UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM FITOTECNIA
DOUTORADO EM FITOTECNIA

ANDRÉIA MITSA PAIVA NEGREIROS

DIVERSIDADE GENÉTICA E ADAPTABILIDADE DE *Monosporascus* E *Macrophomina* ISOLADOS DE PLANTAS DANINHAS EM ÁREAS DE CUCURBITÁCEAS

MOSSORÓ
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Tese apresentada ao Programa de Pós-Graduação em Fitotecnia da Universidade Federal Rural do Semi-Árido como parte dos requisitos para obtenção do título de Doutora em Agronomia: Fitotecnia.

Linha de Pesquisa: Proteção de Plantas

Orientador: Prof. Dr. Rui Sales Junior

Co-orientador: Prof. Dr. Josep Armengol Fortí

MOSSORÓ

2019

Dados Internacionais de Catalogação na Publicação (CIP)
Biblioteca Central Orlando Teixeira (BCOT)
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Setor de Informação e Referência

N385d Negreiros, Andréia Mitsa Paiva.
DIVERSIDADE GENÉTICA E ADAPTABILIDADE DE Monosporascus E Macrophomina ISOLADOS DE PLANTAS DANINHAS EM ÁREAS DE CUCURBITÁCEAS / Andréia Mitsa Paiva Negreiros. - 2019.
90 f. : il.

Orientador: Rui Sales Junior.
Coorientador: Josep Armengol Forti.
Tese (Doutorado) - Universidade Federal Rural do Semi-árido, Programa de Pós-graduação em Fitotecnia, 2019.
1. Filogenia. 2. Fungo. 3. Patogenicidade. 4. taxonomia. I. Sales Junior, Rui, orient. II. Armengol Forti, Josep, co-orient. III. Título.

Bibliotecário-Documentalista
Nome do profissional, Bib. Me. (CRB-15/10.000)

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Defendida em: 22 / 02 / 2019.

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À minha família, pais e irmãos, que me propiciaram uma vida digna, onde eu pudesse crescer, acreditando que tudo é possível, desde que sejamos honestos e íntegros de caráter. Sem eles nada disso seria possível.

AGRADECIMENTOS

À Deus, que iluminou o meu caminho nessa longa jornada e mostrou-se presente em todos os momentos, o que seria de mim sem a fé que eu tenho.

Ao Programa de Pós-Graduação em Fitotecnia da Universidade Federal Rural do Semi-Árido - UFERSA, pela oportunidade de acesso ao ensino público de qualidade.

Ao Professor Dr. Sc. Rui Sales Júnior, orientador, pelos ensinamentos e conselhos que foram fundamentais no percorrer do doutorado.

Ao professor Dr. Sc. Josep Armengol Fortí (Instituto Agroflorestal do Mediterrâneo, na Universidade Politécnica de Valencia, Espanha), pela co-orientação e oportunidade de realizar o período de doutorado "sanduíche" na UPV.

À Dra. Sc. Maela León (Instituto Agroflorestal do Mediterrâneo, na Universidade Politécnica de Valencia, Espanha), por todo ensinamento e boa vontade para o desenvolvimento desta pesquisa, e também pelo apoio e amizade durante meu período de um ano na Espanha.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos durante todo meu doutorado.

Ao Prof Glauber Henrique de Sousa Nunes e a Profª Márcia Michelle de Queiroz Ambrósio pela ajuda e colaboração com os trabalhos desta tese.

Ao pessoal do Laboratório do Instituto Agroflorestal do Mediterrâneo (UPV) pela convivência diária durante um ano do doutorado "sanduíche": Beatriz Sala, Elisa González-Domínguez, Georgina Jimenez, José Manuel, Monica Berbegal, Paloma Abad-Campos, Santiago, Tamiris Rêgo, Tony e Valentino. Ao pessoal do Laboratório de Fitopatologia II da UFERSA: Ana Paula Rodrigues, Andreza Araújo, Antônio Mendonça Jr., Hailton Silva, Hohana Medeiros, Naama Melo, Ricardo Junior, Thomaz Rauan e Wallyson Lima, pela ajuda no desenvolvimento desse trabalho. Aos amigos desde a graduação, Fenando Henrique e Tamires Bessa.

Enfim, agradeço a todos que direta ou indiretamente contribuíram para minha formação profissional, me ajudando a chegar até aqui.

RESUMO

O Brasil é o maior produtor mundial de frutas tropicais, com destaque para o melão e a melancia. Muitos são os problemas ocasionados por patógenos radiculares em ambas as culturas. Dentre eles destacamos os fungos dos gêneros *Monosporascus* e *Macrophomina*. Plantas daninhas presentes nas áreas de cultivo podem atuar como hospedeiros alternativos desses fungos habitantes do solo. Diante disso, este estudo visa conhecer a diversidade genética e a adaptabilidade de *Monosporascus* e *Macrophomina* isolados de plantas daninhas em áreas de cucurbitáceas no Nordeste brasileiro. No primeiro trabalho, uma coleção de 35 isolados de *Monosporascus* spp. provenientes de raízes de duas espécies de plantas daninhas, prevalentes em campos de cultivo de cucurbitáceas no nordeste brasileiro, *Trianthema portulacastrum* e *Boerhavia diffusa* foram utilizados neste estudo. Estes isolados foram identificados com base nas sequências de DNA das regiões dos Espaçadores Internos Transcritos (ITS) do rDNA nuclear, parte do gene do fator de elongação da tradução (*tef-1α*), parte do gene da β-tubulina (*tub*), parte do rDNA nuclear de subunidade pequena (SSU) e parte do rDNA nuclear de subunidade grande (LSU). Cinco novas espécies de *Monosporascus* foram identificadas em nível mundial, sendo *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus* e *M. semiaridus*. *Monosporascus brasiliensis*, *M. nordestinus* e *M. semiaridus* foram isoladas de ambas as espécies de plantas daninhas, enquanto *M. caatinguensis* somente de *T. portulacastrum* e *M. mossoroensis* apenas de *B. diffusa*. O presente estudo confirma que *Monosporascus* spp. pode colonizar raízes de *T. portulacastrum* e *B. diffusa*, e revela que existe diversidade de espécies. No segundo trabalho, foi utilizada uma coleção de 94 isolados de *Macrophomina* spp. obtidos de raízes de *T. portulacastrum* e *B. difusa*, onde foram caracterizados utilizando técnicas moleculares e patogenicidade. A análise filogenética do gene *tef-1α*, amplificado com os primers EF728F e EF986R, permitiu a identificação de 32 isolados como *M. phaseolina* e 62 isolados como *M. pseudophaseolina*. Resultados do teste de patogenicidade realizado em mudas de meloeiro 'Gladial' revelaram que ambas as espécies são patogênicas a esta cucurbitácea, tendo *M. phaseolina* causando uma maior incidência e severidade da doença. Este estudo representa o primeiro relato de *M. pseudophaseolina* em plantas de *T. portulacastrum* e *B. difusa* no Brasil.

Palavras-chave: Filogenia. Fungo. Patogenicidade. Taxonomia.

ABSTRACT

Brazil is the world's largest producer of tropical fruits, highlighting melon and watermelon. Umpteen problems are caused by root pathogens in both cultures. Among them we highlight fungi of the genera *Monosporascus* and *Macrophomina*. Weeds present in the cultivated areas can act as alternative hosts of these soilborne fungi. Therefore, the objective of this work was to know the genetic diversity and adaptability of *Monosporascus* and *Macrophomina* isolated from weeds in melon production fields in Northeastern Brazil. In the first work, a collection of 35 isolates of *Monosporascus* spp. from roots of two weed species prevalent in cucurbits fields of cultivation in Northeastern Brazil, *Trianthema portulacastrum* and *Boerhavia diffusa*, were used in this study. These isolates were identified based on DNA sequences of Internal Transcribed Spacer regions (ITS) of the nuclear rDNA, part of the translation elongation factor gene (*tef-1α*), part of the β-tubulin gene (*tub*), part of the nuclear small subunit rDNA (SSU), and part of the large subunit rDNA (LSU). Five new species of *Monosporascus* were identified worldwide, being *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus* and *M. semiaridus*. *Monosporascus brasiliensis*, *M. nordestinus* and *M. semiaridus* were isolated from both weed species, while *M. caatinguensis* only from *T. portulacastrum* and *M. mossoroensis* only from *B. diffusa*. The present study confirms that *Monosporascus* spp. can colonize roots of *T. portulacastrum* and *B. diffusa*, and reveals that there is a high diversity of species. In the second work, a collection of 94 isolates of *Macrophomina* spp. obtained from roots of *T. portulacastrum* and *B. diffusa* were used, where they were characterized using molecular techniques and pathogenicity. Phylogenetic analysis of the *tef-1α* gene, amplified with the EF728F and EF986R primers, allowed the identification of 32 isolates as *M. phaseolina* and 62 isolates as *M. pseudophaseolina*. Results of the pathogenicity test performed on 'Gladial' melon seedlings revealed that both species are pathogenic to this cucurbitaceae, with *M. phaseolina* presenting a higher incidence and severity of the disease. This study represents the first report of *M. pseudophaseolina* in plants of *T. portulacastrum* and *B. diffusa* in Brazil.

Keywords: Phylogeny. Fungus. Pathogenicity. Taxonomy.

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CHAPTER I

1 GENERAL INTRODUCTION

The Cucurbitaceae family include worldwide economically important fruit crops. According to the FAO (2018), the world production of the main cucurbits species surpassed 255.3 million (mi) tons (t) in 2016. This year, watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) was at the top of the production ranking with 117.0 mi t, followed by cucumber (*Cucumis sativus* L.) (80.6 mi t), melon (*Cucumis melo* L.) (31.2 mi t) and pumpkins (*Cucurbita moschata* Duchesne et Poir., *C. maxima* Duchesne e *C. pepo* L.) (26.5 mi t).

In Brazil, the most cultivated Cucurbitaceae crops are: watermelon, squash, melon, and cucumber. However, watermelon and melon are the ones that present greater economic expression, when compared to other cucurbits.

Brazilian watermelon production in 2017 reached 2.3 mi t, totalizing U$$ 36.3 mi in exportation. The main producing regions, the Northeast and South, were responsible for 28.7% (663.4 thousand t) and 21.8% (504.9 thousand t), respectively. Among the main watermelon producing states in the Northeast, Bahia (239.177 t) and Rio Grande do Norte-RN (199.192 t) stood out (IBGE, 2018).

On the other hand, with a production of 540.2 thousand t in 23.4 thousand hectares, the melon is the second most exported fruit by the Brazilian trade balance, with an export value of U$$ 162.9 million, which corresponds to 21.7% of the Brazilian's fresh fruit exportation. The main melon producing states are Rio Grande do Norte (RN) (338.7 thousand t) and Ceará (CE) (70.6 thousand t), both located in Northeastern Brazil. It should be noted that this region accounts for up to 95.2% of the national production (ANUÁRIO, 2017; IBGE, 2018).

Despite the importance of these crops to the states of RN and CE, the productive sector faces several problems, which is rooted in phyto-technical, phytosanitary and administrative origins. Within the phytosanitary problems, we highlight diseases caused by fungal root pathogens: *Fusarium solani* (Mart.) Sacc. f. sp. *cucurbitae* Snyder & Hansen, *Monosporascus cannonballus* Pollack & Uecker, *Macrophomina phaseolina* (Tassi) Goid., *Rhizoctonia solani* Kühn and *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley (SANTOS et al., 2000; MARINHO et al., 2002; ANDRADE et al., 2005). All of

these pathogens may attack, alone or together with others, plants of many species of cucurbitaceae family, with melon and watermelon the most attacked species, causing symptom such as "vine decline". Among these pathogens, *M. cannonballus* and *M. phaseolina* stand out due to their wide host range and worldwide distribution, besides the fact that they are thermotolerant (MARTYN; MILLER, 1996; TONIN et al., 2013).

Up to this date, *M. cannonballus*, a soil-borne ascomycete, characterized by the formation of black perithecium in the roots, where asci are produced with one big ascospore (rarely two) in a spherical shape (SIVANESAN, 1991, COHEN et al., 2012), has been reported in 22 countries (SALES JUNIOR et al., 2018) as the causal agent of the disease named “Monosporascus root rot and vine decline” (MRRVD) (MARTYN; MILLER, 1996; EL-DESOUKY; EL WAKIL, 2003; BEN SALEM et al., 2015; ALEANDRI et al., 2017; MARKAKIS et al., 2018). Currently, it is one of the main root pathogens associated with the "vine decline" of melon and watermelon worldwide (GAYTÁN-MASCORRO et al., 2012; YAN et al., 2016; MARKAKIS et al., 2018). In Brazil, the pathogen was reported affecting melon roots (SALES JÚNIOR et al., 2004) and watermelon plants (SALES JÚNIOR et al., 2010). This disease may result in severe losses in melon fields, as the fungus is favored by hot, arid or semiarid climates, with saline and alkaline soils (COHEN et al., 2012), such as those in the main melon producing region of Brazil.

The main symptoms of MRRVD shown by infected plants include yellowing, followed by wilting ("vine decline"), which may cause, in more severe attacks, plant death (COHEN et al., 2012). The “vine decline” in melon and watermelon is more frequently observed near the harvest period. This is due to the water imbalance that exists between the water demand of the plant and the insufficient root system uptake since it is rotted due to the attack of the phytopathogen. A more severe attack may result in 100 % loss (GARCÍA-JIMÉNEZ et al., 1993).

In a root system infected by *M. cannonballus*, we may see the presence of perithecia, where ascospores are produced (reproductive structures of the fungus), and acting as the primary structure for survival and inoculum for infection of plant roots (MARTYN; MILLER, 1996; LOUWS et al., 2010). This infection can occur from the fungal mycelium that survived in the soil (SALES JÚNIOR et al., 2018), in weeds (SALES JÚNIOR et al., 2012), in remaining crop residue (PEREIRA et al., 2012) or by ascospores that germinate when stimulated by root exudates or by soil microbiota (MEDEIROS et al., 2006).

Up to this date, five species from the *Monosporascus* genus have been report worldwide: *M. adenantherae* (S.D. Patil & C. Ramesh) A. Pande (PATIL; RAMESH, 1987),

M. cannonballus (POLLACK; UECKER, 1974), *M. eutypoides* (Petrak) von Arx (PETRAK; AHMAD, 1954; BEN SALEM et al., 2013), *M. ibericus* Collado, Ant. González, Stchigel, Guarro & Peláez (COLLADO et al., 2002) and *M. monosporus* (Malloch & Cain) D. Hawksw. & Ciccar (MALLOCH; CAIN, 1971). However, *M. adenantherae* and *M. monosporus* do not have a reference isolate deposited in culture collections or gene sequences available on genetic databases.

Another root pathogen of primary importance for melon and watermelon crops is the *M. phaseolina* fungus. It is a soilborne fungus, belonging to the ascomycete phylum, which produces microsclerotia and pycnidia as structures of asexual reproduction.

In recent years it has been notable for its high frequency of root isolation of these species with symptoms of "vine decline". In recent data, this pathogen has been reported in more than 500 hosts worldwide (HYDER et al., 2018; MEENA et al., 2018; GERIN et al., 2018; NISHAD et al., 2018; FARR; ROSSMAN, 2018; ZIVANOV et al., 2018). Studies of genetic variability detected two new species of *Macrophomina*, *M. pseudophaseolina* (SARR et al., 2014) and *M. euphorbiicola* (MACHADO et al., 2018).

The symptoms caused by *M. phaseolina* in most crops range from seed, roots and stems rot, damping-off, and premature death of seedlings (SHORT; WYLLIE, 1978). After plant death, the fungus continues to colonize host tissues, where it forms microsclerotia, the main source for survival and inoculum of the pathogen. (PAPAVIZAS, 1977; BAIRD et al., 2003). This pathogen may also be transmitted by seeds (REGO et al., 2012), via spores (FUHLBOHM et al., 2013), microsclerotia present in soil (REIS et al., 2014), and may be hosted by weeds (SALES JÚNIOR et al., 2012).

Since they are natural soil-borne pathogens, there is no efficient method for controlling *M. cannonballus* and *M. phaseolina*. The best way to control them is to keep track of the history of the area and use resistant varieties, when they exist (SALES JÚNIOR et al., 2018). So far, there is no registered fungicide in Brazil to use for control of these pathogens in the field. Basically, the registered products are only for seed treatment (AGROFIT, 2019).

Previous knowledge of the participation of weeds as alternative host of fungi of the *Monosporascus* and *Macrophomina* genre is of great relevance for management of "vine decline", especially in the off season. According to Chaves et al. (2003), weeds can act as alternative hosts of plant pathogens, serving as sources of inoculum, playing an important role in the epidemiology of diseases. Some studies show the importance of weeds as alternative hosts and source of inoculum of different phytopathogens, such as fungi, viruses, bacteria, and nematodes. In a study performed by Sales Júnior et al. (2012), the occurrence of *M.*

cannonballus, *M. phaseolina*, and *R. solani* in weeds prevalent in areas of melon in the Northeast of Brazil was observed. In the following year, Rodrigues (2013) reported 14 weed species as hosts of root pathogens that caused "vine decline" in melon and watermelon in the states of RN and CE. In this work, *M. phaseolina* was isolated from 12 of the 14 weed species collected, and *M. cannonballus* from two (2) of the 14 assessed species. Among these weed species, *Boerhavia diffusa* L. and *Trianthema portulacastrum* L. were reported as hosts of both pathogens.

Thus, the objective of this work was to study the genetic diversity and adaptability of *Monosporascus* spp. and *Macrophomina* spp. isolated from weed roots, *T. portulacastrum* and *B. diffusa*, from areas of commercial plantations of cucurbitaceae, with a history of "vine decline" in the Brazilian Northeast.

REFERENCES

AGROFIT. **Sistema de Agrotóxicos Fitossanitários**. 2019. Disponível em: http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. Acesso em: 10 jan. 2019.

ANDRADE, D. E. G. T. et al. Frequência de fungos associados ao colapso do meloeiro e relação com características físicas, químicas e microbiológicas dos solos. **Summa Phytopathologica,** v. 31, n. 4, p. 327-333, 2005.

ALEANDRI, M. P.; et al. Involvement of *Olpidium bornovanus* and *O. virulentus* in the occurrence of melon root rot and vine decline caused by *Monosporascus cannonballus* in Central Italy. **Journal of Plant Pathology**, Bari, v. 99, n. 1, p. 169-176, 2017.

ANUÁRIO – **Anuário Brasileiro da Fruticultura 2017**. Santa Cruz do Sul: Editora Gazeta Santa Cruz, 2017. 88 p.

BAIRD, R. E.; et al. Relative longevity of *Macrophomina phaseolina* and associated mycobiota on residual soybean roots in soil. **Plant Disease**, Saint Paul, v. 87, n. 5, p. 563-566, 2003.

BEN SALEM, I.; et al. *Monosporascus eutypoides*, a cause of root rot and vine decline in Tunisia, and evidence that *M. cannonballus* and *M. eutypoides* are distinct species. **Plant Disease**, Saint Paul, v. 97, n. 6, p. 737-743, 2013.

BEN SALEM, I.; et al. Development of a screening test for resistance of cucurbits and Cucurbita hybrid rootstocks to *Monosporascus cannonballus*. **Tunisian Journal of Plant Protection**, Tunis, v. 10, n.1, p. 23-33, 2015.

CHAVES, A. L. R.; et al. *Erigon bonariensis*: hospedeira alternativa do lettuce mosaic vírus no Brasil. **Fitopatologia Brasileira,** Brasília, v. 28, n. 3, p. 307-311, 2003.

COHEN, R.; PIVONIA, S.; CROSBY, K. M.; MARTYN, R. D. Advances in the Biology and Management of Monosporascus Vine Decline and Wilt of Melons and Other Cucurbits. In: Janick, J. (Editor). **Horticultural reviews**: Wiley-Blackwell. New York, 2012. v. 39, p. 77-120.

COLLADO, J.; et al. *Monosporascus ibericus* sp. nov., an endophytic ascomycete from plants on saline soils, with observations on the position of the genus based on sequence analysis of the 18S rDNA. **Mycological Research**, Cambridge, v. 106, n. 1, p. 118-127, 2002.

EL-DESOUKY, S.M.; et al. Occurrence of *Monosporascus* root rot and vine decline of cantaloupe and watermelon in Egypt. **Egyptian Journal of Phytopathology**, Cairo, v. 31, n. 1-2, p. 141-150, 2003. Disponível em: http://www.ejp.eg.net/vol31_12_2003/vol31_12_2003_11.PDF. Acesso em: 25 abr. 2018.

FAO (Food and Agriculture Organization). **FAOSTAT - Food and agriculture data**. 2018. Disponível em: http://www.fao.org/faostat/en/#home. Acesso em: 23 dec. 2018.

FARR, D. F.; Rossman, A. Y. **Fungal Databases, United States National Fungus Collections, ARS, USDA**. 2018. Disponível em: https://nt.ars-grin.gov/fungaldatabases/. Acesso em: 14 dec. 2018.

FUHLBOHMA, M. J.; et al. Infection of mungbean seed by *Macrophomina phaseolina* is more likely to result from localized pod infection than from systemic plant infection. **Plant Pathology**, Dordrecht, v. 62, p. 1271–1284, 2013.

GARCÍA-JIMÉNEZ, J.; et al. Agentes asociados al "colapso" del melón en distintas zonas españolas. **Boletin Sanidad Vegetal Plagas**, Santiago, v. 19, n. 3, p. 401-423, 1993.

GAYTÁN-MASCORRO, A.; et al. First Report of *Monosporascus cannonballus* on Watermelon in Northern Mexico. **Plant Disease**, Saint Paul, v. 96, n. 7, p. 1068-1068, 2012.

GERIN, D.; et al. First Report of *Macrophomina phaseolina* Causing Crown and Root Rot on Strawberry in Italy. **Plant Disease**, Saint Paul, v. 102, n. 9, p. 1857, 2018.

HYDER, S.; et al. First Report of Charcoal Rot in Tomato Caused by *Macrophomina phaseolina* (Tassi) Goid. from Pakistan. **Plant Disease**, Saint Paul, v. 102, n.7, p. 1459, 2018.

IBGE - INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA. **SIDRA - Sistema IBGE de Recuperação Automática**. Rio de Janeiro, 2018. Disponível em: https://sidra.ibge.gov.br/tabela/5457. Acesso em: 17 dez. 2018.

LOUWS, F. J.; et al. Grafting fruiting vegetables to manage soilborne pathogens, foliar pathogens, arthropods and weeds. **Scientia Horticulturae**, Amsterdam, v. 127, n. 2, p. 127-146, 2010.

MACHADO, A. R.; et al. Bayesian analyses of five gene regions reveal a new phylogenetic species of *Macrophomina* associated with charcoal rot on oilseed crops in Brazil. **European Journal of Plant Pathology**, Dordrechtv, p. 1-12, 2018.

MARINHO, R. E. M.; et al. Identificação da micoflora associada a raízes de meloeiro nos estados do Rio Grande do Norte e Ceará. **Caatinga**, Mossoró, v. 15, n. 1-2, p. 25-28, 2002.

MALLOCH, D.; et al. New cleistothecial Sordariaceae and a new family, Coniochaetaceae. **Canadian Journal of Botany**, Otawa, v. 49, n. 6, p. 869-880, 1971.

MARKAKIS, E. A.; et al. First report of root rot and vine decline of melon caused *Monosporascus cannonballus* in Greece. **Plant Disease**, Saint Paul, v. 102, n. 5, p. 1036-1036 2018.

MARTYN, R. D.; et al. *Monosporascus* root rot and vine decline: an emerging disease of melons worldwide. **Plant Disease**, Saint Paul, v. 80, n. 7, p. 716-725, 1996.

