ALINE VAZ LARANJEIRA DE SOUZA

Fusarium andiyazi SPECIES GROUP:

AN APPLICATION OF THE BIOLOGICAL SPECIES CONCEPT

LAVRAS - MG

2018

ALINE VAZ LARANJEIRA DE SOUZA

Fusarium andiyazi SPECIES GROUP:

AN APPLICATION OF THE BIOLOGICAL SPECIES CONCEPT

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de Mestre.

Orientador

Dr. Ludwig H. Pfenning

LAVRAS - MG

2018

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo próprio autor.

Souza, Aline Vaz Laranjeira de.

Fusarium andiyazi species group: an application of the biological species concept / Aline Vaz Laranjeira de Souza. - 2018.

43 p.: il.

Orientador: Ludwig H. Pfenning.

.

Dissertação (mestrado acadêmico) - Universidade Federal de Lavras, 2018.

Bibliografia.

1. *Fusarium fujikuroi* species complex. 2.*Mating population*. 3.Tropical grasses. I. Pfenning, Ludwig H. II. Título.

ALINE VAZ LARANJEIRA DE SOUZA

Fusarium andiyazi SPECIES GROUP:

AN APPLICATION OF THE BIOLOGICAL SPECIES CONCEPT

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para a obtenção do título de Mestre.

Aprovada em 06 de julho de 2018.

Dr. Eustáquio Souza Dias UFLA

Dr. Lucas Magalhães Abreu UFV

Dr. Ludwig H. Pfenning

Orientador

LAVRAS – MG

2018

Aos meus pais, Ari (in memoriam) e Larbela,

Dedico.

AGRADECIMENTOS

À minha mãe, Larbela, por todo o incentivo durante a minha carreira acadêmica.

À Universidade Federal de Lavras e aos professores do Programa de Pós-Graduação em Microbiologia Agrícola pelos ensinamentos.

À CAPES, pelo financiamento e concessão da bolsa de estudos.

Ao professor Ludwig, pela orientação, disposição e ensinamentos.

Aos colegas do Laboratório de Ecologia e Sistemática de Fungos pela boa convivência em especial à Marileide, sem a qual este trabalho não seria possível.

Aos amigos da UFLA, Tatiana, Lídia, Jéssica, Bárbara, Renan e Thalissa pelos bons momentos.

Ao meu companheiro Anderson, pelo apoio incondicional durante todo o mestrado.

Às amigas de longa data, Bruna e Alessandra, pela preocupação e carinho.

A todos que, de alguma forma, contribuíram para a realização deste trabalho. Muito obrigada.

RESUMO

Fusarium andiyazi é um membro do complexo de espécies *Fusarium fujikuroi* - FFSC, conhecido por causar podridão no sorgo na África e nos Estados Unidos. Há registros de sua associação com outras gramíneas cultivadas e a vegetação natural, como endófito. Em associação com diferentes gramíneas do Brasil, Estados Unidos e países africanos, obteve-se uma coleção de 87 isolados do morfotipo F. andiyazi. A análise da região do gênica TEF mostrou que estes isolados representam *F. andiyazi* e duas outras espécies filogenéticas ainda não descritas, morfologicamente indistinguíveis. Até o momento, a fase sexual de *F. andiyazi* é desconhecida, uma vez que a formação de peritécios férteis não foi observada em cruzamentos laboratoriais. Os objetivos deste estudo foram avaliar a existência da reprodução sexuada de *F. andiyazi* e de suas linhagens irmãs através de cruzamentos laboratoriais, relatar as espécies como possíveis novas *mating populations* do FFSC, avaliar também os marcadores morfológicos da fase sexual. e verificar a presença de uma barreira reprodutiva com outras *mating population* do FFSC. Os isolados depositadas na Coleção Micológica de Lavras - CML, obtidos de arroz, cana-de-açúcar, sorgo, milho, braquiária e milheto do Brasil, Estados Unidos e países africanos, foram incluídas nos experimentos. Entre 87 cepas, 19 representam F. andiyazi, 62 Fusarium sp.1 e seis Fusarium sp.2. Na amplificação de PCR dos *mating types*, para *F. andiyazi*, 12 isolados foram identificadas como *MAT-1* e 7 como *MAT-2*, *Fusarium* sp.1 (21 *MAT-1*, 40 *MAT-2*), para *Fusarium* sp.2 quatro foram identificados como *MAT-2* e dois como *MAT-1*. Três cruzamentos férteis foram obtidos entre as linhagens de *F. andiyazi* e 54 entre as linhagens de *Fusarium* sp.1. Portanto, *F. andiyazi* e *Fusarium* sp.1 representam novas MPs dentro do FFSC. Nos cruzamentos. de *F. andiyazi* e *Fusarium* sp.1 com testadores das outras MPs do FFSC, a produção de peritécio fértil não foi observada, mostrando que existe uma barreira reprodutiva com as outras espécies biológicas do complexo. Muito poucos cruzamentos férteis foram observados entre *F. andiyazi* e *Fusarium* sp.1, uma indicação de que até o momento, nenhuma barreira reprodutiva completa entre as duas espécies foi estabelecida. *Fusarium* sp.2 não produziu cruzamentos férteis entre suas linhagens, com *F. andiyazi*, *Fusarium* sp.1 ou com qualquer outro MP do FFSC. Na caracterização morfológica da fase sexual, as estruturas produzidas por *F. andiyazi* e *Fusarium* sp.1 foram idênticas. O conceito de espécie biológica aplicado ao estudo dessas três espécies dá uma ideia de quão importante o uso desse conceito pode ser na determinação de uma espécie, quando os marcadores morfológicos são insuficientes. A aplicação do conceito de espécie biológica, além de consolidar o conceito de espécie, pode auxiliar na identificação de indivíduos morfologicamente semelhantes.

Palavras-chave: Complexo de espécies *Fusarium fujikuroi*. Gramíneas tropicais. Mating population.

ABSTRACT

Fusarium andiyazi is a member of the *Fusarium fujikuroi* species complex - FFSC, known to cause stem rot in sorghum in Africa and the United States. There are records of its association with other cultivated grasses and the natural vegetation as an endophyte. In association with different grasses from Brazil, the United States and African countries, a collection of 87 isolates of the morphotype *F. andiyazi* were obtained. The analysis of the barcode region TEF showed that these isolates represent *F. andiyazi* and two other, still undescribed phylogenetic species, morphologically indistinguishable. To date, the sexual phase of *F. andiyazi* is unknown, since the formation of fertile perithecia was not observed in laboratory crossings. The aims of this study were to evaluate the existence of the sexual reproduction of *F. andiyazi* and their sibling lineages through laboratory crossings, to report the species as possible new mating populations of the FFSC, to evaluate the morphological markers of the sexual phase as well as to verify the presence of a reproductive barrier with other FFSC mating populations. Strains deposited in the Coleção Micológica de Lavras - CML obtained from rice, sugarcane, sorghum, maize, *Brachiaria* and millet from Brazil, the United States and African countries, were included in the experiments. Amongst 87 strains, 19 represent *F. andiyazi*, 62 *Fusarium* sp.1 and six *Fusarium* sp.2. In the mating type PCR amplification for *F. andiyazi*, 12 strains were identified as MAT-1 and 7 as MAT-2, *Fusarium* sp.1 (21 MAT-1, 40 MAT-2), for *Fusarium* sp.2 two were identified as MAT-1 and four as MAT-2. Three fertile crossings were obtained between strains of *F. andiyazi* and 54 between *Fusarium* sp.1 strains. Therefore, *F. andiyazi* and *Fusarium* sp.1 represent new MPs within the FFSC. In crossings. of *F. andiyazi* and *Fusarium* sp.1 with the other MP of the FFSC, the production of fertile perithecia was not observed, showing that there is a reproductive barrier with the other biological species of the complex. A very few fertile crossings were observed between *F. andiyazi* and *Fusarium* sp.1, an indication that so far, no complete reproductive barrier between the two species has been established. *Fusarium* sp.2 did not produce any fertile crosses between its strains, with *F. andiyazi*, *Fusarium* sp.1 or with any other MP of the FFSC. In the morphological characterization of the sexual phase, the structures produced by *F. andiyazi* and *Fusarium* sp.1 were identical. The biological species concept applied to the study of these three species gives an insight into how important the use of this concept can be in determining a species, when the morphological markers are insufficient. The application of the biological species concept, besides consolidating the concept of species, can help in the identification of morphologically similar individuals.

Keywords: *Fusarium fujikuroi species complex*. Mating population. Tropical grasses.

SUMÁRIO

PRIMEIRA PARTE

1. CARACTERIZAÇÃO DO PROBLEMA

O complexo de espécies *Fusarium fujikuroi* (FFSC) é bastante estudado pela sua associação com patossistemas de importantes plantas cultivadas. Compreende 61 espécies filogenéticas sendo 13 também espécies biológicas, muitas das quais compartilhando características morfológicas entre si. Esta sobreposição dificulta uma identificação precisa da espécie baseando-se unicamente na morfologia (Nirenberg & O'Donnell 1998; O'Donnell et al 1998; Leslie & Summerell 2006).

A espécie *F. andiyazi* pertence ao FFSC e é um importante patógeno da cultura do sorgo (*Sorghum bicolor*) e também é associado à outras culturas, tais como arroz e milho. Foi primeiramente descrita a partir de a partir de caracteres morfológicos e padrões de banda AFLP e apenas em 2016 foram geradas sequencias das regiões genicas TEF, TUB e RPB2 para o isolados tipo da espécie, possibilitando estudos filogenéticos e confirmando *F. andiyazi* também como uma espécie filogenética (Prá et al 2010; Wulff 2010; Zhang et al 2014; Kelly et al 2017; Venturini et al 2017; Marasas et al 2001; Al-Hatmi et al 2015). Análises filogenéticas realizadas para as regiões TEF e RPB2 com amostras do morfotipo *F. andiyazi* coletadas do Brasil, EUA e África demonstraram que os isolados se agrupam em três diferentes clados, um formado com o tipo de *F. andiyazi* e duas outras linhagens irmãs (Figura 2 - Apêndice). Além da filogenia, dados de AFLP sustentam a existência destes três grupos dentro do clado africano do FFSC, cujos isolados apresentam morfologia de *F. andiyazi* e diferentes comportamentos ecológicos, fisiológicos e reprodutivos (Bushula et al 2015).

