará, RS	2015/2016	+
32	CFA 045	Maçambará, RS	2015/2016	-
32	CFA 046	Uruguaiana, RS	2015/2016	+
6	CFA 047	Itaqui, RS	2015/2016	-
NI	CFA 048	Mostardas, RS	2015/2016	
NI	CFA 049	Mostardas, RS	2015/2016	
NI	CFA 050	Mostardas, RS	2015/2016	
NI	CFA 051	Capivari do Sul, RS	2015/2016	
32	CFA 052	Santa Vitória do Palmar, RS	2015/2016	+
29	CFA 053	Arroio Grande, RS	2015/2016	-
32	CFA 054	Arroio Grande, RS	2015/2016	-
26	CFA 055	Sinop, MT	2014/2015	-
26	CFA 056	Sinop, MT	2014/2015	-
26	CFA 057	Sinop, MT	2014/2015	-
NI	CFA 058	União do Sul, MT	2014/2015	
NI	CFA 059	Itajaí, SC	2015/2016	
35	CFA 060	Itajaí, SC	2015/2016	-
NI	CFA 061	Itajaí, SC	2015/2016	
20	CFA 062	Itajaí, SC	2015/2016	-
20	CFA 063	Glorinha, RS	2015/2016	-
NI	CFA 064	Glorinha, RS	2015/2016	
NI	CFA 065	Glorinha, RS	2015/2016	
20	CFA 066	Glorinha, RS	2015/2016	-
26	CFA 067	Santa Maria, RS	2015/2016	-
32	CFA 068	Palma, RS	2015/2016	+
NI	CFA 069	São Sepé, RS	2015/2016	
26	CFA 070	São Sepé, RS	2015/2016	-
NI	CFA 071	Pelotas, RS	2015/2016	
NI	CFA 083	Brazabrantes, GO	2014/2015	
NI	CFA 084	Brazabrantes, GO	2014/2015	
NI	CFA 085	Brazabrantes, GO	2014/2015	
NI	CFA 086	Brazabrantes, GO	2014/2015	
NI	CFA 087	Brazabrantes, GO	2014/2015	
NI	CFA 088	Brazabrantes, GO	2014/2015	

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Table 1 – Continued

Phylogenetic species	Study code	Origin	Crop	Tri5
NI	CFA 089	Brazabrantes, GO	2014/2015	
NI	CFA 091	Brazabrantes, GO	2014/2015	
NI	CFA 092	Brazabrantes, GO	2014/2015	
NI	CFA 093	Brazabrantes, GO	2014/2015	
NI	CFA 094	Brazabrantes, GO	2014/2015	
NI	CFA 095	Brazabrantes, GO	2014/2015	
NI	CFA 096	Brazabrantes, GO	2014/2015	
NI	CFA 097	Brazabrantes, GO	2014/2015	
NI	CFA 098	Brazabrantes, GO	2014/2015	
NI	CFA 099	Brazabrantes, GO	2014/2015	
NI	CFA 100	Sinop, MT	2014/2015	
NI	CFA 101	Sinop, MT	2014/2015	
NI	CFA 102	Sinop, MT	2014/2015	
NI	CFA 103	Sinop, MT	2014/2015	
NI	CFA 104	Sinop, MT	2014/2015	
NI	CFA 105	Sinop, MT	2014/2015	
NI	CFA 106	Sinop, MT	2014/2015	
24	CFA 107	União do Sul, MT	2014/2015	-
26	CFA 108	Tangará da Serra, MT	2014/2015	+
20	CFA 109	Alta Floresta, MT	2014/2015	-
20	CFA 110	Alta Floresta, MT	2014/2015	+
16	CFA 111	Paraibano, MA	2015/2016	-
NI	CFA 112	Paraibano, MA	2015/2016	
26	CFA 113	Paraibano, MA	2015/2016	-
NI	CFA 114	Paraibano, MA	2015/2016	
NI	CFA 115	Formoso do Araguaia, TO	2014/2015	
29	CFA 116	Formoso do Araguaia, TO	2014/2015	+
NI	CFA 117	Formoso do Araguaia, TO	2014/2015	
NI	CFA 118	Formoso do Araguaia, TO	2014/2015	
17	CFA 119	Lagoa da Confusão , TO	2014/2015	-
20	CFA 120	Formoso do Araguaia , TO	2014/2015	-
NI	CFA 121	Itajaí, SC	2015/2016	
NI	CFA 122	Itajaí, SC	2015/2016	
NI	CFA 123	Nova Veneza, SC	2015/2016	
32	CFA 124	Nova Veneza, SC	2015/2016	+
NI	CFA 125	Nova Veneza, SC	2015/2016	
NI	CFA 126	Nova Veneza, SC	2015/2016	
36	CFA 127	Nova Veneza, SC	2015/2016	+
NI	CFA 128	Nova Veneza, SC	2015/2016	
NI	CFA 129	São Borja, RS	2014/2015	