MEDEIROS, E. V., et al. Quantificação de ascósporos de *Monosporascus cannonballus* em solos não cultivados de Caatinga e em áreas de cultivo de melão do Rio Grande do Norte e Ceará. **Fitopatologia Brasileira**, Brasília, v. 31, n. 5, p. 500-504, 2006.

MEENA, B. R; et al. First Report of Charcoal Rot Caused by *Macrophomina phaseolina* in *Basella alba* in India. **Plant Disease**, Saint Paul, v. 102, n. 8, p. 1669, 2018.

NISHAD, I.; et al. First Report of Root Rot of Nepeta cataria Caused by *Macrophomina phaseolina* in India. **Plant Disease**, Saint Paul, v. 102, n. 11, p. 2380, 2018.

PAPAVIZAS, G. C. Some factors affecting survival of sclerotia of *Macrophomina* in soil. **Soil Biology & Biochemistry**, Oxford, v.9, n. 5, p.337-341, 1977.

PATIL, S. D.; et al. Notes on some fungi of Pleosporaceae (Loculoascomycetes) from Maharashtra (India). **Transactions of the Mycological Society of Japan**, Kyoto, v. 28, p. 229-236, 1987.

PEREIRA, R. B.; et al. **Identificação e manejo das principais doenças fúngicas do meloeiro**. Circular Técnica. Brasília: Embrapa Hortaliças, 2012. 8p.

PETRAK, F.; et al. Beiträge zur Pilzflora Pakistans. **Sydowia**, Vienna, v. 8, n. 1-6, p. 162-185, 1954.

POLLACK, F. G.; et al. *Monosporascus cannonballus*, an unusual ascomycete in cantaloupe roots. **Mycologia**, Bronx, v. 66, n. 2, p. 346-349, 1974.

REGO, Suelen Santos; et al. Detection, transmission and pathogenicity of fungi on *Blepharocalyx salicifolius* (H.B.K.) Berg. seeds. **Revista brasileira de sementes**, Londrina, v. 34, n. 1, p. 9-13, 2012.

REIS, E. M.; et al. *Macrophomina phaseolina*: density and longevity of microsclerotia in soybean root tissues and free on the soil, and competitive saprophytic ability. **Summa phytopathologica**, Botucatu, v. 40, n. 2, p. 128-133, 2014.

RODRIGUES, A. P. M. dos S. **Ocorrência de plantas daninhas como hospedeiras de fitopatógenos radiculares e avaliação da patogenicidade sobre as culturas do melão e da melancia.** 2013. 76p. Dissertação (Mestrado em Agronomia: Fitotecnia) – Universidade Federal Rural do Semi-Árido, Mossoró, 2013. Disponível em: http://www2.ufersa.edu.br/portal/view/uploads/setores/82/Disserta%C3%A7%C3%A3o%20(2013)%20ANA%20PAULA%20MEDEIROS%20DOS%20SANTOS%20RODRIGUES.pdf. Acesso em: 30 out. 2018.

SALES JÚNIOR, R.; et al. First report of *Monosporascus cannonballus* on melon in Brazil. **Plant Disease**, Saint Paul, v. 88, n. 1, p. 84, 2004.

SALES JÚNIOR, R.; et al. First report of *Monosporascus cannonballus* on watermelon in Brazil. **Plant Disease**, Saint Paul, v. 94, n. 2, p. 278, 2010.

SALES JÚNIOR, R.; et al. Ervas daninhas como hospedeiras alternativas de patógenos causadores do colapso do meloeiro. **Revista Ciência Agronômica**, Fortaleza, v. 43, n. 1, p. 195-198, 2012.

SALES JÚNIOR, R.; NEGREIROS, A. M. P.; BELTRÁN, R.; ARMENGOL, J. Podridão de raízes por *Monosporascus* e declínio de ramas no meloeiro: grave problema sem solução. In:

LOPES, U. P., MICHEREFF, S. J. (Org.). **Desafios do manejo de doenças radiculares causadas por fungos**. Recife: EDUFRPE, 2018. p. 110-130.

SANTOS, A. A. et al. **Doenças do meloeiro em áreas irrigadas no estado do Ceará.** Fortaleza: Embrapa Agroindústria Tropical, 2000. 11p. (Boletim de Pesquisa, 35).

SARR, M.P.; et al. Genetic diversity in *Macrophomina phaseolina*, the causal agent of charcoal rot. **Phytopathologia Mediterranea**, Bologna, v. 53, n. 2, p. 163-173, 2014.

SIVANESAN, A. IMI Descriptions of Fungi and Bacteria no 1035 *Monosporascus cannonballus*. **Mycopathologia**, Netherlands, v. 114, n. 1, p. 53-54, 1991.

SHORT, G. E.; et al. Inoculum potential of *Macrophomina phaseolina*. **Phytopathology**, Saint Paul, v. 68, p. 742-746, 1978.

TONIN, R. F. B.; et al. In vitro mycelial sensitivity of *Macrophomina phaseolina* to fungicides. **Pesquisa Agropecuária Tropical**, Goiânia, v. 43, n. 4, p. 460-466, 2013.

YAN, L. Y.; et al. First report of root rot and vine decline of melon caused by *Monosporascus cannonballus* in Eastern Mainland China. **Plant Disease**, Saint Paul, v. 100, n. 3, p. 651, 2016.

ŽIVANOV, S. T.; et al. First report of charcoal rot on zebra plant (*Aphelandra squarrosa*) caused by *Macrophomina phaseolina*. **Plant Disease**, Saint Paul, v. 102, n. 11, p. 2377, 2018.

CHAPTER II

PREVALENT WEEDS COLLECTED FROM CUCURBIT FIELDS IN NORTHEASTERN BRAZIL REVEAL NEW SPECIES DIVERSITY IN THE GENUS *Monosporascus*

Abstract

Fungal species belonging to the genus *Monosporascus* have no known asexual morph and the ascocarp is a globose perithecium where asci develop, containing from 1 to 6 spherical ascospores, depending on the species. *Monosporascus cannonballus* is the most well-known species of the genus, and an important root pathogen associated with the vine decline of melon and watermelon crops worldwide. The aim of the present study was to characterize a collection of 35 Monosporascus-like isolates recovered from roots of two weed species prevalent in cucurbit growing fields in Northeastern Brazil: *Boerhavia diffusa* and *Trianthema portulacastrum*. These isolates were identified based on DNA sequences of the Internal Transcribed Spacer regions (ITS) of the nuclear rDNA, part of the translation elongation factor gene (*tef-1α*), part of the β-tubulin gene (*tub*), part of the nuclear small subunit rDNA (SSU), and part of the large subunit rDNA (LSU). Five *Monosporascus* species, namely *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus* and *M. semiaridus*, are newly described. *Monosporascus brasiliensis*, *M. nordestinus* and *M. semiaridus* were isolated from both weed species, while *M. caatinguensis* only from *T. portulacastrum* and *M. mossoroensis* only from *B. diffusa*. The present study confirmed that *Monosporascus* spp. can colonize roots of very diverse hosts, even without causing noticeable disease symptoms, and revealed that the diversity of species in the genus *Monosporascus* is potentially greater than previously expected.

KEYWORDS *Ascomycetes*, *Boerhavia diffusa*, *Monosporascus*, Soilborne pathogens, *Trianthema portulacastrum*.

1 INTRODUCTION

The genus *Monosporascus* Pollack & Uecker 1974, and the type species *M. cannonballus* Pollack & Uecker 1974, were described from a specimen obtained from necrotic melon roots in Arizona (USA) (Troutman & Matejka, 1970; Pollack & Uecker,

1974). To date, five species belonging to this genus have been reported worldwide: *M. adenantherae* (S. D. & C. Ramesh) A. Pande (Patil & Ramesh, 1987), *M. cannonballus* Pollack & Uecker (Pollack & Uecker, 1974), *M. eutypoides* (Petrak) von Arx (Petrak & Ahmad, 1954; Ben Salem *et al*., 2013), *M. ibericus* Collado, Ant. González, Stchigel, Guarro & Peláez (Collado *et al*., 2002), and *M. monosporus* (Malloch & Cain) D. Hawksw. & Ciccar (Malloch & Cain, 1971). However, *M. adenantherae* and *M. monosporus* do not have a reference isolate deposited in culture collections or gene sequences available on genetic databases.

Species belonging to the genus *Monosporascus* share some common features: they are homothallic, there is no asexual morph known and the ascocarp is a globose perithecium where asci develop, containing from 1 to 6 spherical, smooth, reticulate or slightly granulose, brown to black ascospores, depending on the species (Collado *et al*., 2002; Cohen *et al*., 2012). All *Monosporascus* species are soilborne and, in general, they seem to be adapted to hot, arid or semiarid climates, with saline and alkaline soils (Cohen *et al*., 2012).

Monosporascus cannonballus is the most well-known species of the genus, and an important root pathogen associated with the vine decline of melon (*Cucumis melo* L.) and watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai] crops worldwide (Martyn & Miller, 1996; Bruton, 1998; Cohen *et al*., 2012). To date, this pathogen has been reported in cucurbit growing areas of 22 countries (Cohen *et al*., 2012; Al-Mawaali *et al*., 2013; Yan *et al*., 2016, Markakis *et al*., 2018), as the causal agent of the disease named "Monosporascus root rot and vine decline" (MRRVD) (Martyn & Miller, 1996). In Brazil, *M. cannonballus* was reported in 2004 and 2010, affecting the roots of melon and watermelon plants, respectively (Sales Júnior *et al*., 2004; 2010), being the only *Monosporascus* species found in this country.

Currently, melon is the second most exported fresh fruit in Brazil, worth US $ 162.9 million (Anuário, 2018), with the main producing States being Rio Grande do Norte - RN (13,183 ha) and Ceará - CE (3,242 ha), which together represent 95.2 % of the melon produced by the country (IBGE, 2018). Brazil occupies the 11th position (596,430 t) among the world's largest producers of this cucurbit (FAO, 2018). Brazil is the fourth largest watermelon producer in the world, with a production of 2.090 million t in 94.555 ha (IBGE, 2018), with the main producing States being Rio Grande do Sul - RS (15,835 ha) and Bahia - Ba (14,209 ha). However, the watermelons produced in these States are marketed mainly internally (Anuário, 2018).

The production of melon and watermelon in the RN and CE States, located in Northeastern Brazil, is characterized by the use of high yield inputs such as hybrid seeds, high frequency irrigation and mulching, being the cultivation carried out in monoculture with two or more repeated cycles in the same land each growing season (Figuerêdo *et al.*, 2017). According to Bruton *et al*. (1998), these cultural practices may be associated with an increased incidence of MRRVD in cucurbits cultivation. Beltrán *et al*. (2005), studying the population dynamics of *M. cannonballus* ascospores in a field where the monoculture of melon was practiced, concluded that this practice increased the incidence of the disease in the field, as well as the number of ascospores in soil. In Brazil, Medeiros *et al*. (2006) detected the presence of *M. cannonballus* in areas of virgin forest of the Caatinga Biome in the Brazilian Northeast by counting ascospores in soil samples, confirming that this fungus is a natural inhabitant of the soil. It should be noted that these natural areas in the States of the RN and CE are the same that when deforested are used for cultivating melon and watermelon crops.

In addition to root pathogens, weeds also interfere with agricultural production, as they compete directly with the main crop for water, light and nutrients, as well as release allelopathic substances that inhibit plant development and serve as host of microorganisms (Soares *et al*., 2010; Sales Júnior *et al*., 2012; Lemessa & Wakjira, 2014). Recently, Rodrigues (2017) and Sales Júnior *et al*. (2019) evaluated the occurrence of weeds as alternative hosts of root phytopathogenic fungi in cucurbit production areas in the Brazilian states of RN and CE, reporting 13 weed species as hosts of fungal root pathogens associated with vine decline of melon and watermelon such as *Macrophomina phaseolina* (Tassi) Goid. and *Rhizoctonia solani* Kühn. Of these 13 species, two were reported as hosts of *M. cannonballus*: *Boerhavia diffusa* L. and *Trianthema portulacastrum* L. Consequently, additional extensive surveys of these weed species, prevalent in cucurbit growing fields in the RN and CE States in Brazil, were carried out, from which a collection of 35 Monosporascus-like isolates were obtained. Thus, the objective of this work was to determine the identity of these isolates by means of phenotypical characterization (morphology and temperature growth), and DNA sequence analyses of the Internal Transcribed Spacer regions (ITS) of the nuclear rDNA, part of the translation elongation factor gene (*tef-1α*), part of the β-tubulin gene (*tub*), part of the nuclear small subunit rDNA (SSU), and part of the large subunit rDNA (LSU).

2 MATERIALS AND METHODS

2.1 Sampling and isolation

Apparently healthy plants of *T. portulacastrum* and *B. diffusa* were collected from three cucurbits production farms, two located in RN State and one located in CE State (Northeastern Brazil). In each farm three different fields (2 ha each) were surveyed and approximately 25 plants of each species were collected in each field and examined carefully.

Roots were washed under running tap water, surface disinfested for 1 min in a 1.5% sodium hypochlorite solution and washed twice with sterile distilled water. Small pieces of slightly discolored tissues were placed onto potato dextrose agar (PDA) Petri dishes (Merck KGaA, Darmstadt, Germany) amended with 0.5 g L^{-1} of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA) (PDAS). Plates were incubated for 3 to 5 days at 25°C in darkness.

Thirty-five Monosporascus-like isolates, 18 from *T. portulacastrum* and 17 from *B. diffusa* (Table 1) were transferred to PDA, hyphal-tipped, and stored in 15% glycerol solution at -80ºC into 1.5 ml cryovials at the fungal collection of Phytopathogenic Fungi “Prof. Maria Menezes” (CMM) at the Universidade Federal Rural de Pernambuco (Recife, Pernambuco, Brazil).

Table 1 Collection details and GenBank accession numbers of isolates included in this study

Species	Strain number[a]	Host	Collected/isolated by/year	Location	Cordinates	GenBank Accession Numbers.				
						BT[b]	EF	ITS	LSU	SSU
Arecophila bambusae	HKUCC4794	-	-	-		-	-	-	AF452038	AY083802
Arthrinium hysterinum	ICMP 6889	-	-	-		-	-	-	DQ368630	DQ368662
Arthrinium phaeospermum	HKUCC 3395	-	-	-		-	-	-	AY083832	AY083816
Apiospora setosa	ICMP 4207	-	-	-		-	-	-	DQ368631	DQ368661
Diatrype palmicola	MFLUCC 11-0018	-	-	-		-	-	-	KP744481	KP753949
Diatrype palmicola	MFLUCC 11-0020	-	-	-		-	-	-	KP744482	KP753950
Eutypa lata	CBS 208.87	-	-	-		-	-	-	DQ836903	DQ836896
Monosporascus brasiliensis	CMM-4837	*Trianthema portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Assú	5º31'24,7''S 36º54'32''W	MG725315	MG720038	MG735232	MG748801	MG748760
M. brasiliensis	CMM-4838	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Assú	5º31'24,7''S 36º54'32''W	MG725316	MG720039	MG735233	MG748802	MG748761
M. brasiliensis	**CMM-4839**	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Assú	5º31'24,7''S 36º54'32''W	MG725317	MG720040	MG735234	MG748803	MG748762
M. brasiliensis	CMM-4840	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Assú	5º31'24,7''S 36º54'32''W	MG725318	MG720041	MG735235	MG748804	MG748763
M. brasiliensis	CMM-4841	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Assú	5º31'24,7''S 36º54'32''W	MG725319	MG720042	MG735236	MG748805	MG748764
M. brasiliensis	CMM-4842	*Boerhavia diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Assú	5º31'24,7''S 36º54'32''W	MG725320	MG720043	MG735237	MG748806	MG748765

Species	Strain number[a]	Host	Collected/isolated by/year	Location	Cordinates	GenBank Accession Numbers.				
						BT[b]	EF	ITS	LSU	SSU
M. brasiliensis	CMM-4843	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Assú	5º31'24,7''S 36º54'32''W	MG725321	MG720044	MG735238	MG748807	MG748766
M. brasiliensis	CMM-4844	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Assú	5º31'24,7''S 36º54'32''W	MG725322	MG720045	MG735239	MG748808	MG748767
M. brasiliensis	CMM-4845	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Assú	5º31'24,7''S 36º54'32''W	MG725323	MG720046	MG735240	MG748809	MG748768
M. caatinguensis	CMM-4832	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725310	MG720033	MG735227	MG748796	MG748755
M. caatinguensis	**CMM-4833**	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725311	MG720034	MG735228	MG748797	MG748756
M. caatinguensis	CMM-4834	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725312	MG720035	MG735229	MG748798	MG748757
M. caatinguensis	CMM-4835	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725313	MG720036	MG735230	MG748799	MG748758
M. caatinguensis	CMM-4836	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725314	MG720037	MG735231	MG748800	MG748759
M. cannonballus	CMM-2386	*Cucumis melo* L.	-	Brazil, Pau Branco, Rio Grande do Norte		JQ907303	JQ907318	JQ771917	MG748825	MG748784
M. cannonballus	CMM-2429	*C. melo* L.	-	Brazil, Rio Grande do Norte, Mossoró		JQ907311	JQ907315	JQ762366	MG748826	MG748785
M. cannonballus	MC0603	*C. melo* L.	-	Spain, Chilches, Castellón		JQ907307	JQ907314	JQ762364	MG748824	MG748783
M. cannonballus	MC1103	*C. melo* L.	-	Spain, Meliana, Valencia		JQ907302	JQ907317	JQ762369	MG748823	MG748782
M. eutypoides	**MT45**	*Citrullus lanatus* (Thunb.) Matsum. & Nakai	-	Tunisia, Sidi, Bouzid		JQ973834	JQ958959	JQ958963	MG748827	MG748786
M. ibericus	**CBS 110550**	-	-	Spain, Los Alfaques, Tarragona		JQ973833	JQ958958	JQ973832	MG748828	MG748787

Species	Strain number[a]	Host	Collected/isolated by/year	Location	Cordinates	GenBank Accession Numbers.				
						BT[b]	EF	ITS	LSU	SSU
M. mossoroensis	CMM-4856	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º54'2''S 37º24'17''W	MG725334	MG720057	MG735251	MG748820	MG748779
M. mossoroensis	**CMM-4857**	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º54'2''S 37º24'17''W	MG725335	MG720058	MG735252	MG748821	MG748780
M. mossoroensis	CMM-4858	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º54'2''S 37º24'17''W	MG725336	MG720059	MG735253	MG748822	MG748781
M. nordestinus	**CMM-4846**	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4''S 37º26'20,25''W	MG725324	MG720047	MG735241	MG748810	MG748769
M. nordestinus	CMM-4847	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4' S 37º26'20,25''W	MG725325	MG720048	MG735242	MG748811	MG748770
M. nordestinus	CMM-4848	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4''S 37º26'20,25''W	MG725326	MG720049	MG735243	MG748812	MG748771
M. nordestinus	CMM-4849	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4''S 37º26'20,25''W	MG725327	MG720050	MG735244	MG748813	MG748772
M. nordestinus	CMM-4850	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4''S 37º26'20,25''W	MG725328	MG720051	MG735245	MG748814	MG748773
M. nordestinus	CMM-4851	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4''S 37º26'20,25''W	MG725329	MG720052	MG735246	MG748815	MG748774
M. nordestinus	CMM-4852	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4''S 37º26'20,25''W	MG725330	MG720053	MG735247	MG748816	MG748775
M. nordestinus	CMM-4853	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4''S 37º26'20,25''W	MG725331	MG720054	MG735248	MG748817	MG748776
M. nordestinus	CMM-4854	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4''S 37º26'20,25''W	MG725332	MG720055	MG735249	MG748818	MG748777
M. nordestinus	CMM-4855	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º52'53,4''S 37º26'20,25''W	MG725333	MG720056	MG735250	MG748819	MG748778

Species	Strain number[a]	Host	Collected/isolated by/year	Location	Cordinates	GenBank Accession Numbers.				
						BT[b]	EF	ITS	LSU	SSU
M. semiaridus	CMM-4827	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725302	MG720025	MG735219	MG748788	MG748747
M. semiaridus	CMM-4828	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725303	MG720026	MG735220	MG748789	MG748748
M. semiaridus	CMM-4829	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725304	MG720027	MG735221	MG748790	MG748749
M. semiaridus	**CMM-4830**	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725305	MG720028	MG735222	MG748791	MG748750
M. semiaridus	CMM-4831	*T. portulacastrum* L.	R. Sales Junior, 2014	Brazil, Ceará, Limoeiro do Norte	5º11'06,11''S 37º55'2,2''W	MG725306	MG720029	MG735223	MG748792	MG748751
M. semiaridus	CMM-4859	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º54'2''S 37º24'17''W	MG725307	MG720030	MG735224	MG748793	MG748752
M. semiaridus	CMM-4860	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º54'2''S 37º24'17''W	MG725308	MG720031	MG735225	MG748794	MG748753
M. semiaridus	CMM-4861	*B. diffusa* L.	R. Sales Junior, 2014	Brazil, Rio Grande do Norte, Mossoró	4º54'2''S 37º24'17''W	MG725309	MG720032	MG735226	MG748795	MG748754
Seynesia erumpens	SMH 1291	-	-	-		-	-	-	AF279410	AF279409

[a] CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CMM the Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes"of the Universidade Federal Rural de Pernambuco (Recife, Brazil); ICMP: International Collection of Microorganisms from Plants, Auckland, New Zealand; HKUCC: Ecology & Biodiversity, University of Hong Kong, Pokfulam Road, Hong Kong SAR, People's Republic of China; MC and MT Culture collection of the Instituto Agroforestal Mediterràneo, Universitat Politècnica de València, Valencia, Spain; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; SMH: Sabine M. Huhndorf, Dept. of Botany, The Field Museum of Natural History, Chicago, USA. Ex-type culture indicated in bold.

[b] BT (*β-tubulin* gene), EF (*elongation factor* gene/*tef-1α*), ITS (Internal Transcribed Spacer regions of the nuclear rDNA), LSU (part of the large subunit rDNA, and SSU (part of the nuclear small subunit rDNA).

2.2 DNA extraction, PCR amplification and sequencing

Total fungal DNA was extracted using the E.Z.N.A. Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's short protocol instructions with some modifications in the samples preparation step. Briefly, lysis buffer P1 (650 µl) was added to the mycelia in a 2-ml screw-capped conical tubes (Thermo Scientific, San Diego, CA, USA) containing four metal 2.38 mm beads (Qiagen, Hilden, Germany) and two tungsten carbide 3 mm beads (Qiagen) and homogenized twice at 5 m s^{-1} for 20 s using FastPrep-24™5G (MP Biomedicals, Santa Ana, CA, USA).

Five loci were amplified and sequenced: the Internal Transcribed Spacer regions (ITS) of the nuclear rDNA amplified with the primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990), part of the translation elongation factor gene (*tef-1α*) using primers EF1-688F (5'-CGGTCACTTGATCTACAAGTGC-3') and EF1-1251R (5'-CCTCGAACTCACCAGTACCG-3') (Alves *et al*., 2008), part of the β-tubulin gene (*tub*) using primers BtCadF (5'-MATGCGTGAAATYGTAAGT-3') and BtCadR (5'-TCAGCACCCTCAGTGTAATG-3') (Travadon *et al*., 2015), part of the nuclear small subunit rDNA (SSU) using primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') (White *et al*., 1990), and part of the large subunit rDNA (LSU) using primers LROR (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') (Vilgalys & Hester, 1990).