Fusarium andiyazi compartilha caracteres morfológicos com outras espécies filogenéticas do complexo *Fujikuroi*, como *F. verticillioide*s e *F. thapsinum*. Essa sobreposição de características inviabiliza o uso do conceito de espécie morfológico apenas para classificar esta espécie. A aplicação do Conceito de Espécie Biológica (CEB) possibilita a identificação de espécies morfologicamente semelhantes, por meio da técnica de cruzamento (Leslie & Summerell 2006).

Dentro do FFSC foram identificadas treze espécies biológicas isoladas geneticamente, denominadas *mating populations* A-M, todas heterotálicas com um mecanismo de um *locus MAT* e dois idiomorfos, *MAT-1* ou *MAT-2* (Leslie 1995; Leslie & Summerell 2006). No entanto, até o presente momento a fase sexuada *F. andiyazi* não foi descrita, uma vez que que os testes de cruzamento sexual realizados para esta espécie em laboratório não foram bem-sucedidos, considerando que não se observou a formação de peritécios férteis (Marasas et al 2001).

O conceito de espécie biológica possibilita a consolidação de *F. andiyazi* como espécie, além de elucidar a relação com outras linhagens filogeneticamente próximas. Desse modo, *F. andiyazi* é reconhecidamente uma espécie filogenética, que compartilha marcadores morfológicos com outras espécies do FFSC, no entanto ainda não foi identificada como espécie biológica, sendo esta identificação importante para melhor compreensão do seu comportamento reprodutivo, evolução da espécie e relacionamento com outras espécies dentro do FFSC.

2. ESTADO DA ARTE

2.1. Complexo de espécies *Fusarium fujikuroi* (FFSC)

O complexo *Fusarium fujikuroi* (FFSC) é um táxon monofilético que compreende espécies com similaridade morfológica que dificulta a sua diferenciação. Podem ser saprófitas, endófitas, produtoras de importantes micotoxinas, além de patógenos de culturas economicamente importantes, humanos e outros animais. Trabalhos com sistemática molecular revelaram que o complexo possui cerca de 61 espécies filogenéticas distintas e 13 espécies biológicas ou *mating populations* (Tabela 1) (Proctor et al 2004; Leslie et al 2007; Kvas et al 2009; Geiser et al. 2013)

A hipótese biogeográfica que explica a distribuição desses fungos, considera a origem geográfica dos hospedeiros, com base na filogenia obtida a partir do sequenciamento de determinadas regiões gênicas. As espécies dentro do FFSC agruparam-se em três clados bem suportados, os quais receberam as denominações de Africano, Americano e Asiático (O'Donnell et al 1998). Dentro destes clados encontram-se inúmeras espécies associadas a importantes patossistemas e que são produtoras de importantes metabólitos secundários, tais como as micotoxinas fumonisina, fusarina, ácido fusárico e beauvericina, além dos fitormônios giberelina, auxina e citoquininas (Escrivá et al 2014; Niehaus et al 2016).

As espécies do FFSC são responsáveis por grandes perdas em cereais e frutíferas no Brasil e no mundo. *Fusarium fujikuroi*, por exemplo, responsável pela Bakanae disease em arroz (*Oryza sativa*), causa sintomas como podridão do pé e das mudas, esterilidade e descoloração do grão (Leslie et al 2004; Infantino et al 2017). A gomose do abacaxizeiro, importante doença do Brasil, tem como agente causal *F. guttiforme* (Melo et al 2016). A podridão da raiz, espiga e colmo do milho é frequentemente associada a *F. verticillioides* e *F. subglutinans* (Nirenberg & O'Donnell 1998). A podridão da cana-de-açúcar está associada à ocorrência de *F. sacchari*, *F. subglutinans*, *F. verticillioides* e *F. proliferatum*. Em mangueira (*Mangifera indica*), as espécies *F. mangiferae* e *F. sterilihyphosum* são associadas à malformação floral e vegetativa. No Brasil, por meio de estudos filogenéticos, compatibilidade sexual, testes de patogenicidade e descrição da

morfologia determinou-se que *F. tupiense* é também responsável pela malformação da mangueira (Lima et al 2012).

Espécies distintas podem causar sintomas semelhantes em determinado hospedeiro, fazendo-se necessário estudos genéticos mais aprofundados que vão além da análise morfológica, considerando a sobreposição de caracteres morfológicos recorrente em espécies do gênero *Fusarium*.

Tabela 1 – *Mating populations* descritas dentro do complexo de espécies *Fusarium fujikuroi*.

Espécies[a]	MP[b]	Referência[c]
F. verticillioides	A	Kuhlman 1982
F. sacchari	B	Leslie et al. 2005b
F. fujikuroi	C	Kuhlman 1982
F. proliferatum	D	Kuhlman 1982
F. subglutinans	E	Booth 1971
F. thapsinum	F	Klittich et al. 1997
F. nygamai	G	Klaasen e Nelson 1996
F. circinatum	H	Nirenberg e O'Donnell 1998
F. konzum	I	Zeller et al. 2003
F. xylarioides	J	Booth 1971
F. temperatum	K	Scauflaire et al. 2011
F. tupiense	L	Lima et al. 2012
F. musae	M	Van Hove et al. 2011

[a]Espécies biológicas do complexo de espécies *Fusarium fujikuroi*
[b]Designação alfabética para espécies biológicas dentro do FFSC
[c]Referências das descrições das espécies biológicas

2.2. Caracterização e identificação de espécies de *Fusarium*

O gênero *Fusarium* é um grupo monofilético onde atualmente, são reconhecidas mais de 300 espécies recorrentes em todo o mundo em uma ampla diversidade de habitats (Summerell et al 2010; Geiser et al 2013; Moussa et al 2017). A caracterização morfológica é o primeiro passo para que se possa iniciar a identificação de espécies e baseia-se nos caracteres fenotípicos utilizados como marcadores morfológicos definitivos daquele grupo. O FFSC apresenta espécies com sobreposição dessas características, inviabilizando a detecção de um marcador morfológico específico que possa diferenciar as espécies. Muitas vezes a separação sendo possível unicamente através da análise filogenética (Taylor et al 2000; Steenkamp et al 2000; Leslie e Summerell 2006).

Além da caracterização morfológica, o grande número de caracteres adquiridos através das técnicas moleculares, tais como PCR e sequenciamento de DNA, permitiu o surgimento de um conceito complementar ao de espécie morfológica, o conceito de espécie filogenética (CEF). As seqüências dos genes *28S rDNA*, *mtSSU rDNA*, *calmodulina*, *histona-H3*, *fator de elongação-1α* e *β-tubulina* são reconhecidas como eficientes na reconstrução da filogenia do FFSC (O'Donnell 1998; Taylor et al 2000; O'Donnell et al, 2013; Aoki et al 2014).

Adicionalmente, o conceito de espécie biológica é indiscutivelmente apropriado para fungos e a identificação de espécies através deste conceito deve ser priorizada sempre que possível (Leslie 1995; Leslie et al 2001; Summerell et al 2003).

2.3. Conceito de espécie biológica ou *mating populations*

As *mating population* (MP) são espécies biológicas que produzem cruzamentos férteis entre indivíduos de uma mesma MP e inférteis entre MP distintas (Leslie et al 2001). Dentro do complexo de espécies *Fusarium fujikuroi*, por exemplo, existem 13 *mating population* identificadas até o momento, todas heterotálicas.

Os principais genes que regulam os cruzamentos férteis entre fungos filamentosos são os denominados *mating types*, sistema genético que consiste na presença de um único locus *MAT* com dois idiomorfos *MAT-1* e *MAT-2*. Os genes dos locus *MAT* possuem

regiões conservadas entre espécies distantes, que produzem proteínas denominadas HMG-box (high mobility group) e α-box. A diferença sexual entre os fungos consiste na presença ou ausência de determinadas sequências genéticas entre um indivíduo e outro. Em espécies de ascomicetos heterotálicas, só é possível o cruzamento entre dois isolados geneticamente diferentes, ou seja, *mating types* opostos, *MAT-1* e *MAT-2* (Leslie & Summerell 2006).

Em ascomicetos, esse sistema de um locus com dois alelos funcionais serve primariamente na prevenção de autofertilização, favorecendo uma maior recombinação genética na progênie (Leslie & Summerell 2006). Para a identificação dos genes *MAT* foram desenvolvidos *primers* de PCR (reação em cadeira da polimerase), permitindo que para os testes de compatibilidade sexual sejam realizados apenas os cruzamentos entre indivíduos de *mating types* opostos, favorecendo a necessidade de realização de um número menor de cruzamentos (Steenkamp et al 2000). A aplicação do conceito de espécie biológica dentro do FFSC nem sempre possível, uma vez que os isolados frequentemente não possuem a habilidade de servir como fêmea testadora nos cruzamentos, pela baixa produção de peritécios e exsudação de ascósporos (Leslie & Summerell 2006).

No entanto, é importante poder identificar a espécie biológica para melhor compreender os mecanismos de recombinação genética que resultam nas diferentes características encontradas dentro de uma mesma população. Indivíduos de uma mesma espécie que apresentam diferenças quanto à produção de substâncias ou fator de virulência, por exemplo, são resultado da distribuição de alelos durante a meiose, etapa do ciclo sexual, que carregam essas características, resultando na diversidade genética da espécie (Esser 1971; Orr-Weaver & Szostak 1985; Milgroom 1996; McDonald & Linde 2002; Slinski et al 2016).