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Table 1 – Continued

Phylogenetic species	Study code	Origin	Crop	Tri5
NI	CFA 130	Maçambará, RS	2014/2015	
32	CFA 131	São Borja, RS	2014/2015	+
NI	CFA 132	Maçambará, RS	2014/2015	
32	CFA 133	Camaquã, RS	2014/2015	+
32	CFA 134	Mostardas, RS	2014/2015	+
32	CFA 135	Mostardas, RS	2014/2015	+
NI	CFA 136	Mostardas, RS	2014/2015	
20	CFA 137	Arroio Grande, RS	2014/2015	-
NI	CFA 138	Santa Vitória do Palmar, RS	2014/2015	
NI	CFA 139	Glorinha, RS	2014/2015	
NI	CFA 140	Santa Maria, RS	2014/2015	
NI	CFA 141	Santa Maria, RS	2014/2015	
NI	CFA 142	Palma, RS	2014/2015	
NI	CFA 143	Palma, RS	2014/2015	
20	CFA 144	Pelotas, RS	2014/2015	+
NI	CFA 145	Roseira, SP	2015/2016	
26	CFA 146	Boa Vista, RR	2015/2016	+
24	09Ar013	Cachoeirinha, RS	2008/2009	+
20	09Ar023	Palmeira das Missões, RS	2008/2009	-
38	12Ar016	Capivari do Sul, RS	2011/2012	+
F. lacertarum (4)	12Ar093	Pelotas, RS	2011/2012	+
F. lacertarum (4)	12Ar097	Pelotas, RS	2011/2012	+
37	12Ar099	Pelotas, RS	2011/2012	-
26	12Ar100	Brazabrantes, GO	2011/2012	+
32	12Ar101	Pelotas, RS	2011/2012	+
F. lacertarum (4)	12Ar104	Pelotas, RS	2011/2012	-
20	12Ar142	Nova Veneza, SC	2011/2012	-
26	12Ar143	Nova Veneza, SC	2011/2012	-
26	12Ar145	Criciúma, SC	2011/2012	
26	12Ar155	Nova Veneza, SC	2011/2012	

[a] Brazilian States (GO: Goiás, TO: Tocantins, RS: Rio Grande do Sul, MT: Mato Grosso, SC: Santa Catarina, MA: Maranhão, SP: São Paulo, RR: Roraima).

[b] Tri5 genotyping, (+) positive, (-) negative.

Table 2. Colony morphology of phylogenetic species within Fusarium incarnatum-equiseti species complex used in this study.

Species	Colony		Chlamydospores
	Verse / Reverse Color	Radial growth (mm)	
4	White / cream – light-brown	44	Chain
6	White – light-brown / light-brown	45	Chain
16	White / cream – light-brown	49	Solitary
17	White / brownish orange	40	Absent
20	White or cream / cream or brownish orange	49	Absent
24	White or light-brown / orange or brownish orange	45	Absent
26	White – light-brown / brownish orange – brown	52	Solitary
29	White / cream or orange	40	Absent
32	White / orange	50	Absent
33	White / cream	57	Absent
34	White /cream	45	Absent
35	White /orange	35	Absent
36	White / light-brown	48	Absent
37	White / cream to light-brown	43	Absent
38	White / light-brown	48	Absent

Table 3. Morphological characteristics of phylogenetic species within Fusarium incarnatum-equiseti species complex used in this study.