Amplification by polymerase chain reaction (PCR) was performed using Horse-Power™ Taq DNA Polymerase (Canvax Biotech SL, Córdoba, Spain), according to the manufacturer's instructions on a Peltier Thermal Cycler-200 (MJ Research, Wartertown, MA, USA). The thermal cycle consisted of an initial step of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 45 s. A final extension was performed at 72°C for 10 min. PCR products were analyzed by 1% agarose gel electrophoresis and were sequenced by Macrogen Inc. (Madrid, Spain) using both PCR primers. Each consensus sequence was assembled using Sequencher software 5.0 (Gene Codes Corp., Ann Arbor, Michigan).

New sequences were deposited in GenBank and were listed in Table 1 with additional sequences of *M. cannonballus* (CMM2386, CMM2429, MC0603 and MC1103), *M. eutypoides* (MT45), *M. ibericus* (CBS 110550), *Arecophila* (*A.*) *bambusae* (HKUCC4794), *Seynesia erumpens* (SMH 1291), *Arthrinium* (*Ar.*) *hysterinum* (ICMP 6889), *Ar.*

phaeospermum (HKUCC 3395), *Apiospora (Ap.) setosa* (ICMP 4207), *Diatrype palmicola* (MFLUCC 11-0018 and MFLUCC 11-0020) and *Eutypa lata* (CBS 208.87) obtained from GenBank. The alignments were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S22884).

2.3 Phylogenetic analyses

For each of the five loci (LSU, SSU, ITS, *tef1-α* and *tub*), the DNA sequences from this study, together with those retrieved from Genbank (Table 1) were aligned using the ClustalW algorithm (Thompson *et al*., 1994) contained within MEGA7 software package (Kumar *et al*., 2016). The alignments were inspected and corrected manually. Incomplete portions at either end of the alignments were excluded prior to analyses.

The Genealogical Concordance Phylogenetic Species Recognition concept (GCPSR, Taylor *et al*., 2000) was the approach used to identify phylogenetic species based on the existence of statistically supported phylogenetic clades that are present in the majority (at least two of three) of single-locus trees and that are not contradicted by any other single-gene tree(s) determined by the same method.

To determine whether the DNA sequence datasets were congruent, a partition homogeneity test (Farris *et al*., 1994) of all possible combinations was conducted in PAUP 4.0b10 (Swofford, 2003). Two concatenated datasets were built in Sequence Matrix v.1.8 (Vaidya *et al*., 2011). First dataset, LSU/SSU matrix, was used to infer the position and assess the phylogenetic relationships of the genus *Monosporascus* inside the family *Diatrypaceae* and the order *Xylariales*, and to test the monophyly of the genus. For this purpose, some representative species of these family and order were selected. *A. bambusae* (HKUCC4794), *S. erumpens* (SMH 1291), *Ar. hysterinum* (ICMP 6889), *Ar. phaeospermum* (HKUCC 3395), *Ap. setosa* (ICMP 4207) were chosen as outgroups based on Maharachchikumbura *et al*. (2016). Second dataset, ITS/*tef1-α*/*tub* matrix, was used to infer the relative position of species inside the *Monosporascus* genus. In this analysis no outgroup was inserted and the trees generated were midpoint rooted.

Phylogenetic analyses for each locus and concatenated datasets were based on Bayesian inference (BI), Maximum Likelihood (ML) and Maximum Parsimony (MP). Bayesian analyses were performed using MrBayes v 3.2 (Ronquist *et al*., 2012) on the CIPRES Science Gateway V 3.3 (Miller *et al*., 2010). The best-fitting model of nucleotide evolution for each partition was determined by MrModeltest 2.3 (Nylander, 2004) using the

Akaike Information Criterion (AIC). Four simultaneous analysis were run for 100 millions generations, sampling every 1000, with four Markov Chain Monte Carlo (MCMC) chains. The first 25% of saved trees were discarded and posterior probabilities determined from the remaining trees. The ML analyses were done with the tool RAxML - HPC2 on XSEDE (Stamatakis, 2014) implemented on CIPRES Science Gateway V 3.3 (Miller *et al*., 2010). ML tree searches were performed under the GTRGAMMA model with 1000 pseudoreplicates. The other parameters were used as default settings. Phylogenetic analyses consisting of MP were performed in MEGA 7 (Kumar *et al*., 2016) with the Subtree-Pruning-Regrafting (SPR) algorithm, where gaps were treated as missing data. The robustness of the topology was evaluated by 1000 bootstrap replications (Felsenstein, 1985). Measures for the maximum parsimony as tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were also calculate.

Monosporascus spp. are homothallic ascomycetes, thus all progeny from an ascocarp will be genetically identical because they are derived from a single haploid genome, meiosis does not change the multilocus genotype (Kohn, 1995). Nevertheless, for determine the recombination level within phylogenetically closely related species using a five-locus concatenated dataset, a Pairwise homoplasy index (PHI) test (Philippe & Bryant 2006) was performed in SplitsTree4 (Huson & Bryant 2006) (http://ab.inf.uni-tuebingen.de/software/splitstree4/).

2.4 Taxonomy

Agar plugs (6-mm-diam) were taken from the edge of active PDA cultures and transferred onto the centre of 9-cm-diam Petri dishes containing the following culture media: PDA; 2% tap water agar supplemented with sterile melon (*C. melo*) root fragments; potato carrot agar (PCA) (grated potato 20 g, grated carrot 20 g, agar 20 g and tap water 1 l); sugar beet agar (grated sugar beet 25 g, agar 20 g and tap water 1 l); and V-8 juice agar (V-8 juice 200 ml, $CaCO_3$ 2 g, agar 15 g and distilled water 800 ml). Plates were then incubated during two months at 25 and 30ºC in darkness to induce sporulation. Cultures were examined periodically for the development of ascomata and ascospores. Colony colours and pigment production were rated only on PDA after 30 days of incubation according to Rayner (1970). Morphological characteristics were examined by mounting single perithecia in 100% lactic acid v/v and observed using a Zeiss Axio Scope A.1 microscope. The diameter of 50 perithecia and 50 ascospores, and the length and width of 25 asci per isolate were measured

using the imaging device Zeiss AxioVision LE. Photos were captured using a Zeiss AxioCam MRm digital camera from images recorded with the 40x objective. Descriptions, nomenclature and illustrations of taxonomic novelties were deposited in MycoBank (MB826726, MB826728, MB826729, MB826730 and MB826731) (Pollack & Uecker, 1974, Ben Salem et al., 2013, Collado et al., 2002, Crous *et al*., 2004).

The effect of temperature on mycelial growth of selected isolates was measured on PDA. For this purpose, agar plugs (6-mm-diam) obtained from the growing edge of colonies were transferred to the center of PDA plates which were incubated at 10, 15, 20, 25, 30, 35 or 40ºC in darkness. Five replicates for each isolate and temperature combination were used. The diameter of each colony was measured perpendicularly in two directions when the colony reached at least two thirds of the plate diameter, and the mean growth rate was calculated in mm/day. Analyses of variance (ANOVA) were conducted with temperature experiments data to analyse potential trial-by-treatment interactions. ANOVA indicated that the data for the two repetitions were similar for each variable ($P > 0.05$), thus data from both repeats of the experiments were combined. For each isolate, average growth rates at each temperature were adjusted to a regression curve using Statgraphics Plus 5.1 (Manugistics Inc., Rockville, MD), and the best polynomial model was chosen based on parameter significance ($P < 0.05$) and coefficient of determination (R^2) to estimate the optimum growth temperature.

3 RESULTS

3.1 Sequence alignment and phylogenetic analysis

The first approximation to the identification of the 35 isolates, putatively belonging to *Monosporascus* genus, was based on the BLAST analysis of their ITS sequence, showing the highest identities between 92-96% with some accessions of *Monosporascus* species. Subsequently, *Monosporascus* sequence matrices (LSU, SSU, ITS, *tef-1α,* and *tub*) were built. The combined datasets of LSU/SSU and ITS/*tef-1α*/*tub* were used to infer the phylogenetic relationships among known and new *Monosporascus* species.

The results of the partition homogeneity test ($P > 0.05$) for all possible combination of the two (LSU/SSU) and three (ITS/*tef-1α*/*tub*) loci indicated that the datasets were congruent. Phylogenies resulting from the individual locus (Figures S1-S15/Attachment) also were compared visually, and no differences could be detected for the LSU/SSU and ITS/*tef-1α*/*tub*, and therefore the sequences of these two and three regions were combined, respectively. In

ITS, *tef-1α* and *tub* datasets, the terminal clades representing species were the same for all gene regions, supporting the congruency of the different phylogenies. The topology of the trees identified by ML analysis of both concatenated datasets were identical to those obtained by the BI and MP analyses (Figures S16-S19/Attachment), therefore only the ML trees are presented with ML and MP bootstrap support values and BI posterior probability scores at the nodes.

3.2 *Monosporascus* within the family Diatrypaceae

The combined alignment of LSU and SSU used for ML, BI and MP analyses contained 49 taxa, including outgroups, and 1635 base pairs in length (681 base pairs for LSU and 954 for SSU). Sequences of ex-type isolates of *M. cannonballus*, *M. eutypoides*, *M. ibericus*, *A. bambusae*, *S. erumpens*, *Ar. hysterinum*, *Ar. phaeospermum*, *Ap. setosa*, *D. palmicola* and *E. lata* were obtained from GenBank and included in the analysis together with the sequences of isolates generated in this study (Table 1).

Maximum likelihood analysis resulted in a single best ML tree with –lnL = -3779.31365. For the MP analysis 1464 characters were constant, 120 parsimony-informative and 51 were variable and parsimony-uninformative, yielding 10 equally most parsimonious trees (TL = 255; CI = 0.760; RI = 0.897; RC = 0.681). In the BI analysis, the LSU partition had 107 unique site patterns and the SSU partition had 66. The analysis read a total of 40,004 trees, sampling 30,004 of them.

The phylogenies inferred from individual genes (Figures S7-S12/Attachment) and the two-loci phylogeny (Figure 1) showed that our isolates belong to the genus *Monosporascus* with the genera *Diatrype* and *Eutypa* as sister groups inside the family Diatrypaceae belonging to the order Xylariales. The genus *Monosporascus* appeared as a well supported monophyletic clade that is divided into two sub-clades: one includes *M. eutypoides*, *M. cannonballus* and four new *Monosporascus* species (*M. mossoroensis*, *M. nordestinus*, *M. semiaridus* and *M. brasiliensis*), and the other contains *M. ibericus* and another new *Monosporascus* species (*M. caatinguensis*). Pairwise sequence percentage identity among *Monosporascus* species at the LSU and SSU regions is shown in Table 2.

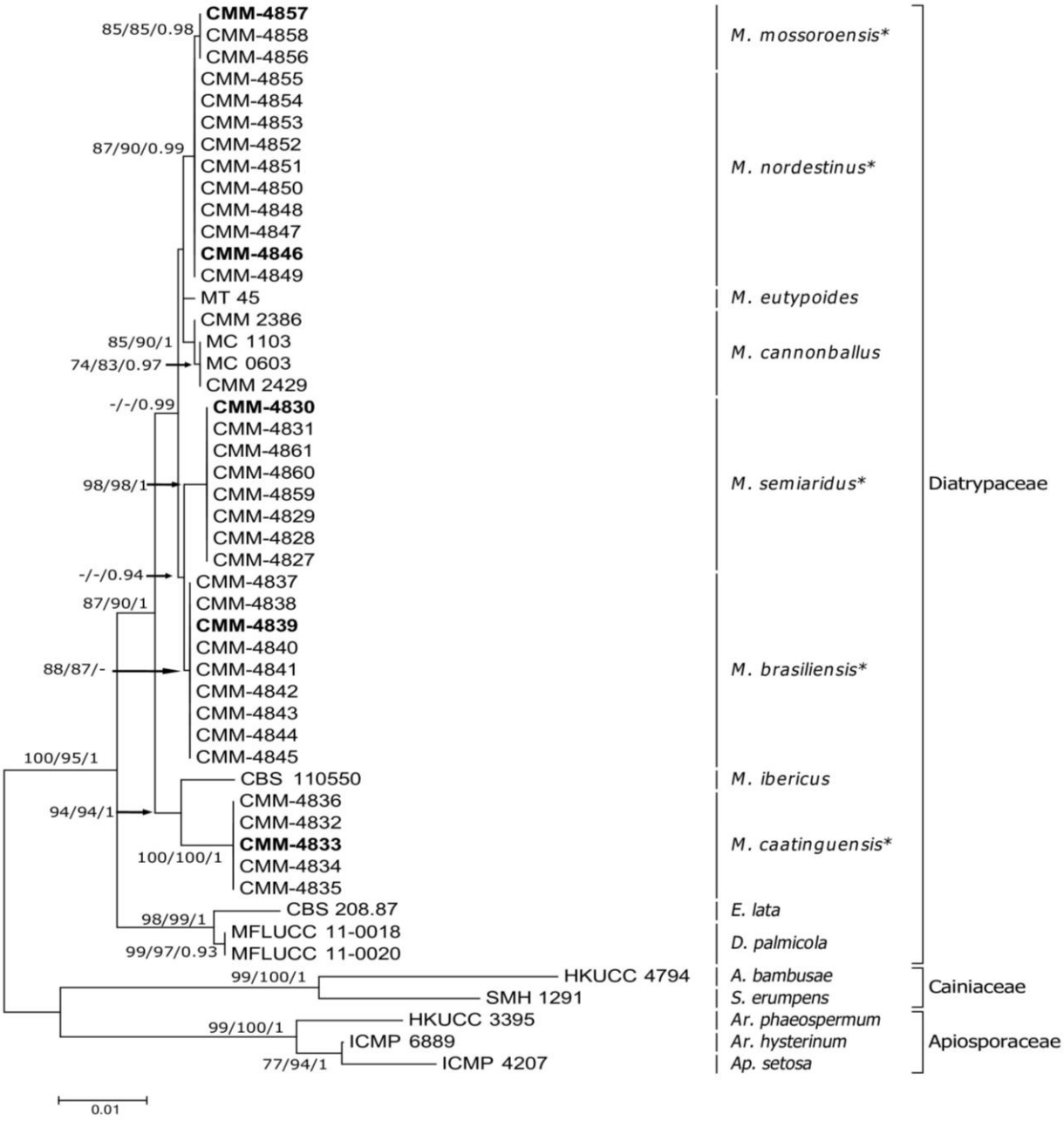

Figure 1 Maximum likelihood phylogeny inferred from the combined LSU and SSU sequence alignments used to infer the phylogenetic relationships of the genus *Monosporascus* inside the family Diatrypaceae and the order Xylariales. Support values (ML bootstrap / MP bootstrap / BI posterior probabilities) are given at the nodes. Bootstrap values less than 70% or posterior probabilities less than 0.9 are indicated with "-". The tree was rooted using *Arecophila bambusae* (HKUCC4794), *Seynesia erumpens* (SMH 1291), *Arthrinium hysterinum* (ICMP 6889), *Ar. phaeospermum* (HKUCC 3395) and *Apiospora setosa* (ICMP 4207) as outgroup sequences. Ex-type strains are indicated in bold. Scale bar shows expected changes per site. New species are indicated with an asterisk.

Table 2 Pairwise sequence percentage identity among *Monosporascus* species at the ITS, *tef1-α*, *tub*, LSU and SSU regions.

M. caatinguensis	80.7	83.0	89.6	67.6	99.5																											
M. cannonballus	93.6	93.5	93.0	99.4	99.8	81.7	84.0	61.4	67.4	99.5																						
M. eutypoides	91.4	93.2	92.8	99.4	99.9	78.6	84.4	88.6	97.1	99.6	94.1	96.6	97.3	99.4	99.9																	
M. ibericus	84.2	81.9	89.5	98.1	99.4	87.3	86.9	91.8	97.8	99.5	85.8	82.0	86.8	97.8	99.4	82.9	82.6	87.4	97.9	99.5												
M. mossoroensis	92.1	92.3	93.2	99.4	99.9	79.9	83.3	88.3	97.4	99.6	94.9	98.1	98.4	99.3	99.9	96.1	96.6	97.8	99.3	100	84.4	81.5	87.1	97.4	99.5							
M. nordestinus	91.9	92.6	93.0	99.3	99.9	80.0	83.8	88.1	97.1	99.6	95.0	97.3	98.7	99.4	99.9	96.3	96.5	97.8	99.4	100	84.2	81.7	87.3	97.6	99.5	98.4	98.9	99.4	99.9	100		
M. semiaridus	90.5	91.0	93.4	99.1	99.9	78.7	82.1	87.5	97.5	99.6	90.3	90.6	90.9	98.5	99.9	91.5	91.4	90.9	98.5	100	82.3	81.2	87.5	97.6	99.5	92.2	90.6	91.1	98.8	100	92.3	91.1	90.8	98.7	100
	ITS	tef	tub	LSU	SSU	ITS	tef	tub	LSU	SSU	ITS	tef	tub	LSU	SSU	ITS	tef	tub	LSU	SSU	ITS	tef	tub	LSU	SSU	ITS	tef	tub	LSU	SSU	ITS	tef	tub	LSU	SSU
	M. brasiliensis					*M. caatinguensis*					*M. cannonballus*					*M. eutypoides*					*M. ibericus*					*M. mossoroensis*					*M. nordestinus*				

3.3 Phylogenetic relationships within the genus *Monosporascus*

The three-loci (ITS/*tef-1α*/*tub*) dataset included 41 sequences (Table 1) from which 35 were of our studied isolates and six of the three *Monosporascus* species with sequences and cultures available: *M. cannonballus* (n = 4), *M. eutypoides* (n = 1) and *M. ibericus* (n = 1) (Fig. 2). The alignment, including gaps, consisted of 1864 characters (519 bp for ITS, 647 for *tef-1α* and 686 for *tub*), of which 1460 were constant, 320 parsimony-informative, and 84 variables and parsimony-uninformative. Parsimony analysis yield 10 most parsimonious trees (TL = 489; CI = 0.878; RI = 0.981 and RC = 0.861). The ML analysis resulted in a single best tree with –lnL = -5107.97577. In the BI analysis, the ITS/*tef-1α*/*tub* partitions had 103/129/98 unique site patterns respectively, and the analysis read a total of 40,004 trees, sampling 30,004 of them.

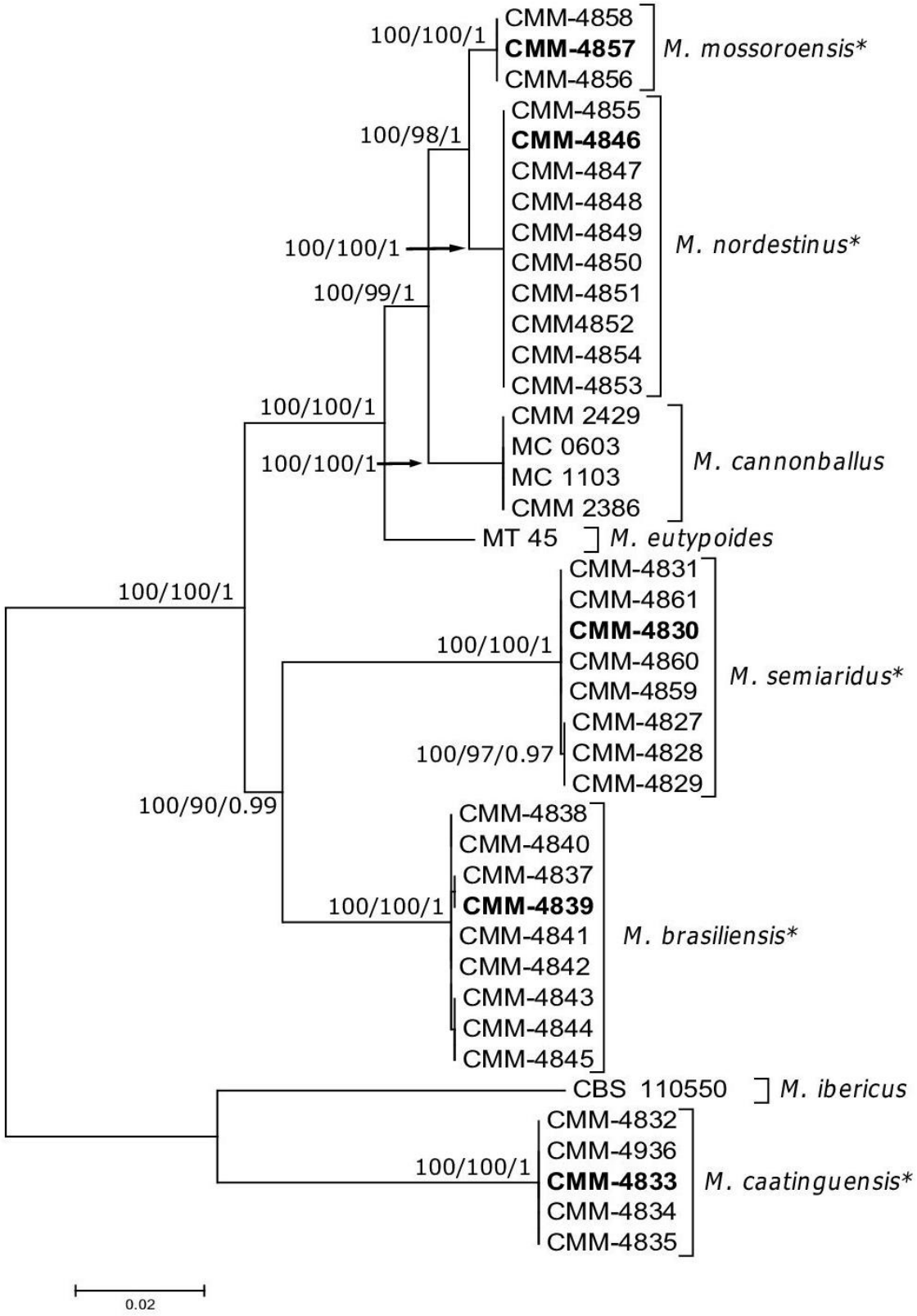

Figure 2 Maximum likelihood phylogeny inferred from the combined ITS, *tef1-α* and *tub* sequence alignments used to infer the relative position of species inside the *Monosporascus* genus. Support values (ML bootstrap / MP bootstrap / BI posterior probabilities) are given at the nodes. The tree was midpoint rooted. Ex-type strains are indicated in bold. Scale bar shows expected changes per site. New species are indicated with an asterisk.

The phylogenetic analysis resolved the dataset into eight clades. Three of them corresponded to previously described *Monosporascus* species, but none of our isolates clustered with them. The other five clades, with 100% bootstrap support for MP and ML and 1 of BI posterior probability, corresponded to the new species of *Monosporascus* (*M. mossoroensis*, *M. nordestinus*, *M. semiaridus*, *M. brasiliensis* and *M. caatinguensis*). These eight clades maintained the same relationship between them presented in LSU-SSU phylogeny.

The *M. mossoroensis* clade, formed by 3 isolates, and the *M. nordestinus* clade, represented by ten isolates, are both phylogeneticaly close to *M. cannonballus* and *M. eutypoides* (Fig. 2). The *M. semiaridus* clade with 8 isolates and the *M. brasiliensis* clade, with 9 isolates, formed a group closely related between them. The *M. caatinguensis* clade, with 5 isolates, is closely related to *M. ibericus*.

The isolates of the *M. semiaridus* clade were divided in two sub-clades by one base transition in ITS region sequences. Moreover, the alignments of *tef-1α* and *tub* of the *M. brasiliensis* clade showed the presence of intraspecific variabilities with four indels and one transition, respectively, resulting also in two sub-clades.

Pairwise sequence percentage identity among *Monosporascus* species at the ITS, *tef-1α* and *tub* regions is shown in Table 2.

The PHI test revealed that there was no significant genetic recombination within this dataset (mean = 0.051, $P = 0.259$).

3.4 Taxonomy

Five new species of *Monosporascus* are described based on the phylogenetic analysis and morphological characters (Fig. 3, Fig. 4, Fig. 5 and Table 3).