2.4. *Fusarium andiyazi*

Fusarium andiyazi foi inicialmente relatado a partir de isolados obtidos em plantas de sorgo (*Sorghum bicolor*) e descrita por Marasas et al. (2001) a partir de características morfológicas e moleculares (perfis de AFLP). *F. andiyazi* também foi

identificado em associação a outras culturas, como milho, cana de açúcar e arroz (Leslie & Summerell 2006; Petrovic et al 2009; Kelly et al 2017). Apenas em 2016 foram geradas sequências da região gênica TEF, possibilitando o reconhecimento de *F. andiyazi* também como espécie filogenética (Al-Hatmi et al, 2016).

As características morfológicas descritas para a identificação do *F. andiyazi* foram descritas a partir de estruturas produzidas em meio CLA e BDA. EM BDA, o micélio se apresenta poroso ou formando flocos, inicialmente branco e podendo tornar-se violeta. Os macroconídios consistem em esporodóquios alaranjados em CLA; hialinos com paredes finas, formato reto a levemente curvado; célula apical é levemente curvada; célula basal pedicilada; septos variando de 3 a 6; geralmente encontrados em esporodóquios em monofiliádes ou conidióforos ramificados. Os microconídios são geralmente clavados a ovóides com uma base achatada, formados em falsas cabeças a partir de longas fiálides e em cadeias longas. A espécie não apresenta clamidósporos. Possuem pseudoclamidósporos de parede lisa e fina são geralmente solitários, mas ocasionalmente podem ser encontrados em cadeias curtas (Marasas et al 2001; Leslie & Summerell 2006).

Fusarium andiyazi compartilha marcadores morfológicos com outras espécies filogenéticas do complexo *Fujikuroi*, como *F. verticillioides* e *F. thapsinum* por exemplo. Essa sobreposição de características inviabiliza somente a utilização do conceito de espécie morfológica para classificar esta espécie (Fandohan et al 2005; Leslie & Summerell 2006; Zhang et al 2014).

Até o presente momento a fase sexuada *F. andiyazi* é desconhecida. Os testes de cruzamento sexual realizados para esta espécie em laboratório não foram bem-sucedidos, uma vez que não se observou a formação de peritécios férteis (Leslie et al 2005).

REFERÊNCIAS

Al-Hatmi AMS, Mirabolfathy M, Hagen F, Normand A, Stielow JB, Karami-Osbo R, Diepeningen AD, Meis JF, De Hoog GS. 2016. DNA barcoding, MALDI-TOF and AFLP data support *Fusarium ficicrescens* as a distinct species within the *F. fujikuroi* species complex. Fungal Biology 120: 265-278

Aoki T, O'Donnell K, Geiser DM. 2014. Systematics of key phytopathogenic *Fusarium* species: current status and future challenges. Journal of Genetic Plant Pathology 80: 189-201.

Bushula V, Leslie JF, Little CR 2015. Genetic diversity in *Fusarium andiyazi* populations from sorghum. Phytopathology 105 (Suppl. 4):S4.21.

Escrivá L, Font G, Manyes L. 2015. In vivo toxicity studies of *Fusarium* mycotoxins in the last decade: A review. Food and Chemical Toxicology 78: 185-206.

Esser K. 1971. Breeding systems in Fungi and their significance for genetic recombination. Molecular General Genetics 110: 86-100.

Fandohan P, Gnonlofin B, Hell K, Marasas WFO, Wingfield MJ. 2014. Natural occurrence of *Fusarium* and subsequent fumonisin contamination in preharvest and stored maize in Benin, West Africa. International Journal of Food Microbiology 99: 173-183.

Geiser et al. 2013. One fungus, one name: defining the genus *Fusarium* in a scientifically robust way that preserves longstanding use. Phytopathology 103: 400-408.

Heng MH, Salleh B, Zakaria L. 2011. Molecular identification of *Fusarium* species in *Giberella fujikuroi* Species Complex from rice, sugarcane and maize from Peninsular Malaysia. International Journal of Molecular Sciences 12: 6722-6732.

Keen NT. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annual Reviews in Genetics 24: 447-463.

Kelly LA, Tan YP, Ryley MJ, Aitken E. 2017. *Fusarium* species associated with stalk rot and head blight of grain sorghum in Queensland and New South Wales, Australia. Plant Pathology.

Kvas M, Marasas WFO, Wingfield BD, Steenkamp ET. 2009. Diversity and evolution of *Fusarium* species in the *Giberella fujikuroi* species complex. Fungal Diversity.34: 1-21.

Leslie JF. 1991. Mating populations in *Giberella fujikuroi* (*Fusarium* section Liseola). Phytopathology 88: 1058-1060.

Leslie JF.; Zeller KA, Wohler M, Summerell BA. 2004. Interfertility of two mating populations in the *Giberella fujikuroi* species complex. European Journal of Plant Pathology 110: 611-618.

Leslie JF.; Zeller KA.; Lamprecht SC, Rheeder JP, Marasas WFO. 2005. Toxicity, pathogenicity, and genetic differentiation of five species of *Fusarium* from sorghum and millet. Phytopathology 95: 275-283.

Leslie JF Summerell, B.A. 2006. The *Fusarium* Laboratory Manual. Malden: Blackwell, USA, 420 p.

Lima CS, Pfenning, LH, Costa SS, Abreu LM, Leslie JF. 2012. *Fusarium tupiense* sp. nov., a member of the *Gibberella fujikuroi* complex that causes mango malformation in Brazil Mycology 104: 1408-1419.

Marasas WFO, Rheeder JP, Lamprecht, SC, Zeller KA, Leslie JF. 2001. *Fusarium andiyazi* sp. nov., a new species from sorghum. Mycologia 93: 1203-1210.

McDonald BA, Linde C. 2002. Pathogen population genetics, evolutionary potential and durable resistance. Annual Review in Phytopathology 40: 349-379.

Milgroom MG. 1996. Recombination and multilocus structure of fungal populations. Annual Review in Phytopathology 34: 457-477.

Melo LGL, Silva EKC, Neto JRMC, Lins SRO, Rodrigues AAC, Oliveira SMA. 2016. Indutores de resistência abióticos no controle da fusariose do abacaxi. Pesquisa Agropecuária Brasileira 51: 1703-1709.

Moussa TAA et al. 2017. Two species of the *Fusarium fujikuroi* species complex isolated from natural environment. Antonie van Leeuwenhoek: 1-14.

Niehaus E, Munsterkotter M, Proctor HR, Brown DW, Sharon A, Idan Y, Oren-Young, Sieber CM, Novák O, Pencik A, Tarkowska D, Hromadova K, Freeman S, Maymon M, Elazar M, Youssef SA, El-Shabrawy ESM, Shalaby ABA, Houterman P, Brock NL, Burkhardrt I, Tsavkelova EA, Dickschat JS, Galuszka P, Guldener U, Tudzynski B. 2016. Comparative "omics" of the *Fusarium fujikuroi* species complex highlights differences in genetic potential and metabolite systems. Genome Biology and Evolution 8: 3574-3599.

Nirenberg HI, O'Donnell K. 1998. New *Fusarium* species and combinations within the Gibberella fujikuroi species complex. Mycologia 90: 434-458.

O`Donnell K, Cigelnik E, Nienberg HI. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90: 465-493.

Orr-Weaver TL, Szostak JW. 1985. Fungal recombination. Microbiological Reviews 49: 33–58.

Petrovic T, Walsh JL, Burgess LW, Summerell B. 2009. *Fusarium* species associated with stalk rot of grain sorghum in the northern grain belt of eastern Australia. Australasian Plant Pathology 38: 373-379.

Prá MD, Tonti S, Pancaldi D, Nipoti P, Alberti, I. 2010. First report of *Fusarium andiyazi* associated with Rice Bakanae in Italy. Plant Disease 94: 1070.

Proctor RH, Plattner RD, Brown DW, Seo J, Lee Y. 2004. Discontinuous distribution of fumosinina biosynthetic genes in the *Giberella fujikuroi* species complex. Mycological Research 108: 815-822.

Rosler SM, Sieber CMK, Humpf H, Tudzynski B. 2016. Interplay between pathway-specific and global regulation of the fumonisin gene cluster in the rice pathogen *Fusarium fujikuroi*. Applied Genetics and Molecular Biotechnology 100: 5869-5882.

Slinski SL, Kirkpatrick SC, Gordon TR. 2016. Inheritance of virulence in *Fusarium circinatum*, the cause of pitch canker in pines. Plant Pathology 65: 1292-1296.

Steenkamp ET, Wingfield BD, Coutinho TA, Zeller KA, Wingfield MJ, Marasas WFO, Leslie JF. 2000. PCR-based identification of *MAT-1* e *MAT-2* in the *Gibberella fujikuroi* species complex. Applied and Environmental Microbiology 66: 4378-4382.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. 2000. Phylogenetic species recognition and species concept in fungi. Fungal Genetics and Biology 31: 21-32.

Venturini G, Toffolatti SL, Quaglino F, Casati P. 2017. First report of *Fusarium andiyazi* causing ear rot on maize in Italy. Plant Disease 101: 839.

Wulff EG, Sorensen JL, Lubeck M, Nielsen KF, Thrane U, Torp J. 2009. *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxicogenicity. Environmental Microbiology 12: 649-657.

Zhang H, Luo W, Pan Y, Xu J, Xu JS, Chen WQ. 2014. First report of *Fusarium* ear rot of maize caused by *Fusarium andiyazi* in China. Plant Disease 98: 1428.

SEGUNDA PARTE – ARTIGO

Fusarium andiyazi species group: an application of the biological species concept

Aline Vaz Laranjeira de Souza, Marileide Moreira Costa, Ludwig H. Pfenning

Laboratório de Ecologia e Sistemática de Fungos, Departamento de Fitopatologia, Universidade Federal de Lavras, Caixa postal 3037, 37200-000 Lavras, MG, Brazil.