Species	Macroconidia					Mesoconidia	Microconidia
	Shape	Septa	Apical cell	Basal cell	Size (µm)	Shape	Shape
F. lacertarum (4)	Dorsiventral curvature	4–5	Tapering and elongate	Prominent foot shape	34.2–65 × 1.3–4.5	Falcate	Absent
6	Falcate	4–5	Hooked	Foot shape	30.8–45 × 2.7–3.5	Curved	Reniform
16	Falcate	4–5	Curved	Foot shape	23.8–35.7 × 2.3–3.9	Absent	Obovoid
17	Falcate	3–5	Hooked	Foot shape	25.4–43.7 × 2.4–5	Absent	Absent
20	Nearly straight	4–6	Slightly curved	Barely notched	22–43.4 × 2.3–4.9	Falcate	Fusiform
24	Falcate	4–5	Hooked	Foot shape	20.4–46.4 × 1.9–5	Curved	Absent
26	Falcate	4–5	Hooked	Distinctly notched	21.5–47.7 × 2.7–4.6	Falcate	Fusiform
29	Nearly straight	4–5	Slightly curved	Barely notched	22.4–46.8 × 1.5–5.9	Falcate	Reniform
32	Falcate	4–7	Curved	Foot shape	20.1–48.9 × 2.2–5.7	Curved	Fusiform
33	Falcate	3–4	Hooked	Foot shape	24.9–47.4 × 2.8–5.7	Straight	Absent
34	Nearly straight	3–5	Blunt	Distinctly notched	21.4–50.6 × 2.5–4.5	Falcate	Absent
35	Nearly straight	3–4	Blunt	Distinctly notched	24.9–43.4 × 2.3–4.3	Straight	Fusiform
36	Nearly straight	3–4	Slightly curved	Barely notched	29.1–39 × 2.8–4.2	Straight	Fusiform
37	Dorsiventral curvature	4–5	Tapering and elongate	Elongated foot shape	24.9–48 × 2.9–4.1	Curved	Absent
38	Falcate	4–6	Curved	Foot shape	18.1–36.6 × 2.6–3.7	Curved	Absent

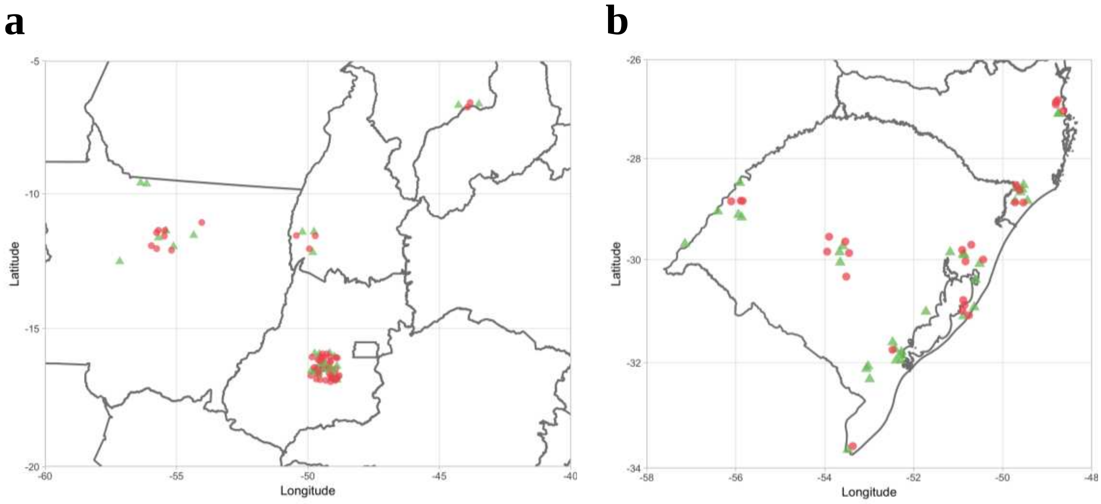

Figure 1. Approximate geographical location of FIESC isolates from northern **(a)** and southern **(b)** rice regions of Brazil and which were not identified (green triangle) or identified (red dot) to species level based on sequences of EF-1α gene.