Monosporascus brasiliensis A. Negreiros, M. León, J. Armengol & R. Sales Júnior, **sp. nov.** MycoBank MB 826726 (Fig. 3A).

Etymology: Name refers to Brazil, where the fungus was isolated.

Diagnosis: Cultures sterile. One hundred and sixty-three polymorphisms can distinguish *M. brasiliensis* from its closest phylogenetic species *M. semiaridus*: 51 (31 indels) in ITS locus; 60 (14 indels) in *tef-1α* locus; 45 (4 indels) in *tub* locus; 6 (2 indels) in LSU locus; and 1 in SSU locus.

Typus: Brazil: Assú, Rio Grande do Norte on *Trianthema portulacastrum* (complete roots), 2014, R. Sales Júnior (holotype; CMM 4839 – ex-type culture).

Culture characteristics: colonies on PDA showed mycelium cottony with average density (Fig. 3). Surface buff without zonation and reverse ochreous to amber. Optimum growth temperature 32.1ºC (Table 3). Growth rate of colonies on PDA at 30 and 35ºC was 8.9 and 9.6 mm per day, respectively. No growth was observed at 10 and 45ºC.

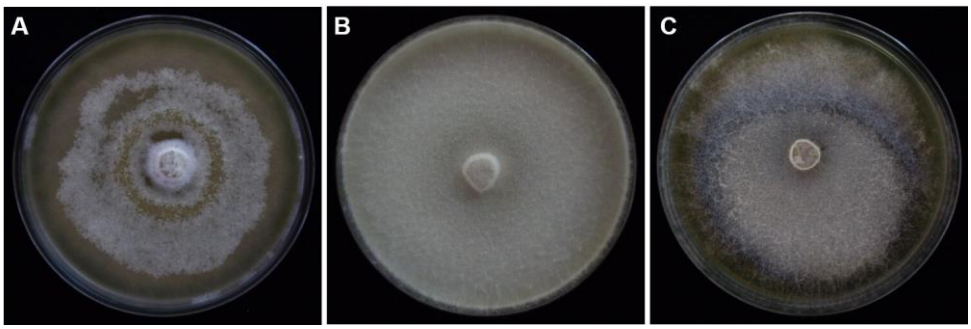

Figure 3 Upper face of 30-days-old colonies of *Monosporascus* spp. grown on PDA culture medium at 25ºC in darkness: A) *M. brasiliensis* CMM 4839; B) *M. caatinguensis* CMM 4833 and C) *M. mossoroensis* CMM 4857.

Table 3 Temperature growth study of *Monosporascus* isolates.

Species name / strain number	Cardinal temperatures for growth (°C)		
	Minimum	Maximum	Optimum
Monosporascus brasiliensis			
CMM 4839	10	45	32.1
CMM 4843	10	45	31.7
Monosporascus caatinguensis			
CMM 4833	10	45	30.7
CMM 4835	10	45	31.2
Monosporascus mossoroensis			
CMM 4857	10	45	31.8
CMM 4858	10	45	31.1
Monosporascus nordestinus			
CMM 4846	10	45	32.4
CMM 4847	10	45	32.1
Monosporascus semiaridus			
CMM 4830	10	45	31.3
CMM 4859	10	45	32.9

Host and distribution: *Boerhavia diffusa* and *Trianthema portulacastrum* (roots) (Brazil, Rio Grande do Norte).

Notes: Isolates of *M. brasiliensis* could not be induced to sporulate on any of the media used in this study, nor on sterilized fragments of melon roots placed on tap water agar,

even after repeated attempts. *Monosporascus brasiliensis* is closely related to *M. semiaridus* based on phylogenetic inference.

Monosporascus caatinguensis A. Negreiros, M. León, J. Armengol & R. Sales Júnior, sp. nov. MycoBank MB 826728 (Fig. 3B).

Etymology: Name refers to Caatinga Biome, where the fungus was isolated. Deforested Caatinga areas are used for intensive cucurbits cultivation.

Diagnosis: Cultures sterile. Two hundred and twenty four polymorphisms can distinguish *M. caatinguensis* from its closest phylogenetic species *M. ibericus*: 60 (29 indels) in ITS locus; 88 (16 indels) in *tef-1α* locus; 59 (13 indels) in *tub* locus; 12 (1 indels) in LSU locus; and 5 in SSU locus.

Typus: Brazil: Limoeiro do Norte, Ceará on *Boerhavia diffusa* (complete roots), 2014, R. Sales Júnior (holotype; CMM 4833 – ex-type culture).

Culture characteristics: colonies on PDA showed mycelium flat with low density (Fig. 3). Surface honey and reverse amber. Optimum growth temperature 30.7ºC (Table 3). Growth rate of colonies on PDA at 30 and 35ºC was 5.3 and 4.5 mm per day, respectively. No growth was observed at 10 and 45°C.

Host and distribution: *Boerhavia diffusa* (roots) (Brazil, Ceará).

Notes: Isolates of *M. caatinguensis* could not be induced to sporulate on any of the media used in this study, nor on sterilized fragments of melon roots placed on tap water agar, even after repeated attempts. *Monosporascus caatinguensis* is closely related to *M. ibericus* based on phylogenetic inference.

Monosporascus mossoroensis A. Negreiros, M. León, J. Armengol & R. Sales Júnior, sp. nov. MycoBank MB 826729 (Fig. 3C).

Etymology: Name refers to Mossoró locality in Rio Grande do Norte State, where the fungus was isolated.

Diagnosis: Cultures sterile. Twenty polymorphisms can distinguish *M. mossoroensis* from its closest phylogenetic species *M. nordestinus*: 8 (2 indels) in ITS locus; 7 in *tef-1α* locus; 4 in *tub* locus; and 1 in LSU locus.

Typus: Brazil: Mossoró, Rio Grande do Norte on *Trianthema portulacastrum* (complete roots), 2014, R. Sales Júnior (holotype; CMM 4857 – ex-type culture).

Culture characteristics: colonies on PDA showed mycelium cottony with low density (Fig. 3). Surface honey and reverse honey to umber. Optimum growth temperature 31.8ºC

(Table 3). Growth rate of colonies on PDA at 30 and 35ºC was 8.6 and 7.4 mm per day, respectively. No growth was observed at 10 and 45ºC.

Host and distribution: *Trianthema portulacastrum* (roots) (Brazil, Rio Grande do Norte).

Notes: Isolates of *M. mossoroensis* could not be induced to sporulate on any of the media used in this study, nor on sterilized fragments of melon roots placed on tap water agar, even after repeated attempts. *Monosporascus mossoroensis* is closely related to *M. nordestinus* based on phylogenetic inference.

Monosporascus nordestinus A. Negreiros, M. León, J. Armengol & R. Sales Júnior, sp. nov. MycoBank MB 826730 (Fig. 4).

Etymology: Name refers to the Brazilian Northeast Region, where the fungus was isolated.

Diagnosis: Asexual morph not seen. Twenty polymorphisms can distinguish *M. nordestinus* from its closest phylogenetic species *M. mossoroensis*: 8 (2 indels) in ITS locus; 7 in *tef-1α* locus; 4 in *tub* locus; and 1 in LSU locus.

Typus: Brazil: Mossoró, Rio Grande do Norte on *Trianthema portulacastrum* (complete roots), 2014, R. Sales Júnior (holotype; CMM 4846 – ex-type culture).

Ascomata superficial to semi-immersed, scattered, globose to hemi-spherical, non-ostiolate, dark brown, (452-) 549 (-668) µm diam. Asci 1- to 3-spored, fasciculate, clavate to subcylindrical, thick-walled, stipitate, rounded at the apex and evanescent: one-spored (76.0-) 85.0 (-114.9) × (40.5-) 46.0 (-52.1) µm, two-spored (95.4-) 100.4 (-137.4) × (39.4-) 45.9 (-50.8) µm, and three-spored (105.2-) 120.4 (-150.4) × (39.0-) 42.5 (-44.7) µm diam. Ascospores one-celled, globose, thick-walled, hyaline when young, becoming dark brown to black when mature, smooth, (35.6-) 42.9 (-48.6) µm diam, without germ pores. Paraphyses numerous, filiform, hyaline. Asexual morph unknown.

Culture characteristics: colonies on PDA showed mycelium cottony with density average to strong (Fig. 4). Surface buff without zonation and reverse luteous to sienna. Optimum growth temperature 32.4ºC (Table 3). Growth rate of colonies on PDA at 30 and 35ºC were 9.2 and 9.6 mm per day, respectively. No growth was observed at 10 and 45ºC. Ascomata were produced on PDA, PCA, sugar beet agar and V-8 juice agar. Ascospore germination was not observed on any of the culture media used and at any of the incubation temperatures tested.

Host and distribution: *Boerhavia diffusa* and *Trianthema portulacastrum* (roots) (Brazil, Rio Grande do Norte).

Notes: *M. nordestinus* is closely related to *M. mossoroensis* based on phylogenetic inference. The morphology of this species is close to *M. eutypoides*, which also presents 1 to 3 ascospores per ascus, but *M. nordestinus* can be distinguished by its higher optimum growth rate temperature, 32.4ºC (this study), when compared to *M. eutypoides*: 29.38 to 29.49ºC (Ben Salem et al., 2013).

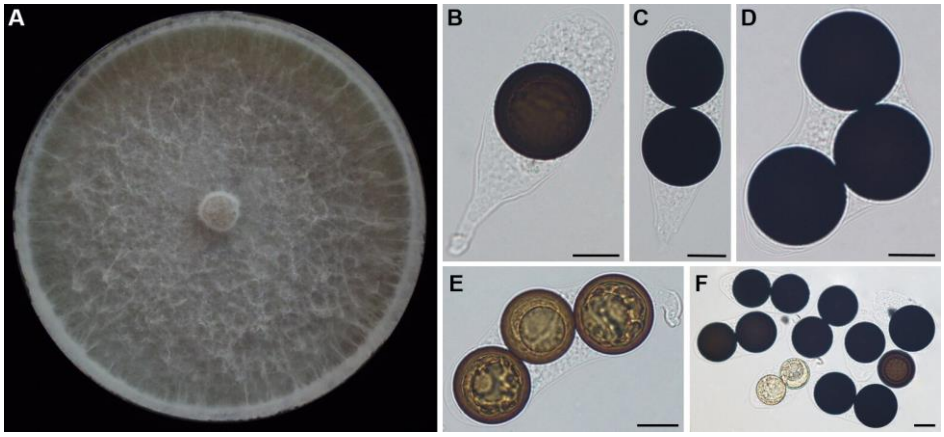

Figure 4 *Monosporascus nordestinus* CMM4846: A) Upper face of a 30-days-old colony grown on PDA culture medium at 25ºC in darkness; B-D) Asci containing 1 (B), 2 (C) and 3 (D) mature ascospores; E) Ascus with 3 inmature ascospores; F) General view of asci and ascospores. Scale bars: B-F = 20 µm.

Monosporascus semiaridus A. Negreiros, M. León, J. Armengol & R. Sales Júnior, sp. nov. MycoBank MB 826731 (Fig. 5).

Etymology: Name refers to the semiarid Brazilian region, where the fungus was isolated.

Diagnosis: Asexual morph not seen. One hundred and sixty-three polymorphisms can distinguish *M. semiaridus* from its closest phylogenetic species *M. brasiliensis*: 51 (31 indels) in ITS locus; 60 (14 indels) in *tef-1α* locus; 45 (4 indels) in *tub* locus; 6 (2 indels) in LSU locus; and 1 in SSU locus.

Typus: Brazil: Limoeiro do Norte, Ceará on *Trianthema portulacastrum* (complete roots), 2014, R. Sales Júnior (holotype; CMM 4830 – ex-type culture).

Ascomata superficial to semi-immersed, scattered, globose to hemi-spherical, non-ostiolate, dark brown, (426-) 546 (-724) µm diam. Asci 1-spored, fasciculate, clavate to subcylindrical, thick-walled, stipitate, rounded at the apex and evanescent, (50.2-) 67.1 (-77.0) × (32.4-) 43.7 (-44.5) µm diam. Ascospores one-celled, globose, thick-walled, hyaline when

young, becoming dark brown to black when mature, smooth, (34.4-) 43.2 (-52.3) µm diam, without germ pores. Paraphyses numerous, filiform, hyaline. Anamorph unknown.

Culture characteristics: colonies on PDA showed mycelium cottony with average density (Fig. 5). Surface buff to honey without zonation and reverse sepia. Optimum growth temperature 31.3ºC (Table 3). Growth rate of colonies on PDA at 30 and 35ºC was 9.6 mm per day at both temperatures. No growth was observed at 10 and 45ºC. Ascomata were produced only on sugar beet agar and V-8 juice agar. Ascospore germination was not observed on any of the culture media used and at any of the incubation temperatures tested.

Host and distribution: *Boerhavia diffusa* and *Trianthema portulacastrum* (roots) (Brazil, Ceará and Rio Grande do Norte).

Notes: *M. semiaridus* is closely related to *M. brasiliensis* based on phylogenetic inference. The morphology of this species is close to *M. cannonballus*, which also presents one ascospore, rarely two, per ascus, but *M. semiaridus* can be distinguished by its slightly shorter asci, 50.2 to 77.0 µm (this study), when compared to *M. cannonballus*: 56 to 90 µm (Sivanesan, 1991a).

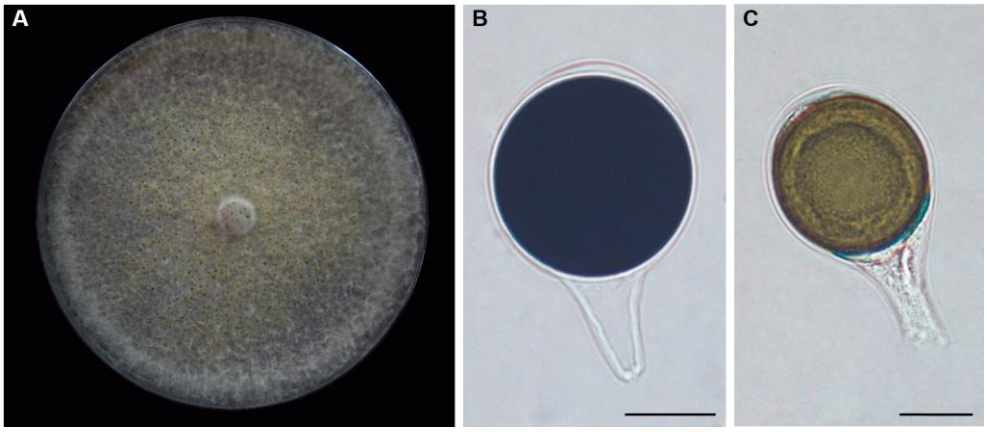

Figure 5 *Monosporascus semiaridus* CMM4830: A) Upper face of a 30-days-old colony grown on PDA culture medium at 25ºC in darkness; B) Ascus containing 1 mature ascospore; C) Ascus containing 1 inmature ascospore. Scale bars: B, C = 20 µm.

4 DISCUSSION

Five species of *Monosporascus*, namely *M. brasiliensis*, *M. caatinguensis*, *M. mossoroensis*, *M. nordestinus* and *M. semiaridus* are here described, all originating from the semi-arid region in Northeastern Brazil, and none of them represent previously described taxa. These fungi were found associated with roots of two native weed species, *B. diffusa* and *T. portulacastrum* collected from cucurbit growing fields. *Monosporascus brasiliensis*, *M.*

nordestinus and *M. semiaridus* were isolated from both weed species, while *M. caatinguensis* only from *T. portulacastrum* and *M. mossoroensis* only from *B. diffusa*. The semi-arid region of Northeastern Brazil is characterized by sandy-alkaline soils and high temperatures during all the year, like other regions where *Monosporascus* spp. have been reported (Cohen *et al*., 2012). Moreover, the optimum growth temperatures of the new *Monosporascus* spp. were over 30ºC. Thus, the environmental conditions required by them are similar to those described for the other species of the genus.

The results of the phylogenetic analyses of the 35 isolates supported the position and evaluation of the phylogenetic relationships of the genus *Monosporascus* inside the family *Diatrypaceae* and the order Xylariales, as suggested by previous molecular studies (Collado *et al*., 2002; Maharachchikumbura *et al*., 2015; 2016), and not to Sordariales as firstly indicated by Hawksworth & Ciccarone (1978). The use of the LSU/SSU loci allowed us to corroborate the phylogenetic placement of the isolates of this study at the taxonomic levels of family and order with strong support (Raja *et al*., 2017). These results confirm that the genera *Monosporascus*, *Diatrype* and *Eutypa* are closely related, proving that they are sister groups (Maharachchikumbura *et al*., 2016).

All *Monosporascus* isolates obtained in this study were phylogenetically related to *M. cannonballus*, *M. eutypoides* and *M. ibericus*, the only species of the genus *Monosporascus* from which nucleotide sequences or living cultures are currently available, and they formed distinct clades. *Monosporascus* species were monophyletic based on the three-gene tree (ITS/*tef-1α*/*tub*) with strong support. The dataset of the three loci showed a close relationship between *M. mossoroensis* and *M. nordestinus*, and both with *M. cannonballus* (Pollack & Uecker, 1974) and *M. eutypoides* (von Arx, 1975). *Monosporascus semiaridus* and *M. brasiliensis* were closely related, while *M. caatinguensis* formed a well-supported monophyletic sister clade with *M. ibericus* (Collado *et al*., 2002).

In our study DNA sequence data have been very useful to determine the identity of *Monosporascus* spp., because it is very difficult to distinguish species within this genus based only on morphology (Cohen *et al*., 2012). The number of ascospores per ascus and ascospores germinability had been traditionally used as the main morphological features for speciation in the genus *Monosporascus* (Cohen *et al*., 2012; Ben Salem *et al*., 2013), while ascospore size has been considered inappropriate, because it may be variable for a single *Monosporascus* strain depending on the growth conditions and the maturity of the spores (Hawksworth & Ciccarone, 1978; Martyn & Miller, 1996).

Collado *et al*. (2002), compared the morphology of *M. ibericus* with that of the three species *M. cannonballus*, *M. eutypoides* and *M. monosporus* based on literature descriptions. These authors indicated that *M. ibericus* was the most distinctive species of the genus, exhibiting a frequent higher number (5 to 6) of ascospores per ascus, whilst *M. eutypoides*, the other multisporous species of the genus, has only up to three ascospores (usually two) (Petrak & Ahmad, 1954), as recently confirmed by Ben Salem *et al*. (2013). *Monosporascus cannonballus* presents one spore per ascus (rarely two) and *M. monosporus* only one (Malloch & Cain, 1971; Pollack & Uecker, 1974; Sivanesan, 1991a, b). Regarding ascospores germination, the ascospores of *M. ibericus* do not germinate in axenic culture (Collado *et al*., 2002), while Sivanesan (1991b) indicated that the ascospores of *M. eutypoides* produce multiple germ tubes readily at temperatures of 30-40ºC. First descriptions of *M. cannonballus* indicated that its ascospores did not germinate *in vitro* (Pollack & Uecker, 1974; Hawksworth & Ciccarone, 1978), but subsequent studies were able to obtain ascospore germination by using thermal treatments at 45ºC (Martyn *et al*., 1992) or in the rhizosphere of melon plants growing in non-autoclaved field soil (Stanghellini *et al*., 2000). This soil methodology was used later by Ben Salem *et al*. (2013), who also obtained germination of *M. eutypoides* ascospores.

Our study adds five new species to the genus *Monosporascus* and, although we had been able to obtain the sexual morph for two of them, *M. nordestinus* and *M. semiaridus*, our results corroborate that, even for these two species, the use of morphological characters alone is insufficient for species delimitation in this genus. For the other three new species, *M. brasiliensis*, *M. caatinguensis* and *M. mossoroensis*, it was not possible to obtain asexual or sexual spores in any of the culture media used. Therefore, the use of DNA sequences analyses, either ITS, *tef-1α* or *tub*, is highly recommended for *Monosporascus* spp. identification.

Currently, only the species *M. cannonballus* and *M. eutypoides* are considered important plant pathogens, both associated with MRRVD disease of cucurbits. For instance, to date in Brazil, only *M. cannonballus* has been reported from watermelon and melon roots (Sales Júnior *et al*., 2004; 2010), but *T. portulacastrum* and *B. diffusa* were already reported as hosts for this pathogen in cucurbit growing areas of Northeastern Brazil (Rodrigues, 2017). In fact, other non-cucurbit plant species have also been reported as hosts of *Monosporascus* spp., these being: *Adenanthera pavonina* L. (Patil & Ramesh, 1987), for *M. adenantherae*; *Medicago sativa* L. (Pollack & Uecker, 1974), *Trifolium pratense* L. (Sivanesan, 1991a), *M. sativa*, *Zea mays* L., *Beta vulgaris* L., *Sorghum bicolor* (L.) Moench, *T. aestivum* L. and

Phaseolus vulgaris L. (Mertely *et al*., 1993), *Lepidium lasiocarpum* Nutt. (Stanghellini *et al*., 1996), and *S. bicolor*, *Solanum lycopersicum* L. and *Z. mays* (Sales Júnior et al., 2018), for *M. cannonballus*; *Achyranthes aspera* L. (Sivanesan *et al*., 1974; Hawksworth & Ciccarone, 1978), *Triticum* sp. (Hawksworth & Ciccarone, 1978), and *Sesamum indicum* L. (Sivanesan, 1991b), for *M. eutypoides*; *Plantago crassifolia* Forssk. and *Atriplex portulacoides* L. (Collado *et al*., 2002), for *M. ibericus*; and *Iris* sp. (Malloch & Cain, 1971) for *M. monosporus*. Overall, this information is an indication that *Monosporascus* spp. may be able to colonize roots of very diverse hosts, even without causing noticeable disease symptoms. In fact, MRRVD is a complex disease and other microorganisms have been reported to play an important role on the occurrence of the disease (Stanghellini and Misaghi, 2011; Aleandri et al., 2017).

Boerhavia diffusa and *T. portulacastrum* plants collected in our surveys were apparently healthy and only slightly root discolorations were observed, from which the new *Monosporascus* species were isolated. In the case of *M. cannonballus* and *M. eutypoides*, this could also partially explain the rapid emergence of MRRVD worldwide when non-cultivated areas are dedicated to cucurbits cultivation as suggested by Cohen *et al*. (2012), as it is the case of the cucurbit growing areas of Northeastern Brazil. Moreover, the exposure of melon and watermelon roots to *Monosporascus* spp. by colonized weeds could also enhance the potential emergence of the new species described here as cucurbit pathogens.

Our findings reveal that the diversity of species in the genus *Monosporascus* is potentially greater than previously expected. Consequently, additional extensive surveys of the roots of weed and crop species should be conducted in other cucurbit growing areas of the world to better understand their role as alternative hosts of *Monosporascus* spp., including pathogenicity tests of the new species detected, in order to determine their host range.

ACKNOWLEDGEMENTS

This research was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

Aleandri M. P., Martignoni D., Reda R., Alfaro-Fernández A., Font M.I., Armengol J., Chilosi G. (2017) Involvement of *Olpidium bornovanus* and *O. virulentus* in the occurrence of melon root and vine decline caused by *Monosporascus cannonballus* in central Italy. *Journal of Plant Pathology*, **99**, 169-176.

Al-Mawaali Q.S., Al-Sadi A.M., Al-Said F.A., Deadman M.L. (2013) Etiology, development and reaction of muskmelon to vine decline under arid conditions of Oman. *Phytopathologia Mediterranea*, **52**, 457-465.

Alves A., Crous P.W., Correia A., Phillips A.J.L. (2008) Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae*. *Fungal Diversity*, **28**, 1-13.

Anuário – Anuário Brasileiro da Fruticultura 2018. (2018), pp. 88. Santa Cruz do Sul, RS, Brazil: Editora Gazeta Santa Cruz.