Manuscript prepared for submission to *Mycologia*.

ABSTRACT

Fusarium andiyazi is a member of the *Fusarium fujikuroi* species complex - FFSC, known to cause stem rot in sorghum in Africa and the United States. There are records of its association with other cultivated grasses and the natural vegetation as an endophyte. In association with different grasses from Brazil, the United States and African countries, a collection of 87 isolates of the morphotype *F. andiyazi* were obtained. The analysis of the barcode region TEF showed that these isolates represent *F. andiyazi* and two other, still undescribed phylogenetic species, morphologically indistinguishable. To date, the sexual phase of *F. andiyazi* is unknown, since the formation of fertile perithecia was not observed in laboratory crossings. The aims of this study were to evaluate the existence of the sexual reproduction of *F. andiyazi* and their sibling lineages through laboratory crossings, to report the species as possible new mating populations of the FFSC, to evaluate the morphological markers of the sexual phase as well as to verify the presence of a reproductive barrier with other FFSC mating populations. Strains deposited in the Coleção Micológica de Lavras - CML obtained from rice, sugarcane, sorghum, maize, *Brachiaria* and millet from Brazil, the United States and African countries, were included in the experiments. Amongst 87 strains, 19 represent *F. andiyazi*, 62 *Fusarium* sp.1 and six *Fusarium* sp.2. In the mating type PCR amplification for *F. andiyazi*, 12 strains were identified as MAT-1 and 7 as MAT-2, *Fusarium* sp.1 (21 MAT-1, 40 MAT-2), for *Fusarium* sp.2 two were identified as MAT-1 and four as MAT-2. Three fertile crossings were obtained between strains of *F. andiyazi* and 54 between *Fusarium* sp.1 strains. Therefore, *F. andiyazi* and *Fusarium* sp.1 represent new MPs within the FFSC. In crossings. of *F. andiyazi* and *Fusarium* sp.1 with the other MP of the FFSC, the production of fertile perithecia was not observed, showing that there is a reproductive barrier with the other biological species of the complex. A very few fertile crossings were observed between *F. andiyazi* and *Fusarium* sp.1, an indication that so far, no complete reproductive barrier between the two species has been established. *Fusarium* sp.2 did not produce any fertile crosses between its strains, with *F. andiyazi*, *Fusarium* sp.1 or with any other MP of the FFSC. In the morphological characterization of the sexual phase, the structures produced by *F. andiyazi* and *Fusarium* sp.1 were identical. The biological species concept applied to the study of these three species gives an insight into how important the use of this concept can be in determining a species, when the morphological markers are insufficient. The application of the biological species concept, besides consolidating the concept of species, can help in the identification of morphologically similar individuals.

Keywords: *Fusarium fujikuroi species complex*. Mating population. Tropical grasses.

1. INTRODUCTION

Fusarium andiyazi was first described by Marasas et al. (2001) as pathogen associated with sorghum rot symptoms (*Sorghum bicolor*) and has been found in most of the sorghum producing regions of the world, as South Africa, Ethiopia, Uganda, Nigeria, Australia, USA and Brazil (Leslie & Summerell 2006; Kelly et al 2017). The species belongs to the *Fusarium fujikuroif* Species Complex - FFSC and it is morphologically similar to other species of the complexcomplex, as *F. verticillioides* and *F. thapsinum*, sharing characters such as the production of microconidia in long chains and monophialides (Marasas et al 2001; Leslie & Summerell 2006).

In 2016, sequences of the gene regions TEF, TUB and RPB2 were provided for the type strain of *F. andiyazi* type, enabling phylogenetic studies and confirming that *F. andiyazi* as a phylogenetic species (Al-Hatmi et al 2016). Phylogenetic analyses performed for the TEF and RPB2 barcode region with *F. andiyazi* morphotype strains collected from Brazil, USA and Africa countries demonstrated that the strains are grouped into three different clades, one grouped together with the *F. andiyazi* type and two other sibling lineages (Figure 2 – Appendix). Besides phylogeny, AFLP data support that there are three clades inside African clade within FFSC with species that present *F. andiyazi* morphology but present different ecological, physiological and reproductive behaviour (unpublished data).

The basis of biological species concept is the ability of individuals to interbreed and to produce fertile offspring, so that they must also be reproductively isolated from different species (Perkins 1994; Leslie 1995; Taylor et al 2000). Biological species can be determined under laboratory crossings with tester strains by identifying crosses that produced fertile perithecia (Leslie & Summerell 2006). Crosses between heterothallic fungi require both MAT-1 and MAT-2 idiomorphs and at least one of the parents to act as a fertile female in the production of sexual structures (Leslie & Klein 1996). The idiomorphs determination by conventional PCR is an extremely useful tool in crossing protocols, once it can reduce by half the number of crossings to be made (Covert et al 1999; Kerényi et al 1999, 2004; Steenkamp et al 2000).

Therefore, this study aims to determine whether *F. andiyazi* and related lineages determined by previous phylogenetic studies, represent new mating populations within the FFSC as well as to determine the ideal crossing conditions. Considerations about how reliable is the biological species concept as a tool in determining these species were also raised.

2. MATERIALS AND METHODS

2.1. Obtention of the strains

A collection of 88 *Fusarium andiyazi* and *Fusarium* sp. strains was obtained from sorghum, rice, sugarcane, *Brachiaria*, maize and millet collected in several regions of Brazil, the United States and African countries and deposited in the Coleção Micológica de Lavras (CML), Departamento de Fitopatologia, Universidade Federal de Lavras, Minas Gerais, Brasil (http://www.dfp.ufla.br/cml/). The strains from Africa and United States were obtained by the Kansas State University (KSU). Also, reference strains from the mating populations of the FFSC were included in the crossings (Table 1).

2.2. Morphological studies

To verify if there are differences in the morphology of the *Fusarium andiyazi* and *Fusarium* sp. strains from Brazil, United States and Africa countries, morphological characterization was performed according to the protocol described by Leslie & Summerell (2006).

Monosporic cultures were grown in Petri dishes containing Potato Dextrose Agar medium (250g potato, 20g dextrose, 20g agar) and incubated at 25 °C in the dark for 4 days for growth rate observation and after 10 days for evaluation of colony staining. Micromorphological characteristics such as size, shape and origin of microconidia and macroconidia; types of phialides; presence of chlamydospores and production of microconidia in chains or false heads were evaluated in Synthetic Nutrient-poor Agar (1g KH_2PO_4, 1g KNO_3, 0,5g $MgSO_4.7H_2O$, 0,5g KCl, 0,2g glucose, 0,2g sucralose, 20g agar) at 20 ºC, under a 12h photoperiod with fluorescent white light, after a period of 10 to 14 days of incubation. For induction of sporulation of the strains, sterile filter paper was deposited on the SNA medium poured in 60 mm diameter Petri dishes.

2.3. Identification of mating type by PCR

To perform the crosses, the idiomorphs MAT-1 and MAT-2 were identified using primers and amplification conditions adapted from Steenkamp et al. (2000). To determine

the ideal annealing conditions, a gradient ranging from 53 to 67 °C was tested. The primers GFmat1a and GFmat1b were used to amplify the MAT-1 idiomorph (200 bp), and GFmat2c and GFmat2d to amplify the idiomorph MAT-2 (800 bp). PCR products were stained with GelRed (Biotium®) and submitted to 1% agarose gel electrophoresis, with subsequent visualization performed on a transilluminator. The length of the amplified fragments was compared to a "ladder" fragment length marker 1 Kb and the PCR were repeated twice for each isolate, and the mating type was confirmed only when the same results were obtained in both reactions.

2.4. Sexual crossings

The crossings were performed according to standard methodology for the study of sexual compatibility in the FFSC determined by Leslie & Summerell (2006). Strains used as a male parent were cultured in a test tube containing complete medium (30g sucrose, 2g $NaNO_3$, 2,5g N-Z amine, 1g yeast extract, 10 ml vitamin stock solution) and incubated at 20 °C under a 12-hour photoperiod for seven days. Strains used as female parent were grown in Petri dishes (60 mm) containing agar carrot medium (400g carrot and 20g agar) and incubated at 25 °C in the dark for seven days.

After the incubation period a spore suspension was prepared using the male parent by the addition of 2 mL Tween 60 solution. Then, the suspension was deposited on the colony surface of the female parental and, using a Drigalsky handle, spread to thoroughly moisten the mycelium. To determine the ideal temperature to induce the production of perithecia and exudation of ascospores, the crossings were incubated at 20, 23 and 25 °C with constant light and 12 h photoperiod of fluorescent white light combined with black light for a period of five weeks, to determine the best conditions of temperature and luminosity. Crossing that produced perithecia and oozed ascospore were considered fertile.

The crossings were repeated at least three times to confirm the results. The structures of the sexual stage were characterized by measuring the diameter of the perithecia, the length and width of the ascus and ascospores, and checking the number of septa. Twenty measurements of each structure were performed, which were used for comparison with the other biological species of FFSC already described in the literature.

To verify reproductive barrier with others FFSC mating populations, crossings were performed with the *Fusarium* sp.1 tester strains, *Fusarium* sp.2 and *F. andiyazi* strains with the female testers of MP-A to MP-M.

2.5. Ascospore viability

The viability of the ascospores was evaluated by the germination test in agar-water 2%. Using a stylet, ascospores cirrus were collected, deposited on the surface of the medium and scattered with a Drigalsky handle. Subsequently, the plates were incubated at 25 °C in the dark for 16h. After the incubation period, the ascospores germination was evaluated under a light microscope.