Beltrán R., Vicent A., Sales Júnior R., García-Jiménez J., Armengol J. (2005) Population dynamics of *Monosporascus cannonballus* ascospores in marsh soils in eastern Spain. *European Journal of Plant Pathololog*y, **113**, 357-365.

Ben Salem I., Correia K.C., Boughalleb N., Michereff S.J., León M., Abad-Campos P., García-Jiménez J., Armengol J. (2013) *Monosporascus eutypoides*, a cause of root rot and vine decline in Tunisia, and evidence that *M. cannonballus* and *M. eutypoides* are distinct species. *Plant Disease*, **97**, 737-743.

Bruton B.D. (1998) Soilborne diseases in Cucurbitaceae: pathogen virulence and host resistance. In Proceedings of Cucurbitaceae ’98. Evaluation and enhancement of cucurbit germplasm, pp. 143–166. Ed J.D. McCreight. Asilomar, CA: 01–04 December 1998. Alexandria, VA, USA: American Society for Horticultural Science Press.

Bruton B.D., Russo V.M., Garcia-Jiménez J., Miller M.E. (1998) Carbohydrate partitioning, cultural practices, and vine decline diseases of cucurbits. In Proceedings of Cucurbitaceae ’98. Evaluation and enhancement of cucurbit germplasm, pp. 189–200. Ed J.D. McCreight. Asilomar, CA: 01–04 December 1998. Alexandria, VA, USA: American Society for Horticultural Science Press.

Cohen R., Pivonia S., Crosby K.M., Martyn R.D. (2012) Advances in the biology and management of *monosporascus* vine decline and wilt of melons and other cucurbits. *Horticultural Reviews*, **39**, 77-120.

Collado J., González A., Platas G., Stchigel A.M., Guarro J., Peláez F. (2002) *Monosporascus ibericus* sp. nov., an endophytic ascomycete from plants on saline soils, with observations on

the position of the genus based on sequence analysis of the 18S rDNA. *Mycological Research*, **106**, 118-127.

Crous P.W., Gams W., Stalpers J.A., Robert V., Stegehuis G. **(**2004) MycoBank: an online initiative to launch mycology into the 21st century. *Studies in Mycology*, **50**, 19-22.

FAO (2018) - Food and Agriculture Organization of the United Nations. URL http://www.fao.org/faostat/en/#home [accessed on September 2018].

Farris J.S., Kallersjo M., Kluge A.G., Bult C. (1994) Testing significance of incongruence. *Cladistics*, **10**, 315-319.

Felsenstein J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783-791.

Figuerêdo M.C.B., Gondim R.S., Aragão F.A.S. (2017) Produção de melão e mudanças climáticas: sistemas conservacionistas de cultivo para redução das pegadas de carbono e hídrica, pp. 302. Brasília, DF, Brazil: Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA-CNPAT.

Gardes M., Bruns T.D. (1993) ITS primers with enhanced specifity for Basidiomycetes: application to identification of mycorrhizae and rusts. *Molecular Ecology*, **2**, 113-118.

Hawksworth D.L., Ciccarone A. (1978) Studies on a species of *Monosporascus* isolated from *Triticum*. *Mycopathologia* **66**, 147-151

Huson D.H., Bryant D. (2006) Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution*, **23**, 254–267

IBGE. (2018) Instituto Brasileiro de Geografia e Estatística. URL https://sidra.ibge.gov.br/pesquisa/pam/tabelas [accessed on September 2018].

Kohn L.M. (1995) The clonal dynamic in wild and agricultural plant pathogen populations. *Canadian Journal of Botany*, **73**, S1231-S1240

Kumar S., Stecher G., Tamura K. (2016) Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, **33**, 1870-1874.

Lemessa F., Wakjira M. (2014) Mechanisms of ecological weed management by cover cropping: a review. *Journal of Biological Sciences*, **14,** 452-459.

Maharachchikumbura S.S.N., Hyde K.D., Jones E.B.G. (2015) Towards a natural classification and backbone tree for Sordariomycetes. *Fungal Diversity*, **72**, 199-301.

Maharachchikumbura S.S.N., Hyde K.D., Jones E.B.G. (2016) Families of Sordariomycetes. *Fungal Diversity*, **79**, 1-317.

Malloch D., Cain R.F. (1971) New cleistothecial Sordariaceae and a new family, Coniochaetaceae. *Canadian Journal of Botany*, **49**, 869-880.

Markakis E.A., Trantas E.A., Lagogianni C.S., Mpalantinaki E., Pagoulatou M., Ververidis F.N., Goumas D.E. (2018) First report of root rot and vine decline of melon caused by *Monosporascus cannonballus* in Greece. *Plant Disease*, **102**, 1036.

Martyn R.D., Miller M.E. (1996) Monosporascus root rot and vine decline: an emerging disease of melons worldwide. *Plant Disease*, **80**, 716-725.

Martyn R.D., Mertely J.C., Miller M.E., Katsar C., Baasiri R. (1992) Morphology and germination of *Monosporascus cannonballus* ascospores. (Abstr.) *Phytopathology*, **82**, 1115.

Medeiros E.V., Sales Júnior R., Michereff S.J., Barbosa M.R. (2006) Quantificação de ascósporos de *Monosporascus cannonballus* em solos não cultivados de Caatinga e em áreas de cultivo de melão do Rio Grande do Norte e Ceará. *Fitopatologia Brasileira*, **31**, 500-504.

Mertely J.C., Martyn R.D., Miller M.E., Bruton B.D. (1993) An expanded host range for the muskmelon pathogen, *Monosporascus cannonballus*. *Plant Disease*, **77**, 667-673.

Miller M.A., Pfeiffer W., Schwartz T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, LA, USA, pp. 1-8.

Nylander J.A. A. (2004) MrAIC.pl. Program distributed by the editor. Evolutionary Biology Centre, Uppsala University

Patil S.D., Ramesh C. (1987) Notes on some fungi of Pleosporaceae (Loculoascomycetes) from Maharashtra (India). *Transactions of the Mycological Society of Japan*, **28**, 229-236.

Petrak F., Ahmad S. 1954. Beiträge zur Pilzflora Pakistans. *Sydowia*, **8**, 162-185.

Philippe H., Bryant D. (2006) A simple and robust statistical test for detecting the presence of recombination. *Genetics*, **172**, 2665–2681

Pollack F.G., Uecker F.A. (1974) *Monosporascus cannonballus* an unusual ascomycete in cantaloupe roots. *Mycologia*, **66**, 346-349

Raja H.A., Miller A.N., Pearce C.J., Oberlies N.H. (2017) Fungal identification using molecular tools: A primer for the natural products research community. *Journal of Natural Product*s, **80**, 756-770.

Rayner R.W. (1970) *A Mycological Colour Chart*, pp. 34. Kew, Surrey, England: Commonwealth Mycological Institute.

Rodrigues A.P.M.S. (2017) Variabilidade de isolados de *Monosporascus cannonballus* oriundos de plantas daninhas em campos de produção de meloeiro. PhD Thesis. Universidade Federal Rural do Semi-Árido, Mossoró, Brazil.

Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu L., Suchard M.A., Huelsenbeck J.P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, **61**, 539-542.

Sales Júnior R., Dias Balbino D.A., Paiva Negreiros A.M., Da Silva Barboza H., Valente de Medeiros E., Armengol J. (2018) Cotton, cowpea and sesame are alternative crops to cucurbits in soils naturally infested with *Monosporascus cannonballus*. *Journal of Phytopathology*, **166**, 396-402.

Sales Júnior R., Nascimento I.J.B., Freitas L.S.R., Beltrán Armengol J., Vicent A., García-Jiménez J. (2004) First report of *Monosporascus cannonballus* on melon in Brazil. *Plant Disease*, **88**, 84.

Sales Júnior R., Oliveira O.F., Medeiros E.V., Guimarães I.M., Correia K.C., Michereff S.J. (2012) Ervas daninhas como hospedeiras alternativas de patógenos causadores do colapso do meloeiro. *Revista Ciência Agronômica*, **43**, 195-198.

Sales Júnior R., Rodrigues A.P.M.S., Negreiros A.M.P., Ambrósio M.M.Q., Barboza H. S., Beltrán R. (2019) Weeds as potential hosts for fungal root pathogens of watermelon. *Revista Caatinga*, **32**, 1-6.

Sales Júnior R., Santana C.V.S., Nogueira D.R.S., Silva K.J.P., Guimarães I.M., Michereff S.J., Abad-Campos P., García-Jiménez J., Armengol J. (2010) First Report of *Monosporascus cannonballus* on Watermelon in Brazil. *Plant Disease*, **94**, 278.

Sivanesan A. (1991a) IMI Descriptions of Fungi and Bacteria n. 1035. *Monosporascus cannonballus*. *Mycopathologia*, **114**, 53-54.

Sivanesan A. (1991b) IMI Descriptions of Fungi and Bacteria n. 1036. *Monosporascus eutypoides*. *Mycopathologia*, **114**, 55-56.

Sivanesan A., Talde U. K., Tilak S.T. (1974) *Bitrimonospora indica* gen. et sp. nov., a new loculoascomycete from India. *Transactions of The British Mycological Society*, **63**, 595-596.

Soares I.A.A., Freitas F.C.L., Negreiros M.Z., Freire G.M., Aroucha E.M.M., Grangeiro L.C., Lopes W.A.R., Dombroski J.L.D. (2010) Interferência das plantas daninhas sobre a produtividade e qualidade de cenoura. *Planta Daninha*, **28**, 247-254.

Stamatakis A. **(**2014) – RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, **30**, 1312-1313.

Stanghellini M.E., Kim D.H., Rasmussen S.L. (1996) Ascospores of *Monosporascus cannonballus*: Germination and distribution in cultivated and desert soils in Arizona. *Phytopathology*, **86**, 509-514.

Stanghellini M.E., Kim D.H., Waugh M. (2000) Microbe-mediated germination of ascospores of *Monosporascus cannonballus*. *Phytopathology*, **90**, 243-247.

Swofford D.L. (2003) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0. Sinauer Associates, Sunderland, MA, USA.

Stanghellini M.E, Misaghi I.J. (2011). *Olpidium bornovanus*-mediated germination of ascospores of *Monosporascus cannonballus*: a host-specific rhizosphere interaction. *Phytopathology*, **101**, 794-796.

Taylor J.W., Jacobson D.J., Kroken S., Kasuga T., Geiser D.M., Hibbett D.S., Fisher M.C. (2000) Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology*, **31**, 21-32.

Thompson J.D., Higgins D.G., Gibson T.J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673-4680.

Travadon R., Lawrence D., Rooney-Latham S., Gubler W.D., Wilcox W.F., Rolshausen P.E., Baumgartner K. (2015) *Cadophora* species associated with wood-decay of grapevine in North America. *Fungal Biology*, **119**, 53-66.

Troutman J., Matejka J.C. (1970) Fungi associated with cantaloupe in Arizona. (Abstr.). *Phytopathology*, **60**, 1317

Vaidya G., Lohman D.J., Meier R. (2011) SequenceMatrix: Concatenation software for the fast assembly of multi-gene datasets with character set and codon information. *Cladistics*, **27**, 171-180.

Vilgalys R., Hester M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology*, **172**, 4238-4246.

von Arx J.A. **(**1975) On *Thielavia angulata* and some recently described *Thielavia* species. *Kavaka*, **3**, 33-36.

White T.J., Bruns T.D., Lee S.B., Taylor J.W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A Guide to Methods and Applications. Volume 18, pp. 315–322. Eds M. Innis, D. Gelfand, J. Sninsky and T.J. White. Orlando, FL, USA: Academic Press.

Yan L.Y., Zang Q.Y., Huang Y.P., Wang Y.H. (2016) First report of root rot and vine decline of melon caused by *Monosporascus cannonballus* in Eastern Mainland China. *Plant Disease*, **100**, 651.

ATTACHMENT – Supporting Information

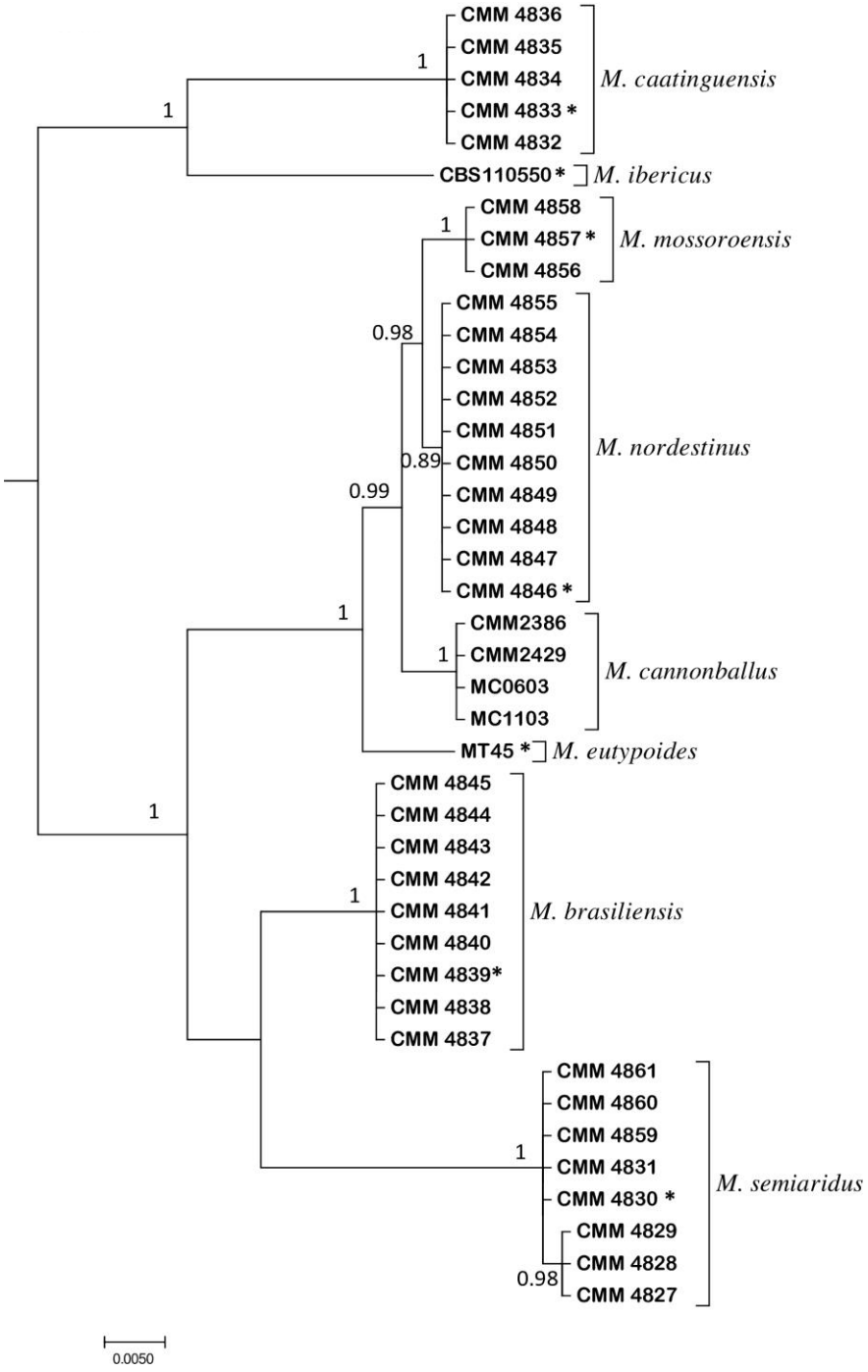

Figure S1 Fifty percent majority rule consensus tree derived from Bayesian analysis of the *tub* alignment dataset used to infer the relative position of species inside the *Monosporascus* genus. Bayesian posterior probabilities higher than 0.90 are indicated, at nodes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk. Scale bar shows expected changes per site.

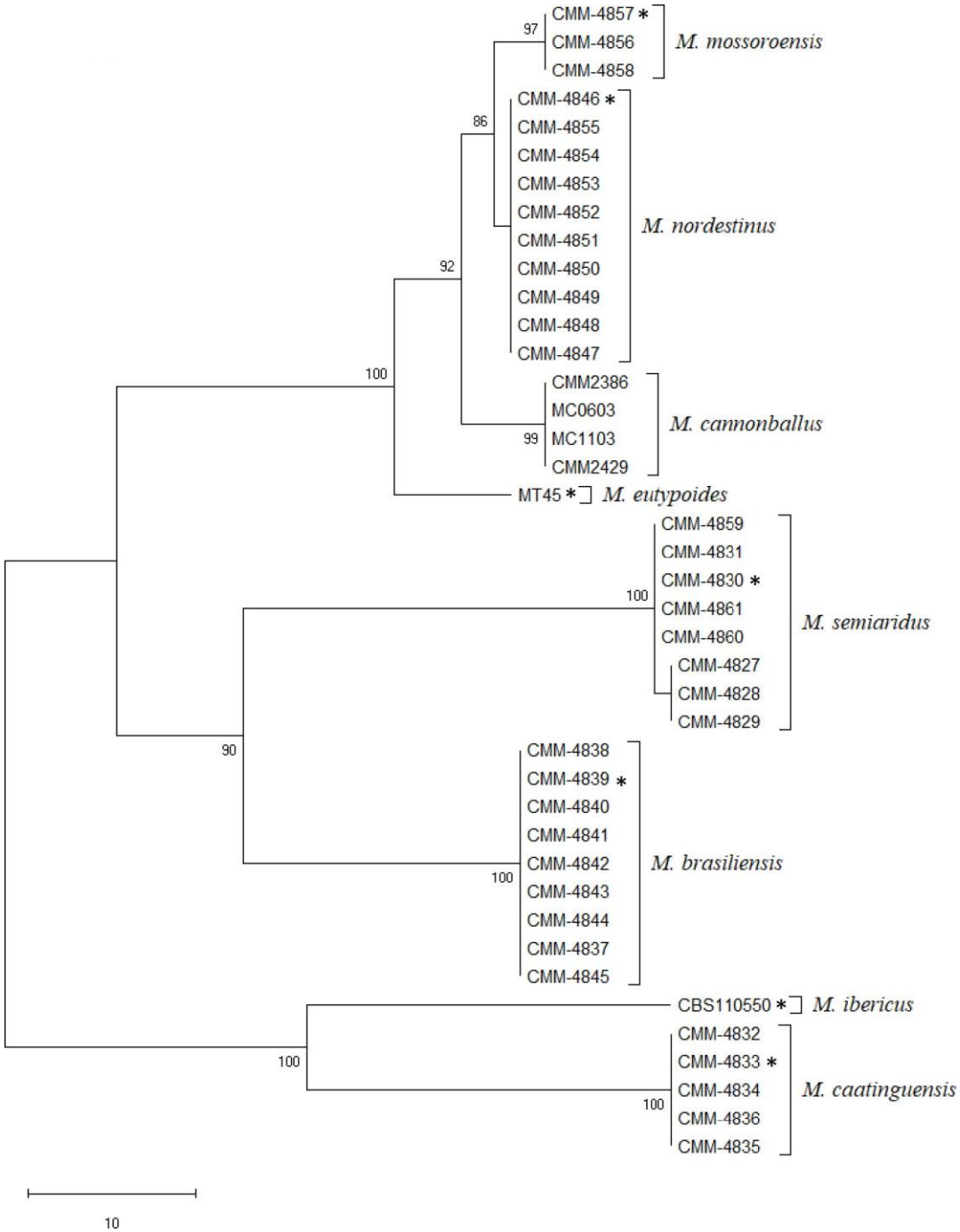

Figure S2 The first of 10 equally most parsimonious trees (TL = 156; CI = 0.895; RI = 0.986) resulting from a parsimony analysis of the tub sequence alignment, used to infer the relative position of species inside the *Monosporascus* genus. The bootstrap support values (≤ 70%) are indicated at the nodes and the scale bar represents the number of changes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk.

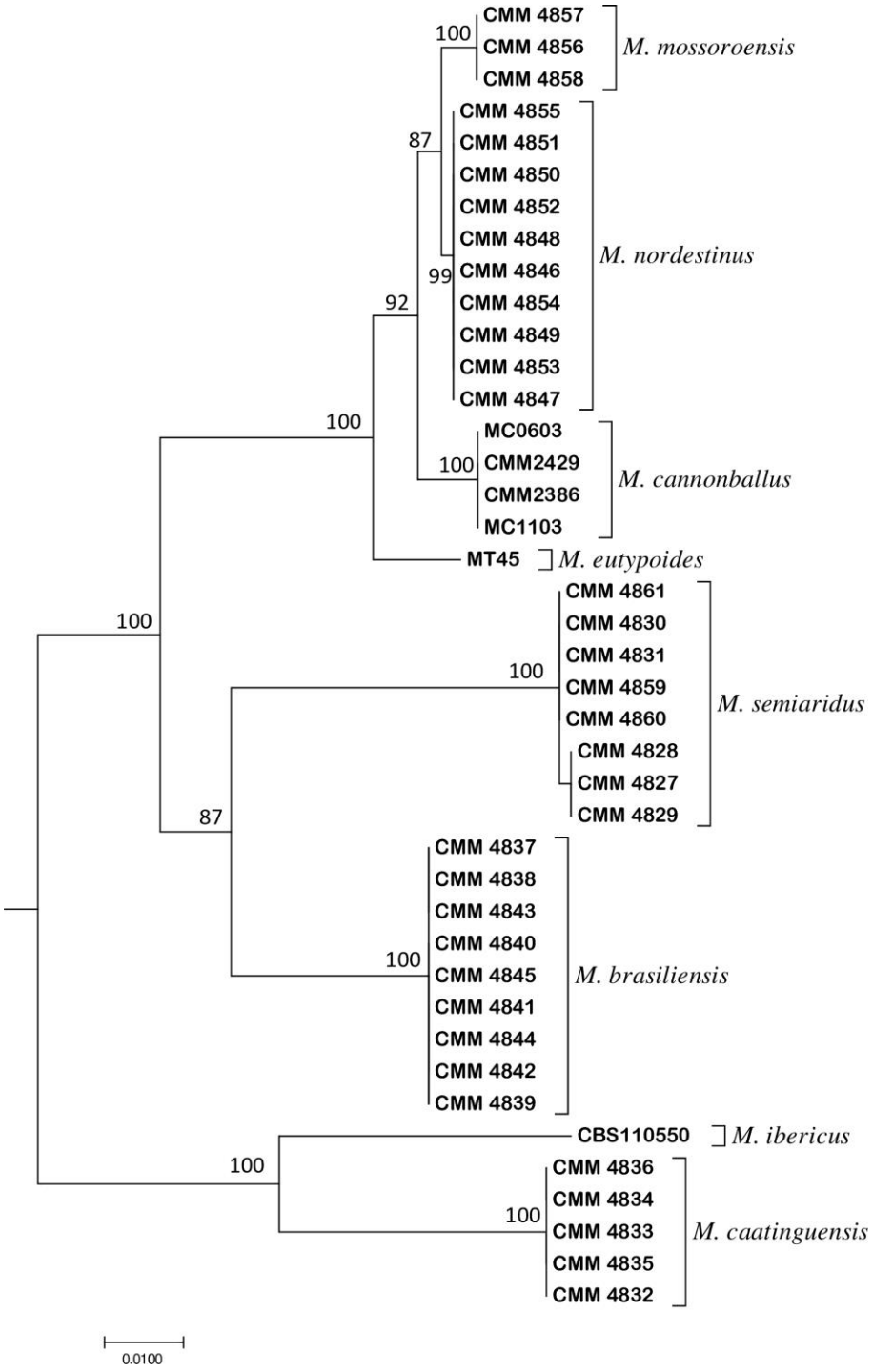

Figure S3 Maximum likelihood phylogeny inferred from the *tub* sequence alignment used to infer the relative position of species inside the *Monosporascus* genus. Support values (ML bootstrap) are given at the nodes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk. Scale bar shows expected changes per site.