3. RESULTS

3.1. Identification of mating type

Through the different protocols tested in the determination of the mating type through the conventional PCR technique, the ideal temperature set determined for primers Gfmat1a/Gfmat1b and Gfmat1c/Gfmat1d was 65 °C and extension time of 30 seconds. At temperatures below 59 °C unspecific amplification occurred for both primers. The mating types the strains were confirmed by crossing protocols, when it was possible. Amongst the twenty strains of *F. andiyazi*, seven was identified as MAT-1 idiomorph and the 13 as MAT-2. Twenty-one strains of *Fusarium* sp. 1, presented MAT-1 idiomorph and 40 strains the idiomorph MAT-2 and one remain unknown. Four strains of *Fusarium* sp.2 were identified as MAT-2 and two as MAT-1 (Table 1).

3.2. Crossing experiments and ascospore viability test

Nearly 900 crossings were performed for the isolates of *F. andiyazi*, *Fusarium* sp.1 and *Fusarium* sp.2. All strains were tested as both female and male. So that all strains of both mating type, at some point, were in the Petri dish containing carrot-agar medium or in the test tube, containing complete medium. The ideal crossing conditions determined in this study to induce the production of perithecia, exudation and germination of ascospores, were the temperature of 20 °C and 12 h photoperiod of fluorescent white light combined with black light. All crossings were performed three times for confirmation of the results.

Table 1. *Fusarium andiyazi* and related lineages strains.

N°	Species	Cod CML[a]	Cod KSU	Phylogenetic Group	Host	Origin	*MAT*[c]
1	*F. andiyazi*	2740		1	*Sorghum bicolor*	Sete Lagoas, MG[b]	1
2	*F. andiyazi*	2746		1	*Sorghum bicolor*	Sete Lagoas, MG	1
3	*F. andiyazi*	2756		1	*Sorghum bicolor*	Sete Lagoas, MG	1
4	*F. andiyazi*	2758		1	*Sorghum bicolor*	Sete Lagoas, MG	1
5	*F. andiyazi*	2763		1	*Sorghum bicolor*	Uberlândia, MG	1
6	*F. andiyazi*	2815		1	*Sorghum bicolor*	Jardinópolis, SP	1
7	*F. andiyazi*	2817		1	*Sorghum bicolor*	Jardinópolis, SP	1
8	*F. andiyazi*	884	4647	1	*Sorghum bicolor*	Etiópia	2
9	*F. andiyazi*	3854	3860	1	*Sorghum bicolor*	Kansas, USA	1
10	*F. andiyazi*	3862	4807	1	*Sorghum bicolor*	Kansas, USA	1
11	*F. andiyazi*	3868	10771	1	*Sorghum bicolor*	Kansas, USA	1
12	*F. andiyazi*	3869	11149	1	*Sorghum bicolor*	Kansas, USA	1
13	*F. andiyazi*	3852	4645	1	*Sorghum bicolor*	Kansas, USA	2
14	*F. andiyazi*	3856	4648	1	*Sorghum bicolor*	Kansas, USA	2
15	*F. andiyazi*	3857	4650	1	*Sorghum bicolor*	Kansas, USA	2
16	*F. andiyazi*	3864	2791	1	*Sorghum bicolor*	Uganda, África	1
17	*F. andiyazi*	3867	9504	1	*Sorghum bicolor*	Uganda, África	2
18	*F. andiyazi*	3876	15137	1	*Sorghum bicolor*	Uganda, África	1
19	*F. andiyazi*	3877	16115	1	*Sorghum bicolor*	Uganda, África	2
20	*Fusarium* sp.1	2744		2	*Sorghum bicolor*	Sete Lagoas, MG	1
21	*Fusarium* sp.1	3044		2	*Brachiaria brizantha*	Lavras, MG	1
22	*Fusarium* sp.1	2754		2	*Sorghum bicolor*	Sete Lagoas, MG	2
23	*Fusarium* sp.1	2755		2	*Pennisetum amaericanum*	Luiz Ed. Magalhães, BA	Nd[d]
24	*Fusarium* sp.1	2762		2	*Sorghum bicolor*	Sete Lagoas, MG	1
25	*Fusarium* sp.1	2768		2	*Sorghum bicolor*	Indianápolis, MG	2
26	*Fusarium* sp.1	2780		2	*Sorghum bicolor*	Paracatu , MG	1
27	*Fusarium* sp.1	2783		2	*Sorghum bicolor*	Lavras, MG	1
28	*Fusarium* sp.1	2791		2	*Sorghum bicolor*	Sete Lagoas, MG	2
29	*Fusarium* sp.1	2793		2	*Sorghum bicolor*	Indianápolis, MG	2
30	*Fusarium* sp.1	2796		2	*Sorghum bicolor*	Chapadão do Sul, MS	1
31	*Fusarium* sp.1	2797		2	*Sorghum bicolor*	Birigui, SP	1
32	*Fusarium* sp.1	2800		2	*Sorghum bicolor*	Luiz Ed. Magalhães, BA	1
33	*Fusarium* sp.1	2801		2	*Sorghum bicolor*	Jardinópolis, SP	1
34	*Fusarium* sp.1	2802		2	*Sorghum bicolor*	Maurilândia, GO	2
35	*Fusarium* sp.1	2805		2	*Sorghum bicolor*	Rio verde, GO	2
36	*Fusarium* sp.1	2807		2	*Sorghum bicolor*	Lavras, MG	1
37	*Fusarium* sp.1	2809		2	*Sorghum bicolor*	Indianápolis, MG	1
38	*Fusarium* sp.1	2812		2	*Zea mays*	Rio verde, GO	2

N°	Species	Cod CML[a]	Cod KSU	Phylogenetic Group	Host	Origin	*MAT*[c]
39	*Fusarium* sp.1	2813		2	*Zea mays*	Rio verde, GO	2
40	*Fusarium* sp.1	2821		2	*Sorghum bicolor*	Chapadão do Sul, MS	2
41	*Fusarium* sp.1	2822		2	*Sorghum bicolor*	Chapadão do Sul, MS	1
42	*Fusarium* sp.1	2823		2	*Sorghum bicolor*	Jardinópolis, SP	1
43	*Fusarium* sp.1	3041		2	*Brachiaria brizantha*	Lavras, MG	2
44	*Fusarium* sp.1	3040		2	*Brachiaria decumbens*	Lavras, MG	1
45	*Fusarium* sp.1	3578		2	*Saccharum officinarum*	Araras, SP	2
46	*Fusarium* sp.1	3579		2	*Saccharum officinarum*	Araras, SP	2
47	*Fusarium* sp.1	3580		2	*Saccharum officinarum*	Araras, SP	2
48	*Fusarium* sp.1	3581		2	*Saccharum officinarum*	Araras, SP	2
49	*Fusarium* sp.1	3582		2	*Saccharum officinarum*	Araras, SP	2
50	*Fusarium* sp.1	3583		2	*Saccharum officinarum*	Piracicaba, SP	2
51	*Fusarium* sp.1	3584		2	*Saccharum officinarum*	Piracicaba, SP	1
52	*Fusarium* sp.1	3585		2	*Saccharum officinarum*	Piracicaba, SP	2
53	*Fusarium* sp.1	3586		2	*Saccharum officinarum*	Araras, SP	2
54	*Fusarium* sp.1	3561		2	*Saccharum officinarum*	União, PI	1
55	*Fusarium* sp.1	3648		2	*Oryza sativa*	Itajaí, SC	2
56	*Fusarium* sp.1	3649		2	*Oryza sativa*	Itajaí, SC	2
57	*Fusarium* sp.1	3650		2	*Oryza sativa*	Nova Veneza, SC	2
58	*Fusarium* sp.1	3651		2	*Oryza sativa*	Brazabantes, GO	2
59	*Fusarium* sp.1	3680		2	*Oryza sativa*	Itajaí, SC	2
60	*Fusarium* sp.1	3681		2	*Oryza sativa*	Itajaí, SC	2
61	*Fusarium* sp.1	3682		2	*Oryza sativa*	Brazabantes, GO	2
62	*Fusarium* sp.1	3780		2	*Oryza sativa*	Brazabantes, GO	2
63	*Fusarium* sp.1	3787		2	*Oryza sativa*	Brazabantes, GO	1
64	*Fusarium* sp.1	3793		2	*Oryza sativa*	Itajaí, SC	2
65	*Fusarium* sp.1	3809		2	*Oryza sativa*	Nova Veneza, SC	2
66	*Fusarium* sp.1	3810		2	*Oryza sativa*	Itajaí, SC	1
67	*Fusarium* sp.1	3812		2	*Oryza sativa*	Tangará da Serra, MT	1
68	*Fusarium* sp.1	3814		2	*Oryza sativa*	Itajaí, SC	2
69	*Fusarium* sp.1	3816		2	*Oryza sativa*	Garuva, SC	2
70	*Fusarium* sp.1	3818		2	*Oryza sativa*	Itajaí, SC	2
71	*Fusarium* sp.1	3819		2	*Oryza sativa*	Brazabantes, GO	2
72	*Fusarium* sp.1	3822		2	*Oryza sativa*	Gariva, SC	2
73	*Fusarium* sp.1	3824		2	*Oryza sativa*	Garuva, SC	2
74	*Fusarium* sp.1	3874	15118	2	*Sorghum bicolor*	Camarões, África	2
75	*Fusarium* sp.1	3875	15131	2	*Sorghum bicolor*	Camarões, África	2
76	*Fusarium* sp.1	3894	4846G-27	2	*Sorghum*	África	1
77	*Fusarium* sp.1	3853	3089G-1	2	*Sorghum*	África	2
78	*Fusarium* sp.1	3895	8436	2	*Sorghum*	Tanzania	1
79	*Fusarium* sp.1	3860	4739	2	*Sorghum*	Egypt	2

N°	Species	Cod CML[a]	Cod KSU	Phylogenetic Group	Host	Origin	*MAT*[c]
80	*Fusarium* sp.1	3865	9040	2	*Sorghum bicolor*	Uganda-África	2
81	*Fusarium* sp.1	3866	9052	2	*Sorghum bicolor*	Uganda- África	2
82	*Fusarium* sp.2	3870	15071	3	*Sorghum bicolor*	Camarões, África	2
83	*Fusarium* sp.2	3871	15074	3	*Sorghum bicolor*	Camarões, África	1
84	*Fusarium* sp.2	3872	15077	3	*Sorghum bicolor*	Camarões, África	1
85	*Fusarium* sp.2	3873	15082	3	*Sorghum bicolor*	Camarões, África	2
86	*Fusarium* sp.2	3859	4735	3	*Sorghum bicolor*	Kansas, USA	2
87	*Fusarium* sp.2	3858	4734	3	*Sorghum bicolor*	Kansas, USA	2

[a] CML: Coleção Micológica de Lavras, Universidade Federal de Lavras, Lavras, Minas Gerais, Brasil; [b] Brazilian state: BA: Bahia, GO: Goiás, MG: Minas Gerais, MS: Mato Grosso do Sul, SC: Santa Catarina, PI: Piauí, SP: São Paulo; [c]*MAT*: *Mating type* identified by PCR: *MAT-1* = 1; *MAT-2* = 2; [d] Nd :not determined.

The crossings between the members of *F. andiyazi* present extremely low fertility in a manner that may have not been possible to determine the testers (Table 2). Crossings produced perithecia from the second week and ascospores exudation occurred within six weeks after. Amongst the crossings of all MAT-1 *F. andiyazi* strains with all MAT-2, as well as each being tested as a female or male, only three were considered fertile. Crossings between the strains CML 2756, MAT-1, with MAT-2 strains CML 884, from Ethiopia, CML 3867 and CML 3877, from Uganda, Africa produced fertile perithecia from the second week and the exudation occurred within six weeks. After 24h incubation the ascospores presented almost 100% germination ratio, thus this may be an evidence to support that *F. andiyazi* can be describe as a new mating population within FFSC.

Table 2. Fertile crossings ratio amongst *Fusarium andiyazi* strains evaluated after incubation at 20ºC plus photoperiod during at least five weeks.

♀ (*MAT-1*)[a]		♂ (*MAT-2*)[b]	♀ (*MAT-2*)[c]	♂ (*MAT-1*)[d]
CML2756	X	CML884		
CML2756	X	CML3867		
CML2756	X	CML3877		

[a] ♀ **(MAT-1)** indicates strains mating type MAT-1 fertile as female.
[b] ♂**(MAT-2)** indicates strains mating type MAT-2.
[c] ♀ **(MAT-2)** indicates strains mating type MAT-2 fertile as female.
[d] ♂ **(MAT-1)** indicates strains mating type MAT-1.

Strains of *Fusarium* sp. 1 obtained in different hosts in Brazil and Africa present a high reproduction rate, as so was possible to determine female tester strains for both mating type, being CML 3584 (MAT-1) and CML 2793 (MAT-2) (Table 3). 54 fertile crossings were obtained among these testers and most of strains from respective opposite mating type, producing a large amount or fertile perithecia up to one week after the crossing. Exudation of ascospores occurred within two weeks and the ascospores germinated after 16h incubation. Furthermore, were obtained at least two fertile crossings between CML 3584 and CML 2793 *Fusarium* sp.1 with CML 884 and CML2758 of *F. andiyazi* (Table 3). Fertile peritecia were obtained only with *Fusarium* sp.1 as female parentparent. All strains from *F. andiyazi* and *Fusarium* sp. 1 are reproductive isolated from others mating population of the FFSC. Crossings between strains from *Fusarium* sp. 2 did not produce fertile perithecia.

Table 3. Fertile crossings ratio amongst *Fusarium* sp.1 strains evaluated after incubation at 20 ºC plus photoperiod during at least five weeks.

♀ (*MAT-1*)[a]		♂(*MAT-2*)[b]	♀ (*MAT-2*)[c]		♂(*MAT-1*)[d]
CML2780	X	CML2813	CML2768	X	CML2807
CML2780	X	CML2793	CML2793	X	CML3584
CML2801	X	CML2821	CML2793	X	CML2744
CML3584	X	CML3586	CML2793	X	CML2780
CML3584	X	CML2754	CML2793	X	CML2783
CML3584	X	CML2768	CML2797	X	CML2796
CML3584	X	CML2793	CML2793	X	CML2797
CML3584	X	CML2805	CML2793	X	CML2800
CML3584	X	CML2812	CML2793	X	CML2807
CML3584	X	CML2813	CML2793	X	CML2809
CML3584	X	CML3579	CML2793	X	CML2823
CML3584	X	CML3580	CML2793	X	CML3044
CML3584	X	CML3558	CML2793	X	CML3895
CML3584	X	CML3582	CML2793	X	CML2758
CML3584	X	CML3787			
CML3584	X	CML3810			
CML3584	X	CML3809			
CML3584	X	CML3814			
CML3584	X	CML3816			
CML3584	X	CML3818			
CML3584	X	CML3819			
CML3584	X	CML3822			
CML3584	X	CML3824			
CML3584	X	CML3583			
CML3584	X	CML3585			
CML3584	X	CML3853			
CML3584	X	CML3865			
CML3584	X	CML3866			
CML3584	X	CML3860			
CML3584	X	CML3648			
CML3584	X	CML3649			
CML3584	X	CML3650			
CML3584	X	CML3680			
CML3584	X	CML3681			
CML3584	X	CML3682			
CML3584	X	CML3853			
CML3584	X	CML3873			
CML3584	X	CML3866			
CML3584	X	CML3865			
CML3584	X	CML3860			
CML3584	X	CML884			

[a] ♀ **(MAT-1)** indicates strains mating type MAT-1 fertile as female.

[b] ♂**(MAT-2)** indicates strains mating type MAT-2.

[c] ♀ **(MAT-2)** indicates strains mating type MAT-2 fertile as female.

[d] ♂ **(MAT-1)** indicates strains mating type MAT-1.

3.3. Morphological characteristics of asexual stage

The strains of the three populations presented macroscopic and micromorphological characteristics described by Marasas et al. (2001) for the species *F. andiyazi*. There were no differences in the strains obtained from Brazil those from African countries or the United States.

In PDA (Potato-Dextrose-Agar) the strains presented flocculent and powdery mycelium with initially white and ranging from violet to purple. The observed micromorphological characteristics in SNA were aerial mycelium presenting abundant microconidia in long chains. Microconidia shape ranging from nailed to ovoid with slightly flattened base with usually 0-1 septa. Macroconidia hyaline and slightly curved, presenting up to 6 septa.

3.4. Description of sexual stage

Fertile crosses were obtained between F. *andiyazi* isolates, between *Fusarium* sp. 1 isolates and between the two species. The ascospores oozed by perithecia of all fertile crossings were viable, germinating after 16 hours incubation. The structures of the sexual stage were characterized by measuring the diameter of the perithecia, the length and width of the ascus and ascospores and checking the number of septa. Thirty measurements of perithecia and ascus and 30 measurements of ascospores were performed for each species, which were used for comparison with the FFSC biological species already described in the literature.

The *F. andiyazi* and *Fusarium* sp.1 sexual stage presented typical morphological characteristics of other FFSC species, as black perithecia varying from rounded to oval, measuring 200-425 x 190-400 μm, generally single but occasionally clustered. Fusiform ascus with usually 7-8 ascospores, measuring 62,5-100 x 6,25-12,5 μm.

Ascospores oozed in cirrus, hyaline, presenting the shapes varying from clavate to ovoid, with 1 to 3 septa. In *F. andiyazi* ascospores similar to microconidia are more frequent and *Fusarium* sp.1, the clavate shape. In both *F. andiyazi* and *Fusarium* sp.1 occur the three ascospores format. with both rounded end cells, usually one with a slight constriction and measuring 11,5-20 x 4-7,5 μm (Figure 1).

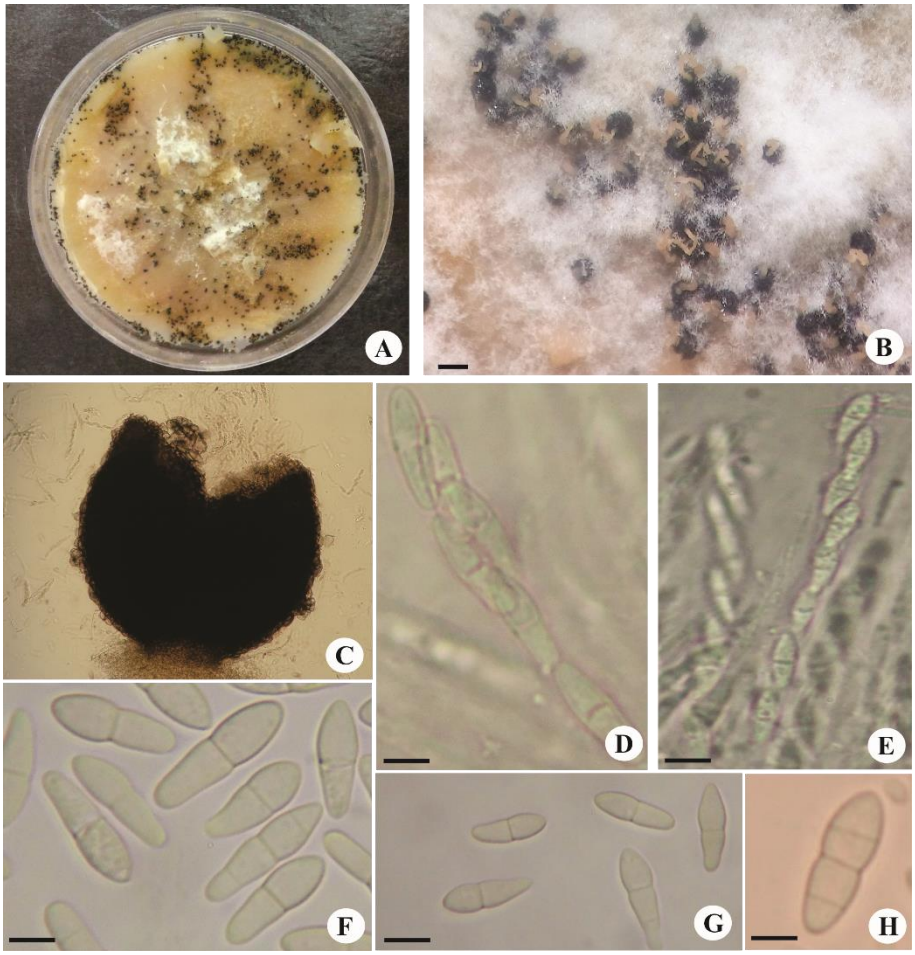

Figure 1. Morphological characteristics of the sexual stage of *Fusarium andiyazi* and *Fusarium* sp.1 **A.** Culture with fertile perithecia. **B.** Perithecia oozing ascospores. Bar = 300 µm **C.** Perithecia releasing ascii. Bar = 100 µm **D** Ascus Bar = 20 µm **E.** Ascus with eight ascospores Bar = 20 µm **F, G, and H.** Ascospores. Bar = 10 µm.