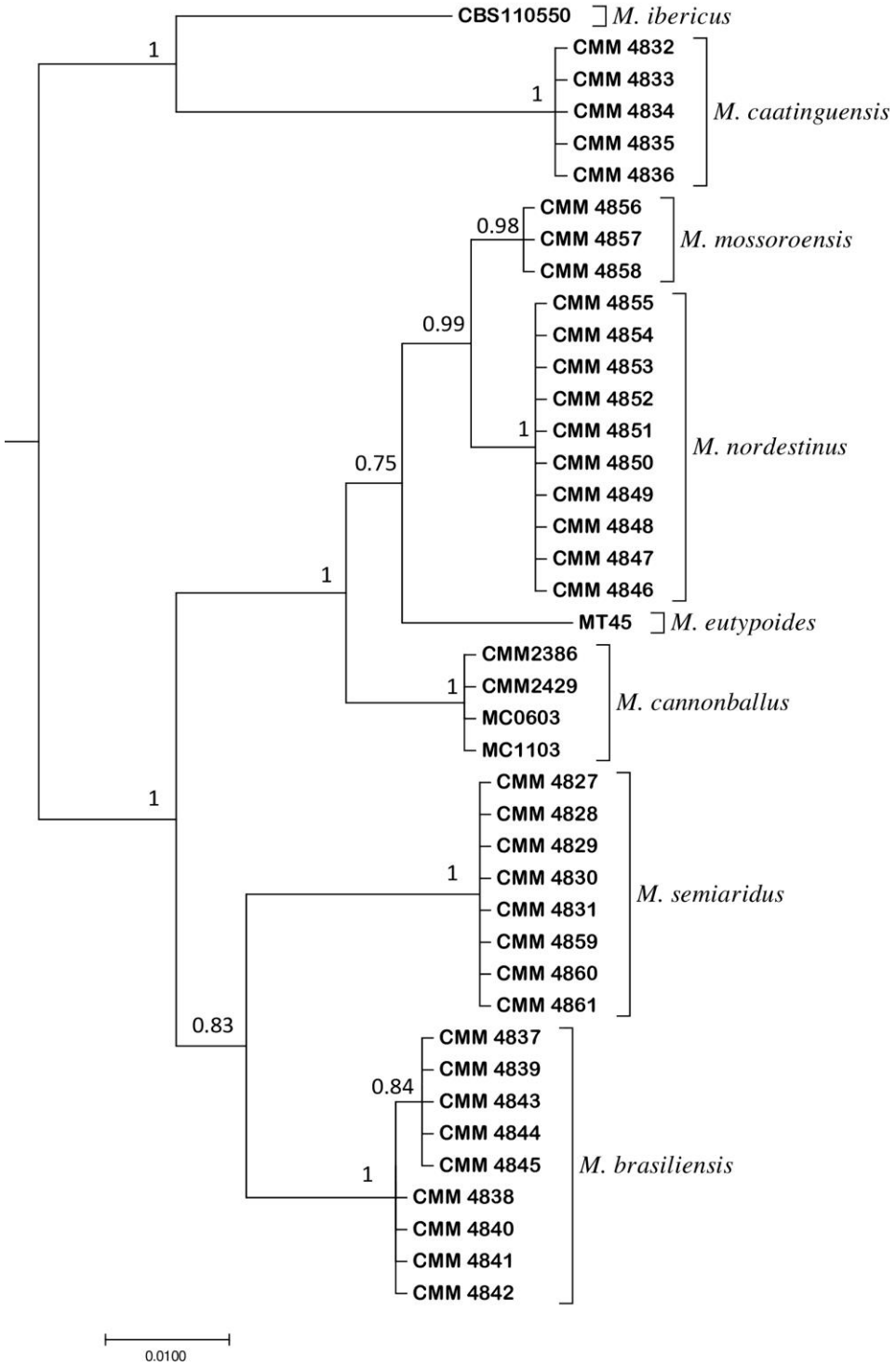

Figure S4 Fifty percent majority rule consensus tree derived from Bayesian analysis of the ITS alignment used to infer the relative position of species inside the *Monosporascus* genus. Bayesian posterior probabilities higher than 0.90 are indicated, at nodes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk. Scale bar shows expected changes per site.

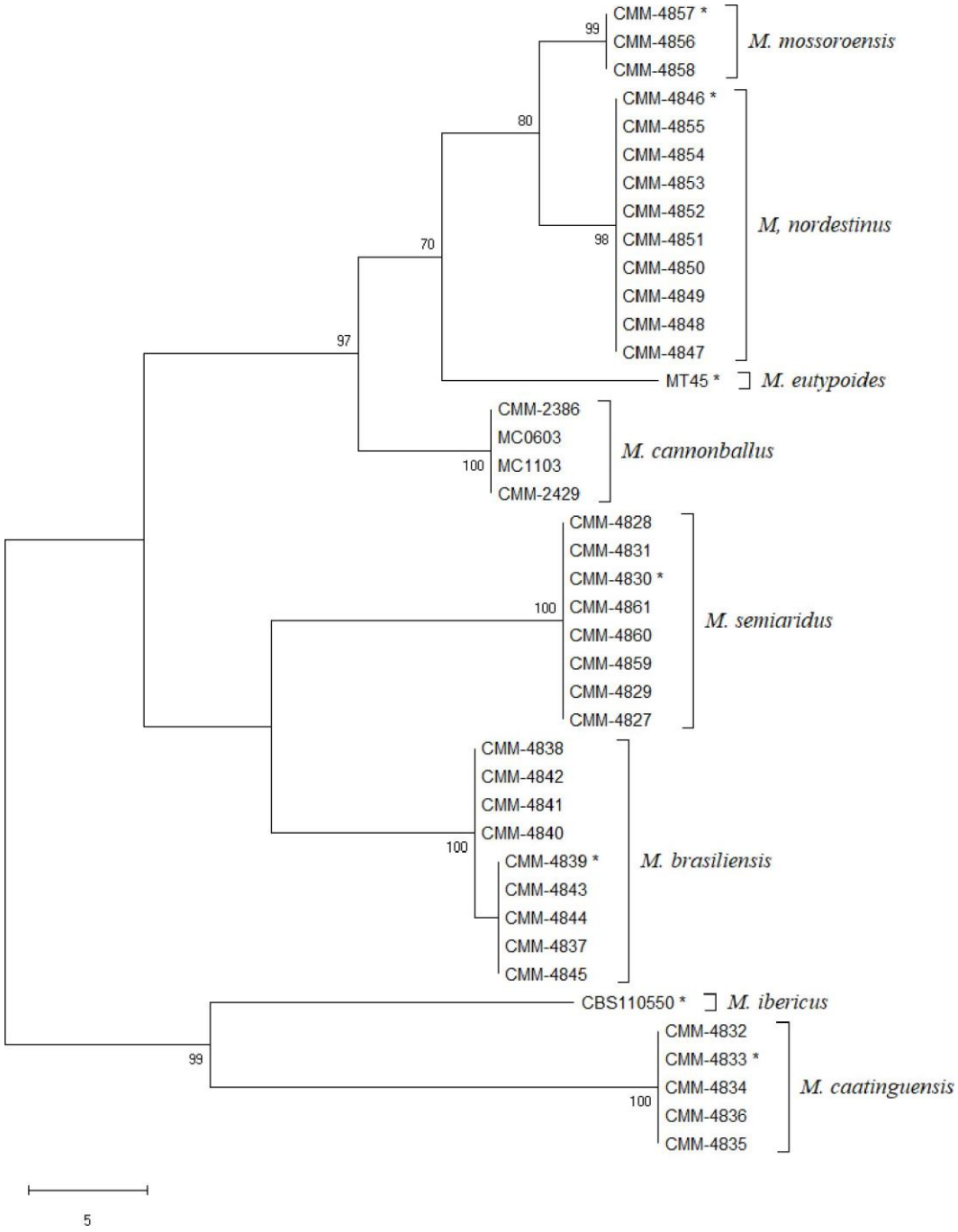

Figure S5 The first of 10 equally most parsimonious trees (TL = 113; CI = 0.853; RI = 0.975) resulting from a parsimony analysis of the ITS sequence alignment, used to infer the relative position of species inside the *Monosporascus* genus. The bootstrap support values (≤ 70%) are indicated at the nodes and the scale bar represents the number of changes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk.

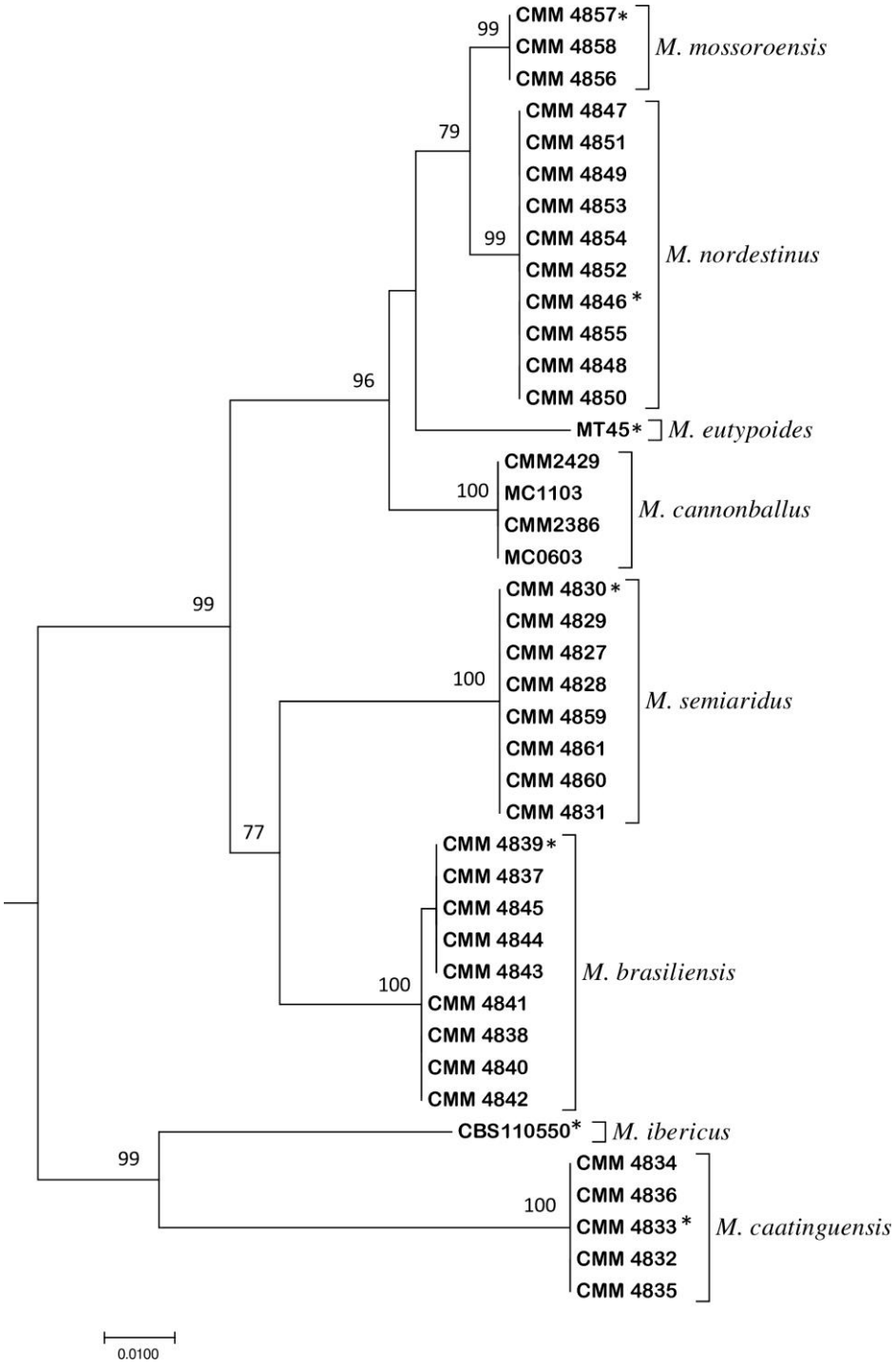

Figure S6 Maximum likelihood phylogeny inferred from the Internal Transcribed Spacer (ITS) sequence alignment used to infer the relative position of species inside the *Monosporascus* genus. Support values (ML bootstrap) are given at the nodes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk. Scale bar shows expected changes per site.

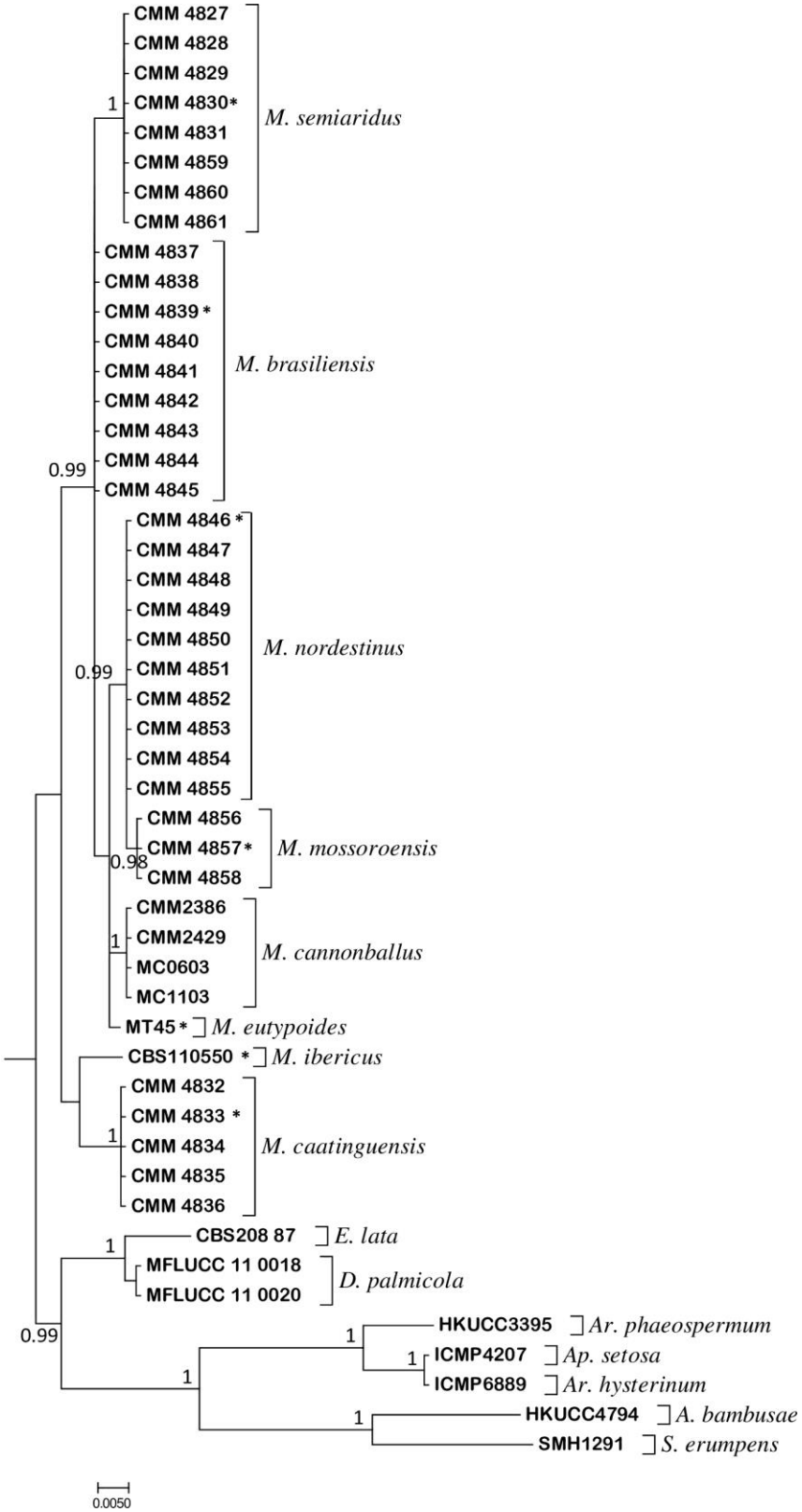

Figure S7 Fifty percent majority rule consensus tree derived from Bayesian analysis of the LSU alignment used to infer the phylogenetic relationships of the genus *Monosporascus* inside the family Diatrypaceae and the order Xylariales. The tree was rooted using *Arecophila bambusae* (HKUCC4794), *Seynesia erumpens* (SMH 1291), *Arthrinium hysterinum* (ICMP 6889), *Ar. phaeospermum* (HKUCC 3395) and *Apiospora setosa* (ICMP 4207) as outgroup sequences. Ex-type strains are indicated with an asterisk. Bayesian posterior probabilities higher than 0.90 are indicated at nodes. Scale bar shows expected changes per site.

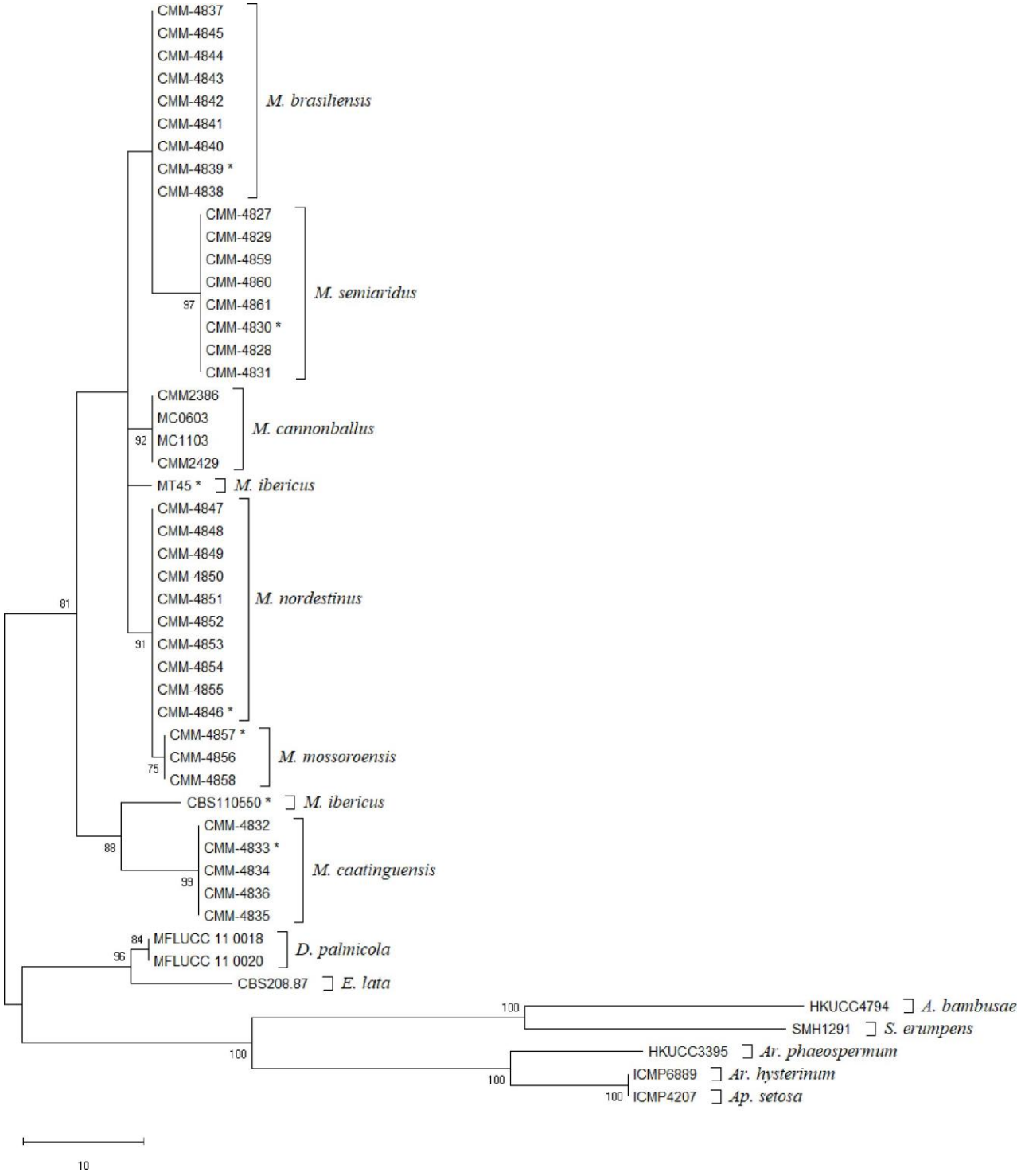

Figure S8 The first of 10 equally most parsimonious trees (TL = 188; CI = 0.772; RI = 0.908) resulting from a parsimony analysis of the LSU sequence alignment, used to infer the phylogenetic relationships of the genus *Monosporascus* inside the family Diatrypaceae and the order Xylariales. The bootstrap support values (≤ 70%) are indicated at the nodes and the scale bar represents the number of changes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk.

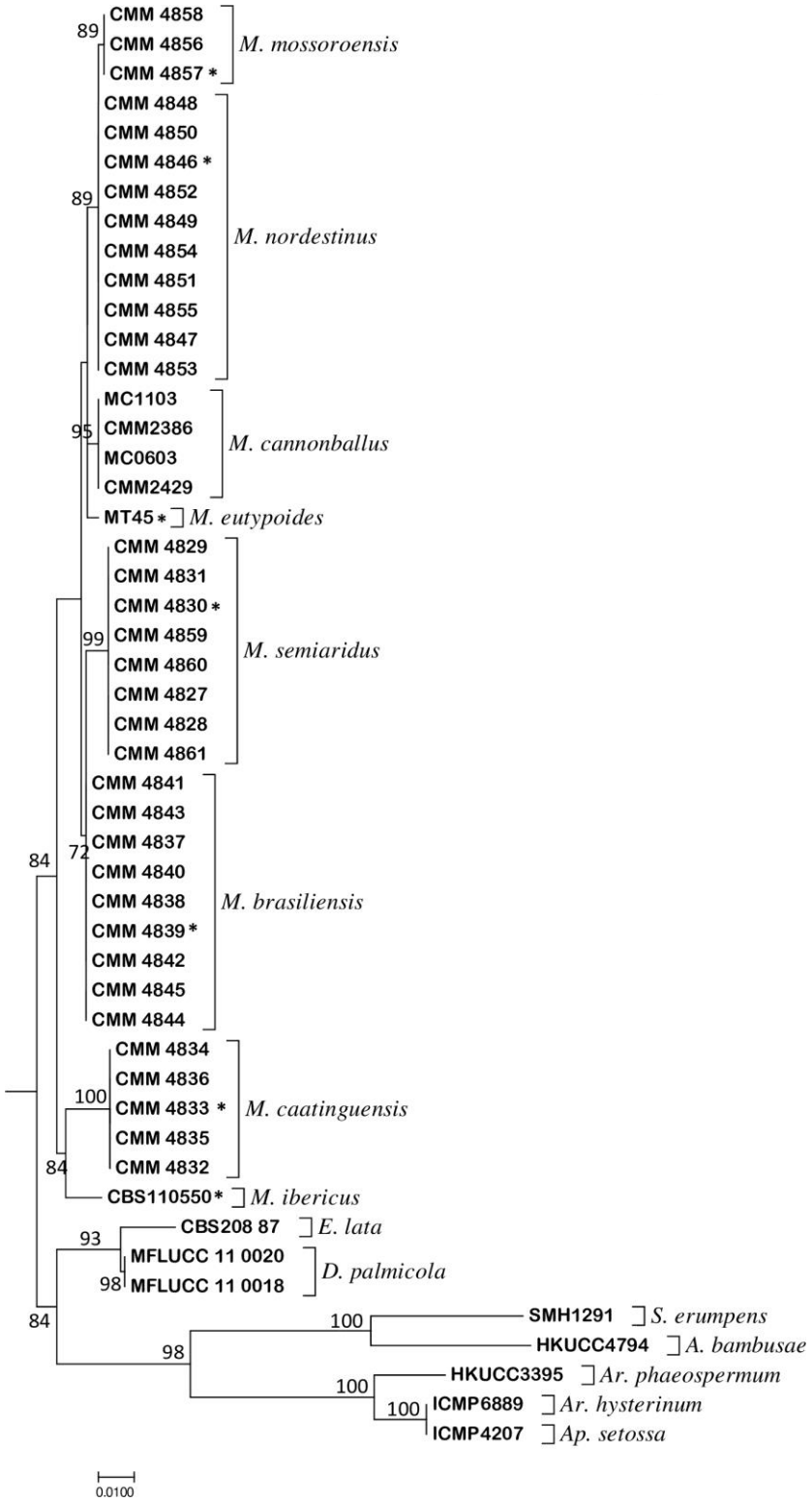

Figure S9 Maximum likelihood phylogeny inferred from the LSU sequence alignment used to infer the phylogenetic relationships of the genus *Monosporascus* inside the family Diatrypaceae and the order Xylariales. Support values (ML bootstrap) higher than 70% are given at the nodes. The tree was rooted using *Arecophila bambusae* (HKUCC4794), *Seynesia erumpens* (SMH 1291), *Arthrinium hysterinum* (ICMP 6889), *Ar. phaeospermum* (HKUCC 3395) and *Apiospora setosa* (ICMP 4207) as outgroup sequences. Ex-type strains are indicated with an asterisk. Scale bar shows expected changes per site.

Figure S10 Fifty percent majority rule consensus tree derived from Bayesian analysis of the SSU alignment used to infer the phylogenetic relationships of the genus *Monosporascus* inside the family Diatrypaceae and the order Xylariales. The tree was rooted using *Arecophila bambusae* (HKUCC4794), *Seynesia erumpens* (SMH 1291), *Arthrinium hysterinum* (ICMP 6889), *Ar. phaeospermum* (HKUCC 3395) and *Apiospora setosa* (ICMP 4207) as outgroup sequences. Ex-type strains are indicated with an asterisk. Bayesian posterior probabilities higher than 0.90 are indicated at nodes. Scale bar shows expected changes per site.

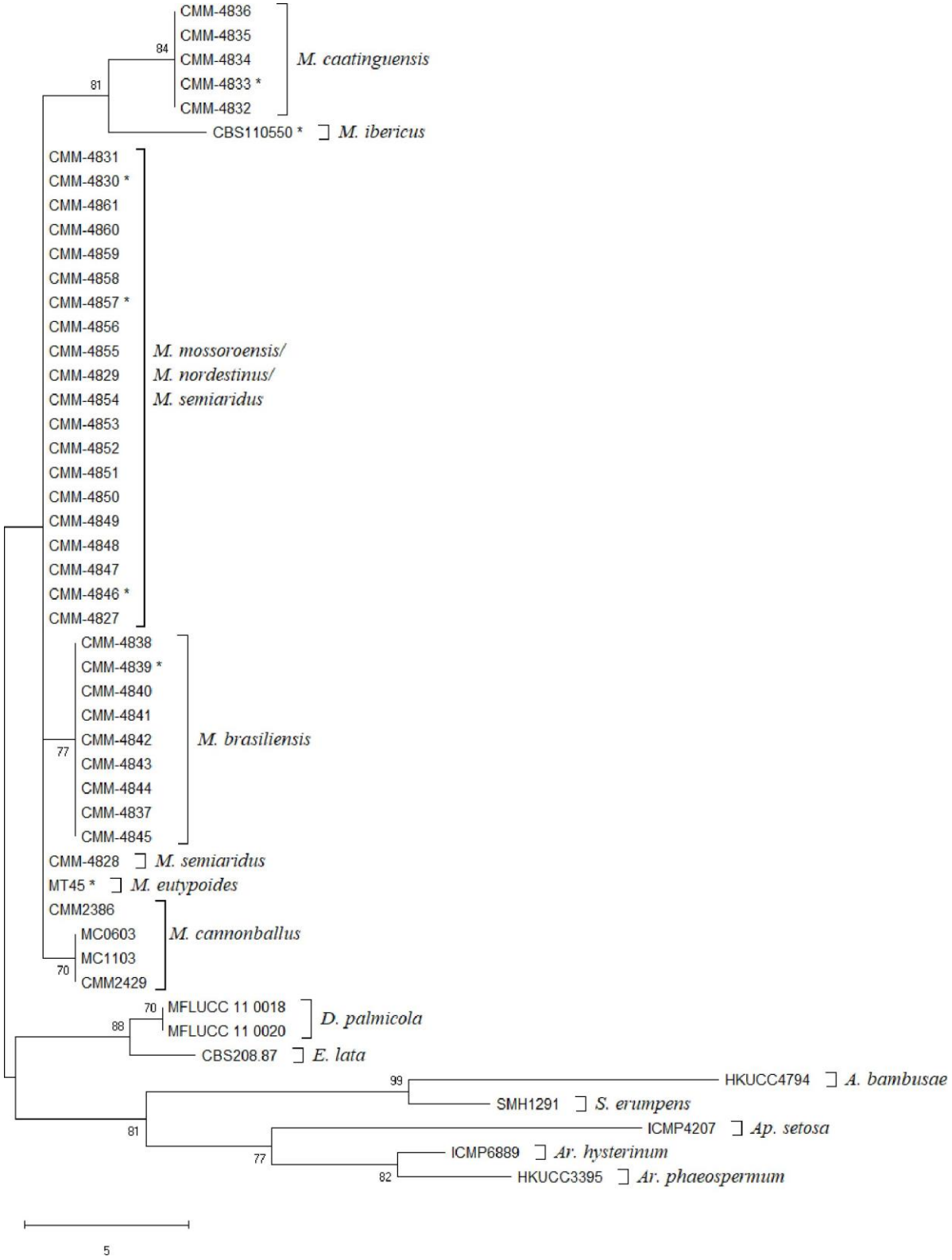

Figure S11 The first of 8 equally most parsimonious trees (TL = 65; CI = 0.75; RI = 0.87) resulting from a parsimony analysis of the SSU sequence alignment, used to infer the phylogenetic relationships of the genus *Monosporascus* inside the family Diatrypaceae and the order Xylariales. The bootstrap support values (≤ 70%) are indicated at the nodes and the scale bar represents the number of changes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk.

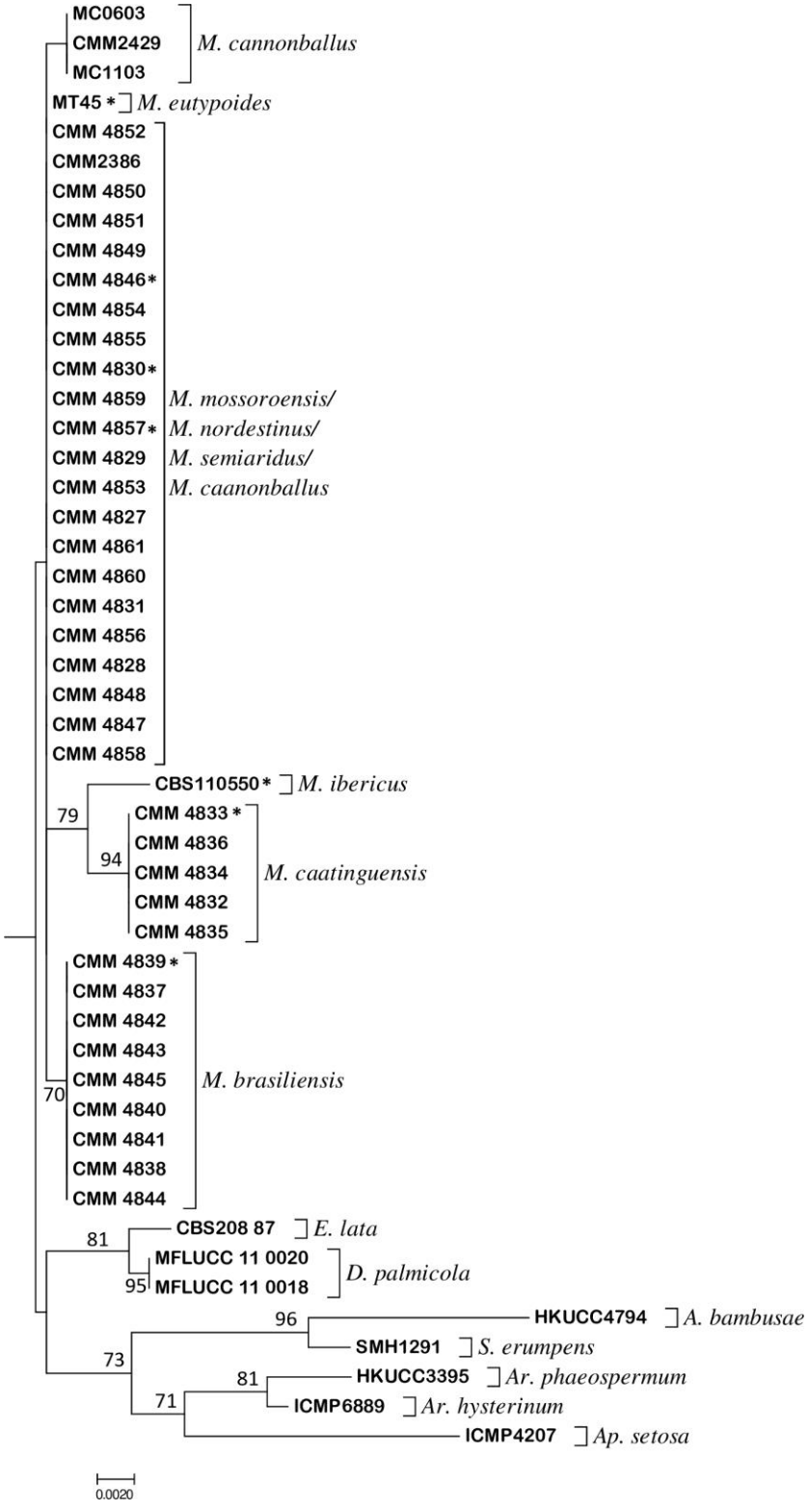

Figure S12 Maximum likelihood phylogeny inferred from the SSU sequence alignment used to infer the phylogenetic relationships of the genus *Monosporascus* inside the family Diatrypaceae and the order Xylariales. Support values (ML bootstrap) higher than 70% are given at the nodes. The tree was rooted using *Arecophila bambusae* (HKUCC4794), *Seynesia erumpens* (SMH 1291), *Arthrinium hysterinum* (ICMP 6889), *Ar. phaeospermum* (HKUCC 3395) and *Apiospora setosa* (ICMP 4207) as outgroup sequences. Ex-type strains are indicated with an asterisk. Scale bar shows expected changes per site.

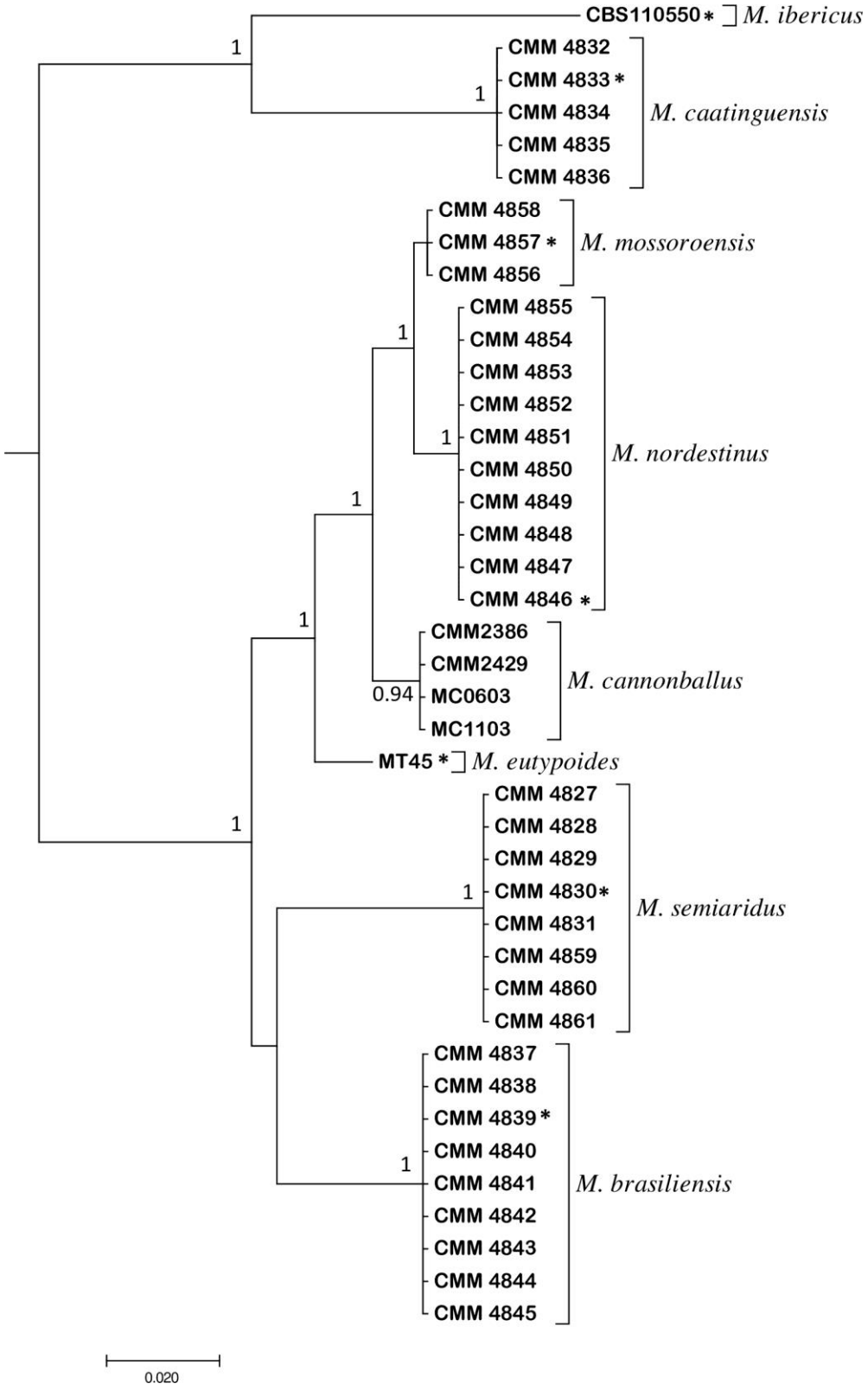

Figure S13 Fifty percent majority rule consensus tree derived from Bayesian analysis of the *tef1-α* alignment used to infer the relative position of species inside the *Monosporascus* genus. Bayesian posterior probabilities higher than 0.90 are indicated, at nodes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk. Scale bar shows expected changes per site.

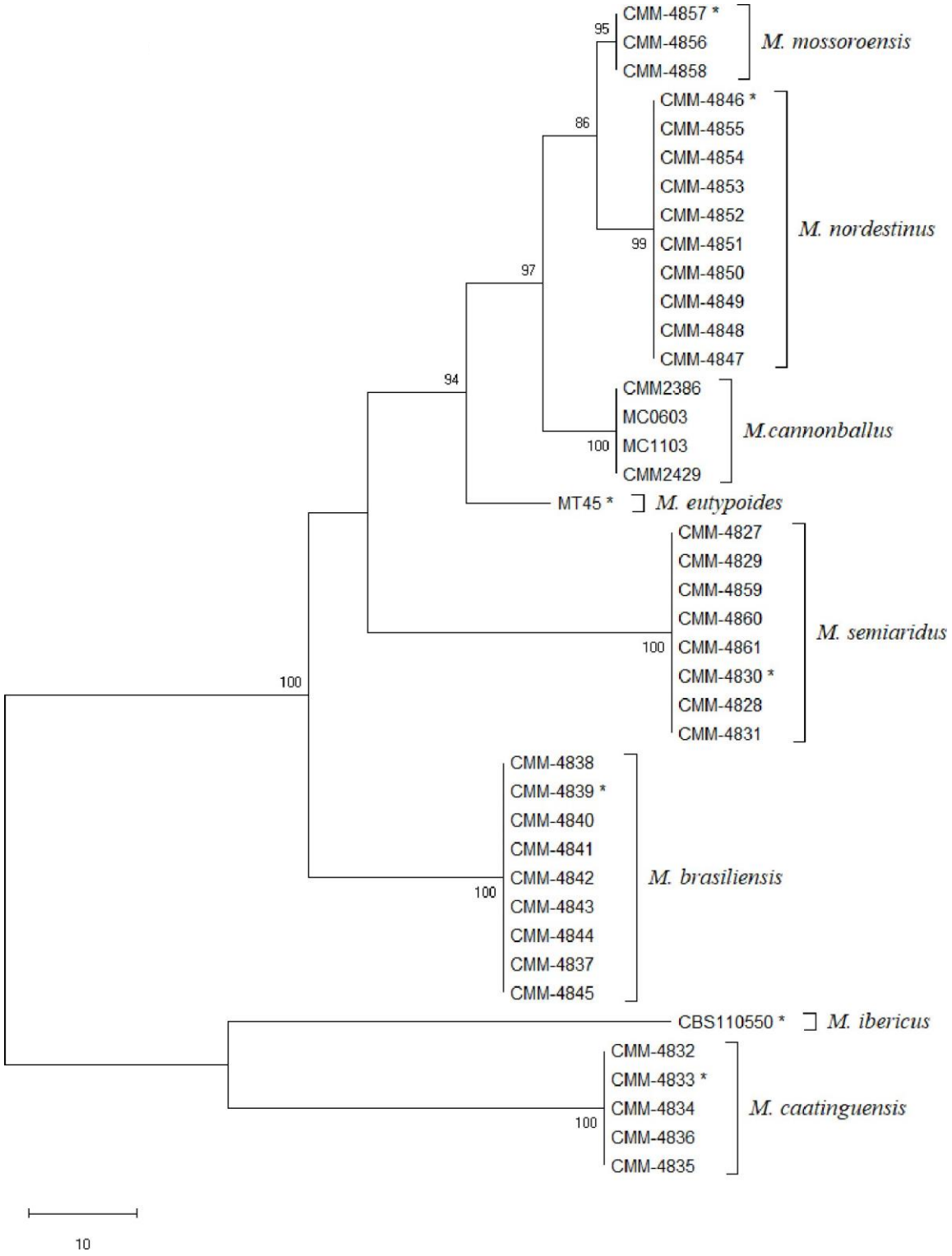

Figure S14 The first of 10 equally most parsimonious trees (TL = 218; CI = 0.887; RI = 0.98) resulting from a parsimony analysis of the tef1-α sequence alignment, used to infer the relative position of species inside the *Monosporascus* genus. The bootstrap support values (≤ 70%) are indicated at the nodes and the scale bar represents the number of changes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk.

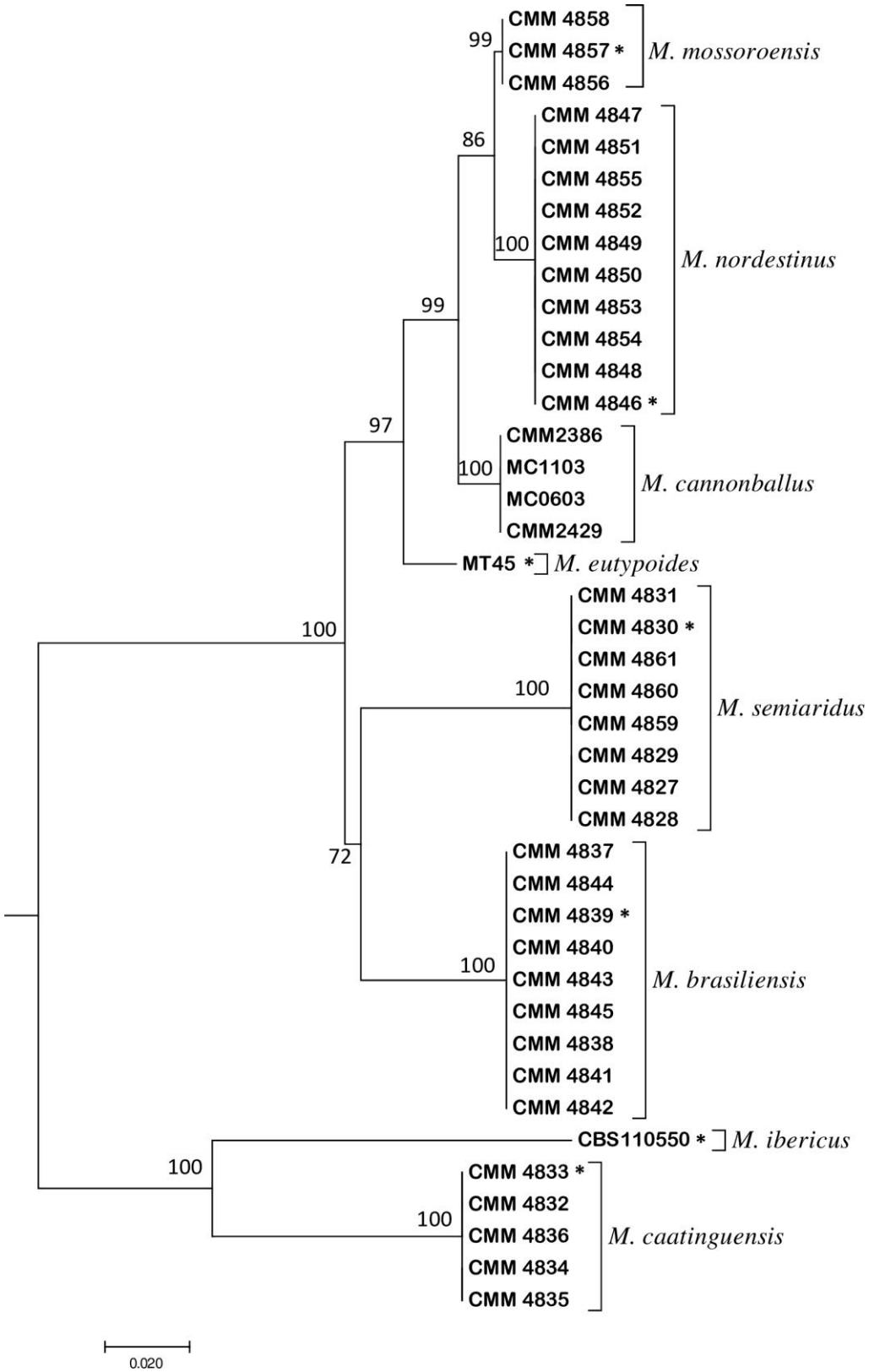

Figure S15 Maximum likelihood phylogeny inferred from the *tef1-α* sequence alignment used to infer the relative position of species inside the *Monosporascus* genus. Support values (ML bootstrap) are given at the nodes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk. Scale bar shows expected changes per site.