4. DISCUSSION

The results obtained from the crosses showed that *Fusarium andiyazi* and *Fusarium* sp.1 represent biological species corresponding to mating populations N and O, respectively, within the *Fusarium fujikuroi* species complex. For the 88 isolates used in this study the mating type identification was determined by PCR or, when possible, confirmed by laboratory sexual crossing. The specific primers developed for determination of mating type of the FFSC species through conventional PCR, allows the reduction of the number of crosses to be performed almost by half (Covert et al 1999; Kerényi et al 1999, 2004; Steenkamp et al 2000). The annealing temperature was adjusted so that there was specific amplification of the idiomorph relative to the primer used in the reaction. For Gfmat1a/Gfmat1b and Gfmat1c/Gfmat1d primers the ideal annealing temperatures was 65 ºC. Lower temperatures allowed non-specific amplification although Palacios et al. (2015) obtained specific amplification at the condition of 53 °C for *MAT-2* of *F. proliferatum* strains.

Marasas et al. (2001) identified 13 strains as MAT-1 and 15 as MAT-2 of *F. andiyazi* and by performing crosses between they but did not obtain any fertile perithecium. The authors suggest that the occurrence of isolates that behave as fertile females in natural populations are less frequent, or that adequate conditions to induce the sexual phase have not been determined yet. In contrast to Marasas, studies with four *F. andiyazi* obtained from rice report that fertile crossings were obtained when these strains were crossed with *F. verticillioides* testers (mating population A). However, some of the strains obtained in Brazil came from rice and other hosts than sorghum was identified by tef sequences as *Fusarium* sp.1., a sister species of *F. andiyazi*, which suggests that could take place a misidentification when reports of *F. andiyazi* occur in other cultures and actually the *Fusarium* sp.1 species is erroneously characterized as *F. andiyazi*. Furthermore, crossings between all strains of *F. andiyazi* and FFSC mating populations tester strains did not produce any fertile perithecia.

Since its first description, *F. andiyazi* has been reported in other cultures. Govender et al. (2010) studying South Africa strains identified the species in sugarcane by analysing microsatellite sequences (ISSR), in addition to associating it as causal agent of pokkah boeng. Studies in Africa, Malaysia and Southeast Asia reported the occurrence

of *F. andiyazi* associated with Bakanae disease in rice, whose molecular identification of the strains was performed through phylogenetic analysis of *EF-1α* region (Prá et al 2010; Wulff et al 2010; Hsuan et al 2011; Choi et al 2018). In northern Mexico, Italy and Korea by phylogenetic analysis, the authors identified strains of *F. andiyazi* associated with maize rot (Zhang et al 2014; Levya-Madrigal et al 2015, Venturini et al 2017; Choi et al 2018). Also, there are recent reports of *F. andiyazi* in barley, soybean and melon (Choi et al 2018; Seo & Kim 2017). However, considering unpublished data by our research group, it can be hypothesized that the strains identified as *F. andiyazi* from other crops as cited may be the new species *Fusarium* sp.1, which in Brazil was found in some hosts that are reported in the works cited above, such as sugarcane, rice, maize and others.

By comparison, it is possible to note the differences between the reproductive behaviour of *F. andiyazi* and his two sibling lineages. *Fusarium* sp.1 exhibits a high fertility rate so that the female tester strains of both mating type were quickly determined. The MAT-1 female tester presented high fertility by crossings with most of MAT-2 strains. In contrast, *F. andiyazi* presents low fertility with a median production of perithecia, and it takes 5 times more longer than Fusarium sp.1 takes to the perithecia oozed ascospores. Only CML 2756, MAT-1, presented a fertile crossing, being a potential tester, however, this strain interbreeds only with three of *F. andiyazi* MAT-2. Also, neither MAT-2 strain presented characteristics of fertile female or produced any fertile perithecia. Due to the low number of fertile crossings obtained and the absence of MAT-2 strains from Brazil, it can be correlated to the difficulty in finding a fertile female for *F. andiyazi* with the low occurrence of fertile females in natural populations, as suggested by Marasas et al. (2001).

No fertile crossings obtained between *Fusarium* sp.2 strains could be an indicative which this species reproduces only asexually, however the sample number used in this study is very limited and a larger number of individuals would be necessary to come up with a more precise inference about the fertility of this group. Something important to emphasize is that this lineage have not been identified in sorghum in Brazil yet and only in Africa it has been found so far.

Despite the low fertility observed in the intraspecific crossings of *F. andiyazi*, fertile crossings were observed between two strains of *Fusarium* sp.1 and two *F. andiyazi* being fertile CML 2793 x CML 2758 and CML 3584 x CML 884. Crossings were

obtained only with the tester strains of *Fusarium* sp.1 on plate, evidencing that the interbreed between these two phylogenetic distinct species shows the absence of reproductive barrier and an incomplete speciation process how so that the two crossings were obtained only in one way, with *Fusarium* sp.1 as female. Also, the crossings between these two species took up to six weeks to perithecia exudate, which may be an evidence that these interbreed are not common to occur in nature. Besides, AFLP data, phylogenetic studies and ecological behaviour support that *F. andiyazi* and *Fusarium* sp.1 are two different species with morphological similarity and an incomplete reproductive barrier, considering that sporadic crossings occur.

A similar case was reported by Leslie et al. (2004), the occurrence of fertile crosses and viable progeny production with reduced germination rate amongst few strains of mating population C and D of FFSC, corresponding to *F. fujikuroi* and *F. proliferatum*, respectively. According to the authors, the fact that these two species interbreed would be sufficient evidence that both are interfertile enough to belong to the same biological species, however, more evidence would be needed of the natural occurrence of these crossings.

Moreover, the proportion of the mating type distribution among the strains may be an indicative that the occurrence of sexual reproduction of *F. andiyazi* in nature is rare, to date possible only in laboratory conditions. The lack of testers and the low fertility would be a limiting factor for the determination of *F. andiyazi* as a new biological species. As far as concerned *F. andiyazi*, obtaining more isolates from Brazil and identified as MAT-2 could provide more support to elucidate if the species is a new mating population of FFSC.

Considering that there are 61 phylogenetic species and thirteen biological species in the FFSC, among which only *F. verticillioides* and *F. udum* have records of perithecia occurring associated with its hosts, in nature (Edwards 1935; Rai et al 1982), it is not possible to affirm whether this greater adaptation to different cultures may be related to some advantage gained during the genetic recombination resulting from meiosis sexual recombination through gene flow and chromosomal crossing-over, but it is interesting to suppose that sexual reproduction may bring some advantage to the species (Esser 1971; Heitman et al 2006). *Fusarium* sp.1 have affinity to other cultures besides sorghum, such as sugarcane, rice and maize whereas *F. andiyazi* presents an affinity only to sorghum.

Furthermore, the fertility of these two species are considerable different and genetic data shows that they are distinct phylogenetically (Figure 2). Considering these facts, *Fusarium andiyazi* and *Fusarium* sp.1 represent new mating populations within the FFSC, but do not developed a complete reproductive barrier yet, as sporadic crossings may occur under laboratory conditions.

The biological species concept is a tool that can be used to distinguish *F. andiyazi* and *Fusarium* sp.1 from other mating populations within the FFSC as both did not produce any fertile crossing with none A-M mating populations. Additionalphylogenetic analyses, molecular characterization and metabolite production can help to elucidate the differences between these species, as both can interbreed and have no distinct morphological traits. To increase the number of *Fusarium andiyazi* strains from Brazil and other sorghum producing regions in the world may help to consolidate the concept of *F. andiyazi* as a biological species, once more crossings can be performed enhancing the chance to find a tester. Also, since a few fertile crossings were detected in this study, backcrossing procedures can be tested to determine female tester strains for both mating types, as more research time is required. Formally describe *Fusarium* sp.1 as possible mating population along with *F. andiyazi* are the next steps of this study.

REFERENCES

Britz H, Coutinho TA, Wingfield MJ, Marasas WFO, Gordon TR, Leslie JF. 1999. *Fusarium subglutinans* f. sp. *pini* represents a distinct *mating population* in the *Gibberella fujikuroi* species complex. Applied and Environmental Microbiology 65: 1198-1201.

Choi J, Lee S, Nah J, Kim H, Paek J, Lee S, Ham H, Hong SK, Yun S, Lee T. 2018. Species composition of and fumonisin production by the *Fusarium fujikuroi* species complex isolated from Korean cereals. International Journal of Food Microbiology 267: 62-69.

Covert SF, Briley A, Wallace MM, McKinney T. 1999. Partial MAT-2 gene structure and the influence of temperature on mating success in *Gibberella circinata*. Fungal Genetics Biology 28:43–54.

Govender P, McFarlane SA, Rutherford RS. 2010. *Fusarium* species causing pokkah boeng and their effect on *Eldana saccharina* walker (Lepidoptera: Pyralidae). Proceedings South African Sugar Technology Association 83: 267-270.

Edwards ET. 1935. Studies on *Giberella fujikuroi* var. *subglutinans* the hitherto undescribed ascigerous stage of *Fusarium moniliforme* var. *subglutinans* and on its pathogenicity on maize in New South Wales. Scientific Bulletin Department Agriculture New South Wales 49: 5-68.

Heng MH, Salleh B, Zakaria L. 2011. Molecular identification of *Fusarium* species in *Giberella fujikuroi* species complex from rice, sugarcane and maize from Peninsular Malaysia. International Journal of Molecular Sciences 12: 6722-6732.

Kelly LA, Tan YP, Ryley MJ, Aitken E. 2017. *Fusarium* species associated with stalk rot and head blight of grain sorghum in Queensland and New South Wales, Australia. Plant Pathology 66: 1413-1423.

Kérenyi Z, Zeller K, Hornok L, Leslie JF. 1999. Molecular standardization of mating type terminology in the *Gibberella fujikuroi* species Complex. Applied and Environmental Microbiology 65: 4071-4076.

Kérenyi Z, Moretti A, Waalwijk C, Oláh B, Hornok L. 2004. Mating type sequences in asexually reproducing *Fusarium* species. Applied and Environmental Microbiology 70: 4419-4423.

Leslie JF. 1995. *Gibberella fujikuroi*: available populations and variable traits. Canadian Journal of Botany 73: S282–S291.

Leslie JF, Klein KK. 1996. Female fertility and mating type effects on effective population size and evolution in filamentous fungi. Genetics 144:557–567.

Leslie JF, Zeller KA, Wohler M, Summerell BA. 2004. Interfertility of two mating populations in the *Giberella fujikuroi* species complex. European Journal of Plant Pathology 110: 611-618

Leslie JF, Summerell BA. 2006. The *Fusarium* Laboratory Manual. Malden: Blackwell, USA, 420 p.

Levya-Madrigal KY, Larralde-Corona CP, Apódaca-Sanchez MA, Quiroz-Figueroa FR, Mexia-Bolaños PA, Portillo-Valenzuela S, Ordaz-Ochoa J, Maldonado-Mendoza IE. 2015. *Fusarium* species from the *Fusarium fujikuroi* species complex involved in mixed infections of maize in Northern Sinaloa, Mexico. Journal of Phytopathology 163: 486-497.

Lima CS, Pfenning LH, Costa SS, Abreu LM, Leslie JF. 2012. *Fusarium tupiense* sp. nov., a member of the *Gibberella fujikuroi* complex that causes mango malformation in Brazil. Mycology 104: 1408-1419.

Marasas WFO, Rheeder JP, Lamprecht, SC, Zeller KA, Leslie JF. 2001. *Fusarium andiyazi* sp. nov., a new species from sorghum. Mycologia 93: 1203-1210.

Palacios SA, Susca A, Haidukowski M, Stea G, Cendoya E, Ramírez ML, Chulze SN, Farnochi MC, Moretti A, Torres AM. 2015. Genetic variability and fumonisin production by *Fusarium proliferatum* isolated from durum wheat grains in Argentina. International Journal of Food Microbiology 201: 35-41

Perkins D. 1994. How should the interfertility of interspecies crosses be designated? Mycologia 86: 758-761.

Prá MD, Tonti S, Pancaldi D, Nipoti P, Alberti, I. 2010. First report of *Fusarium andiyazi* associated with rice bakanae in Italy. Plant Disease 94: 1070.

Rai B, Upadhyay RS. 1992. *Gibberella indica*: the perfect state of *Fusarium udum.* Mycologia 94: 343–346.

Raju MB, Perkins DD. 1994. Diverse programs of ascus development in pseudohomothallic species of *Neurospora*, *Gelasinospora* and and *Podospora*. Developmental Genetics 15: 104-118.

Seo Y, Kim YH. 2017. Potential Reasons for Prevalence of *Fusarium* Wilt in Oriental Melon in Korea. The Plant Pathology Journal 33: 249-263.

Steenkamp ET, Wingfield BD, Coutinho TA, Zeller KA, Wingfield MJ, Marasas WFO, Leslie JF. 2000. PCR-based identification of *MAT-1* e *MAT-2* in the *Gibberella fujikuroi* species complex. Applied and Environmental Microbiology 66: 4378-4382.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. 2000. Phylogenetic species recognition and species concept in fungi. Fungal Genetics and Biology 31: 21-32.

Venturini G, Toffolatti SL, Quaglino F, Casati P (2017) First report of *Fusarium andiyazi* causing ear rot on maize in Italy. Plant Disease 101: 839.

Wulff EG, Sorensen JL, Lubeck M, Nielsen KF, Thrane U, Torp J. 2009. *Fusarium* spp. associated with rice bakanae: ecology, genetic diversity, pathogenicity and toxicogenicity. Environmental Microbiology 12: 649-657.

Zhang H, Luo W, Pan Y, Xu J, Xu JS, Chen WQ. 2014. First Report of Fusarium Ear Rot of Maize Caused by *Fusarium andiyazi* in China. Plant Disease 98: 1428.

APPENDIX

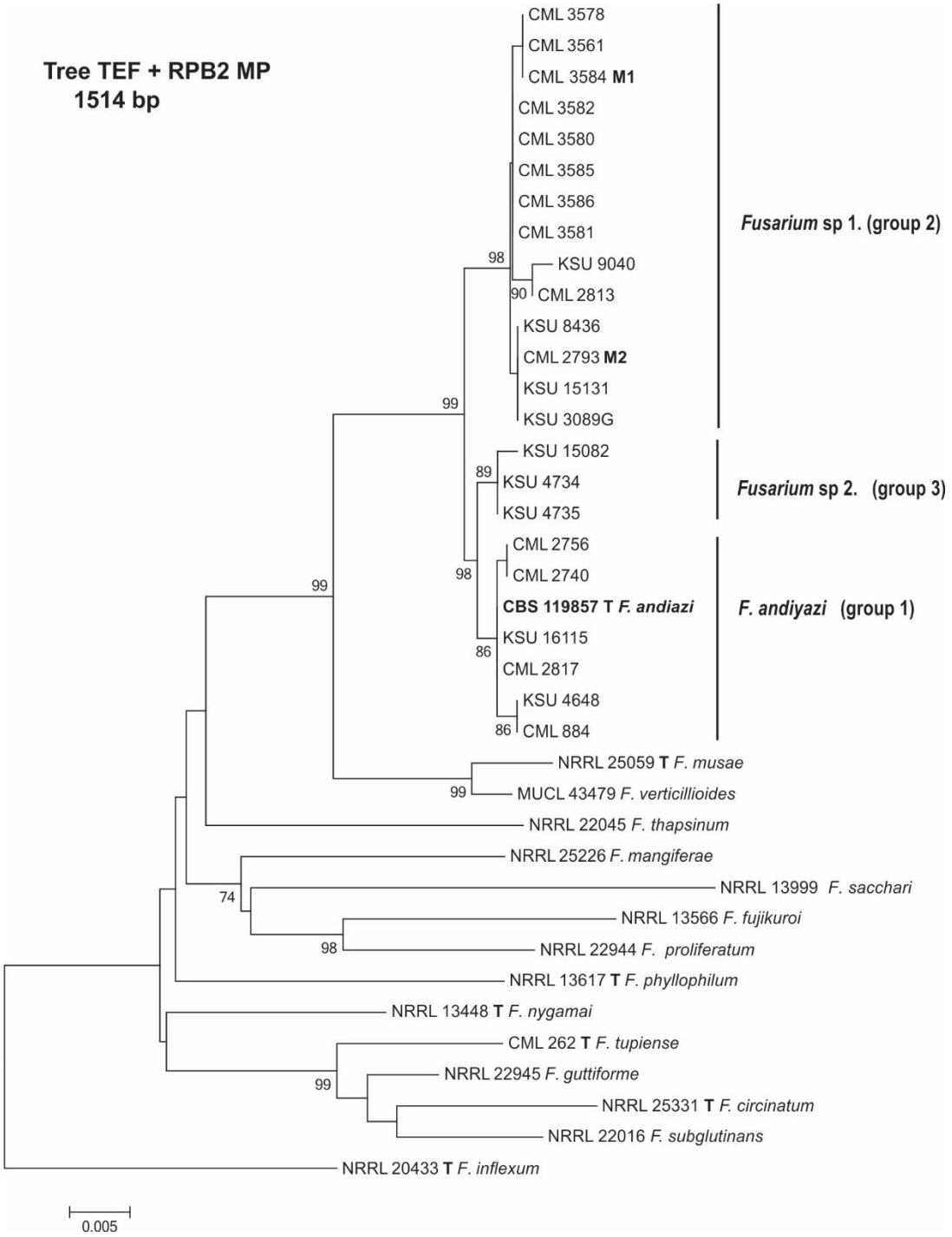

Figure 2. Phylogenetic tree generated by Maximum parsimony analysis from sequences of the *EF-1α* and *RPB2* genes from *Fusarium andiyazi* from *Sorghum bicolor* and *Fusarium* sp. strains from *Brachiaria* sp., *Sorghum bicolor*, *Zea mays*, *Pennisetum americanum*, *Saccharum officinarum* and reference strains belonging to the *Fusarium fujikuroi* species complex. Abbreviations: CBS Centraalbureau voor Schimmelcultures CML: Coleção Micológica de Lavras; MUCL: Mycotheque de l'Universite Catholique de Louvain, Louvain-la-Neuve, Belgium: NRRL: Northern Regional Research Laboratory, Peoria IL, USA. Source: Costa et al. 2018, in prep.