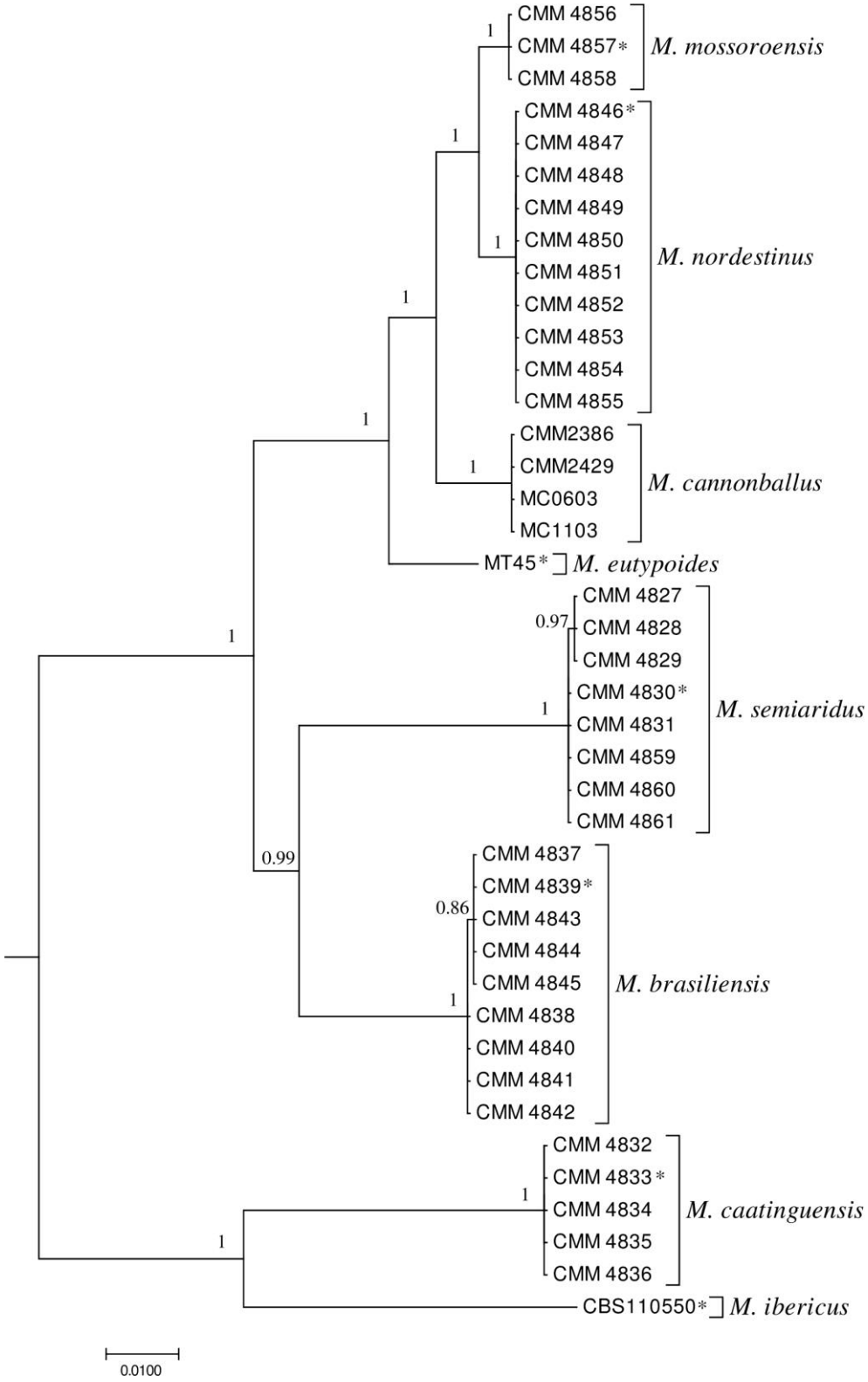

Figure S16 Fifty percent majority rule consensus tree derived from Bayesian analysis of the combined ITS, *tef1-α* and *tub* sequence alignments used to infer the relative position of species inside the *Monosporascus* genus. Bayesian posterior probabilities higher than 0.90 are indicated, at nodes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk. Scale bar shows expected changes per site.

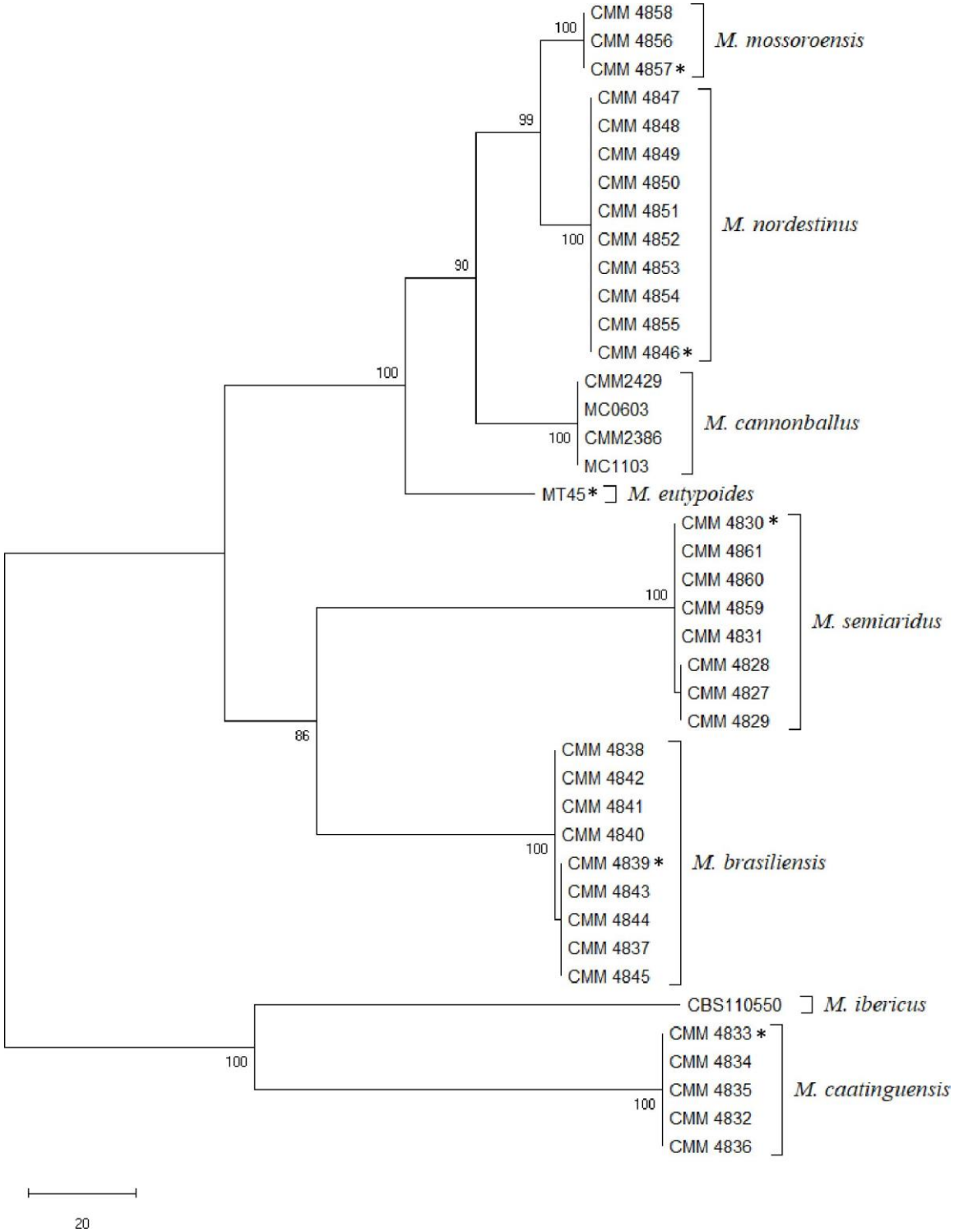

Figure S17 The first of 10 equally most parsimonious trees (TL = 489; CI = 0.878; RI = 0.981) resulting from a parsimony analysis of the combined (ITS, *tef-1α* and *tub*) sequence alignment, used to infer the relative position of species inside the *Monosporascus* genus. The bootstrap support values (≤ 70%) are indicated at the nodes and the scale bar represents the number of changes. The tree was midpoint rooted. Ex-type strains are indicated with an asterisk.

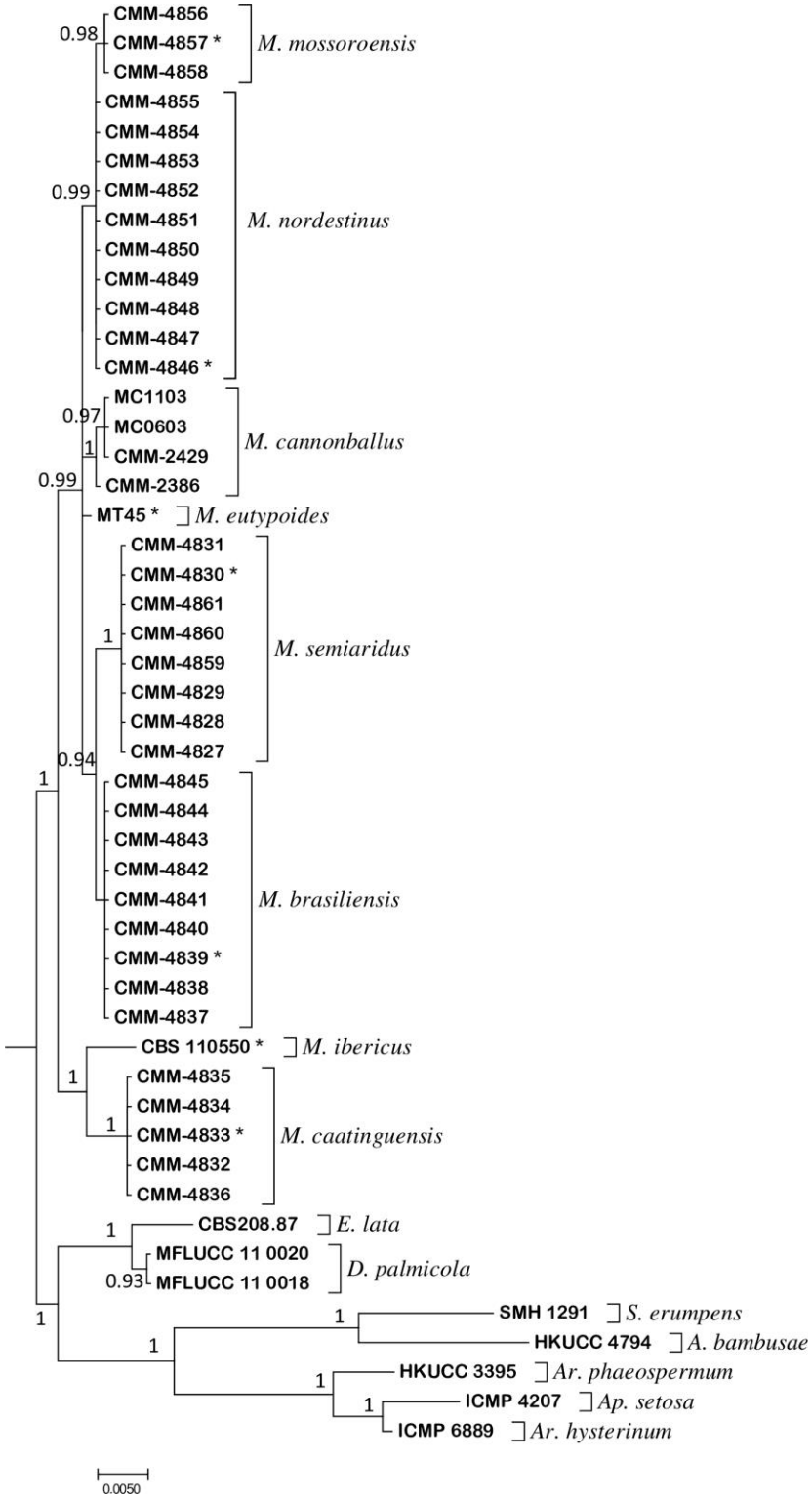

Figure S18 Fifty percent majority rule consensus tree derived from Bayesian analysis of the combined LSU and SSU sequence alignments used to infer the phylogenetic relationships of the genus *Monosporascus* inside the family Diatrypaceae and the order Xylariales. The tree was rooted using *Arecophila bambusae* (HKUCC4794), *Seynesia erumpens* (SMH 1291), *Arthrinium hysterinum* (ICMP 6889), *Ar. phaeospermum* (HKUCC 3395) and *Apiospora setosa* (ICMP 4207) as outgroup sequences. Ex-type strains are indicated with an asterisk. Bayesian posterior probabilities higher than 0.90 are indicated at nodes. Scale bar shows expected changes per site.

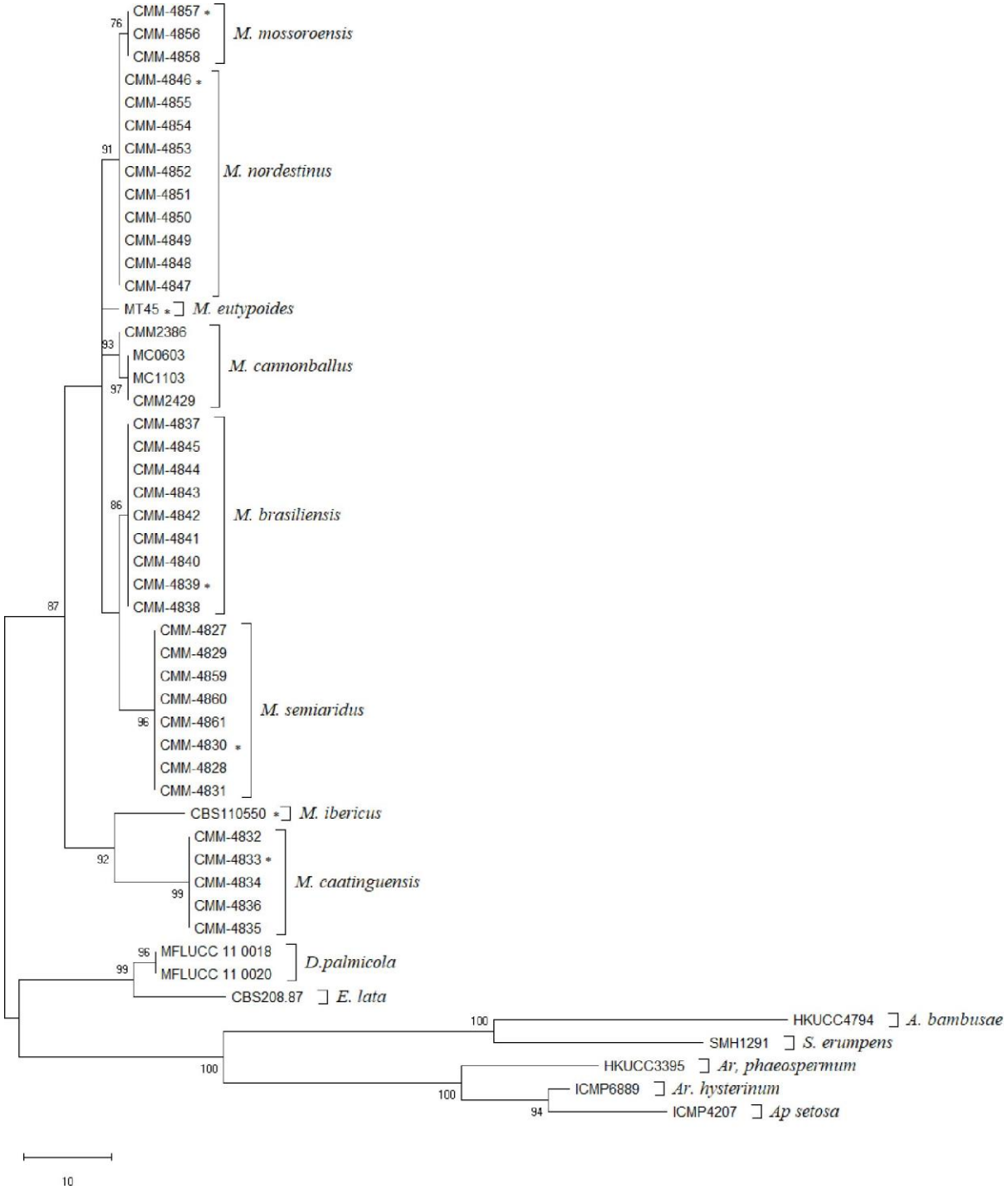

Figure S19 The first of 10 equally most parsimonious trees (TL = 255; CI = 0.76; RI = 0.90) resulting from a parsimony analysis of the combined (LSU and SSU) sequence alignment, used to infer the phylogenetic relationships of the genus *Monosporascus* inside the family Diatrypaceae and the order Xylariales. The bootstrap support values (≤ 70%) are indicated at the nodes and the scale bar represents the number of changes. The tree was rooted using *Arecophila bambusae* (HKUCC4794), *Seynesia erumpens* (SMH 1291), *Arthrinium hysterinum* (ICMP 6889), *Ar. phaeospermum* (HKUCC 3395) and *Apiospora setosa* (ICMP 4207) as outgroup sequences. Ex-type strains are indicated with an asterisk.

CHAPTER III

IDENTIFICATION AND PATHOGENICITY OF *Macrophomina* SPECIES COLLECTED FROM WEEDS IN MELON FIELDS IN NORTHEASTERN BRAZIL

Abstract

In this work, a collection of 94 *Macrophomina* isolates obtained from roots of two symptomless weed species, *Trianthema portulacastrum* and *Boerhavia diffusa*, collected during surveys conducted during 2015 and 2016 in melon production fields in Northeastern Brazil, were characterized by using phenotypical and molecular techniques. Phylogenetic analysis of the EF1-α gene, allowed the identification of 32 isolates as *M. phaseolina* and 62 isolates as *M. pseudophaseolina*. Results of a pathogenicity test performed on melon seedlings of the cv. 'Gladial' revealed that all *M. phaseolina* isolates inoculated were able to cause disease to melon seedlings, but only some *M. pseudophaseolina* isolates were able to infect them. This study represents the first report of *M. pseudophaseolina* in both *T. portulacastrum* and *B. diffusa* weeds, which are prevalent in the main Brazilian melon producing and exporting regions. Information about the biology and epidemiology of *M. pseudophaseolina* is scarce because of its recent description, thus further research is needed for a better understanding of this fungus as a potentially emerging pathogen of melon and other crops.

KEYWORDS: *Boerhavia diffusa*, *Macrophomina phaseolina*, *Macrophomina pseudophaseolina*, *Trianthema portulacastrum*, soilborne pathogen.

1 INTRODUCTION

Brazil is the 11th largest world producer of melon (*Cucumis melo*), with a production of 596,000 t in 2016 (FAOSTAT, 2018). Melon is currently the second most exported fruit in Brazil, generating an income of US$ 162.9 million (Anuário, 2018). The main melon producing states are Rio Grande do Norte (RN) and Ceará (CE) located in Northeastern Brazil, which account for 95.2 % of the total production (IBGE, 2018).

One of the main diseases of melon and watermelon (*Citrullus lanatus*) crops in Northeastern Brazil is root rot and vine decline (RRVD) caused by a complex of different soilborne pathogens such as *Monosporascus cannonballus* Pollack & Uecker, *Rhizoctonia*

solani Kühn and *Macrophomina phaseolina* (Tassi) Goid. (Andrade et al., 2005). *Macrophomina phaseolina* has been also reported as an important cucurbit pathogen in other countries of the world such as Iran (Salari, Panjehkeh, Nasirpoor, & Abkhoo, 2012), Israel (Cohen, Omari, Porat, & Edelstein, 2012; Reuveni, Krikun, Nachmias, & Schlevin, 1982), Chile (Jacob, Krarup, Díaz, & Latorre, 2013) and Egypt (El-Kolaly & Abdel-Sattar, 2013).

Fungi of the genus *Macrophomina* are members of the family Botryosphaeriaceae, belonging to the class Dothideomycetes. Currently, there are three species of *Macrophomina* reported in the world: *M. phaseolina*, *M. pseudophaseolina* Crous, Sarr & Ndiaye (Sarr, Ndiaye, Groenewald, & Crous, 2014), and *M. euphorbiicola* A.R. Machado, D.J. Soares & O.L. Pereira (Machado, Pinho, Soares, Medeiros-Gomes, & Pereira, 2018). These species are soilborne fungi but, *M. phaseolina* has a wider host range, being pathogenic to more than 500 crops and in non-cultivated species (Farr & Rossman, 2018), including economically important hosts, such as common bean, cotton, sorghum and soybean (Baird & Brock, 1999; Baird, Watson, & Scruggs, 2003; Cruciol & Costa, 2017; Funnell-Harris, O'neill, Sattler, & Yerka, 2016; Rusuku, Buruchara, Gatabazi, & Pastor-Corrales, 1997). *Macrophomina phaseolina* has a worldwide distribution, but it is considered economically more important in subtropical and tropical countries with semi-arid climate (Wrather et al., 1997; Wrather et al., 2001). On the contrary, *M. euphorbiicola* has been described affecting only *Jatropha gossypifolia* and *Ricinus communis* in Brazil (Machado, Pinho, Soares, Medeiros-Gomes, & Pereira, 2018), and *M. pseudophaseolina* affecting *Abelmoschus esculentus*, *Arachis hypogaea*, *Hibiscus sabdarifa* and *Vigna unguiculata* in Senegal (Sarr, Ndiaye, Groenewald, & Crous, 2014) and *A. hypogaea*, *Gossypium hirsutum* and *R. communis* in Brazil (Machado, Pinho, Soares, Medeiros-Gomes, & Pereira, 2018).

According to Agustí-Brisach, Gramaje, León, García-Jiménez, & Armengol (2011) & Chaves, Braun, Eiras, Colariccio, & Galleti (2003), weeds can act as secondary hosts of phytopathogens, serving as potential sources of inoculum. Fuhlbohm, Ryley, & Aitken (2012) isolated *M. phaseolina* from the roots of symptomless plants of 23 weed species found in Australian mung bean (*V. radiata*) fields, and all isolates were pathogenic on mung bean seedlings. In similar studies, Sales Júnior et al. (2012) & Rodrigues (2013) confirmed the occurrence of *M. cannonballus*, *M. phaseolina* and *Rhizoctonia solani*, causal agents of RRVD, on melon and on roots of several weed species prevalent in melon cultivation areas in Northeastern Brazil. More specifically, Rodrigues (2013) isolated *M. phaseolina* from 85.7% of the analyzed weed species. Among these, *Trianthema portulacastrum* L. and *Boerhavia diffusa* L. were confirmed as hosts of *M. phaseolina*.

Claudino & Soares (2014) hypothesized that in addition to *M. phaseolina*, other species of *Macrophomina* could be present in Brazil. This was recently confirmed by the report of *M. euphorbiicola* and *M. pseudophaseolina* associated with charcoal rot of oilseed crops in this country (Machado, Pinho, Soares, Medeiros-Gomes, & Pereira, 2018). In this context, the increasing economic importance of RRVD of melons associated with *M. phaseolina* in Northeastern Brazil, as well as the existing reports of weeds as hosts of this fungus (Fuhlbohm, Ryley, & Aitken, 2012; Rodrigues, 2013; Sales Júnior et al., 2012), suggest that more than one species of *Macrophomina* may be also present on weeds growing in melon fields in this region. Thus, the objective of this work was to characterize a wide collection of *Macrophomina* isolates obtained from roots of *T. portulacastrum* and *B. diffusa* weeds growing in melon production fields in Northeastern Brazil by using molecular techniques, as well as to evaluate its pathogenicity to melon seedlings.

2 MATERIALS AND METHODS

2.1 Sampling and fungal isolation

Field surveys were conducted during 2015 and 2016 in eight major commercial melon cropping areas, located in the agricultural centers of Mossoró and Assú (RN state) and Icapuí (CE state), Northeastern Brazil (Figure 1). Symptomless *T. portulacastrum* and *B. diffusa* weed species were selected based on their prevalence in commercial melon fields in RN and CE states and previous reports confirming its role as alternative hosts of *M. phaseolina* (Rodrigues, 2013). Two fields (2 ha each) were surveyed per area and thirty plants of each weed species were collected per field.

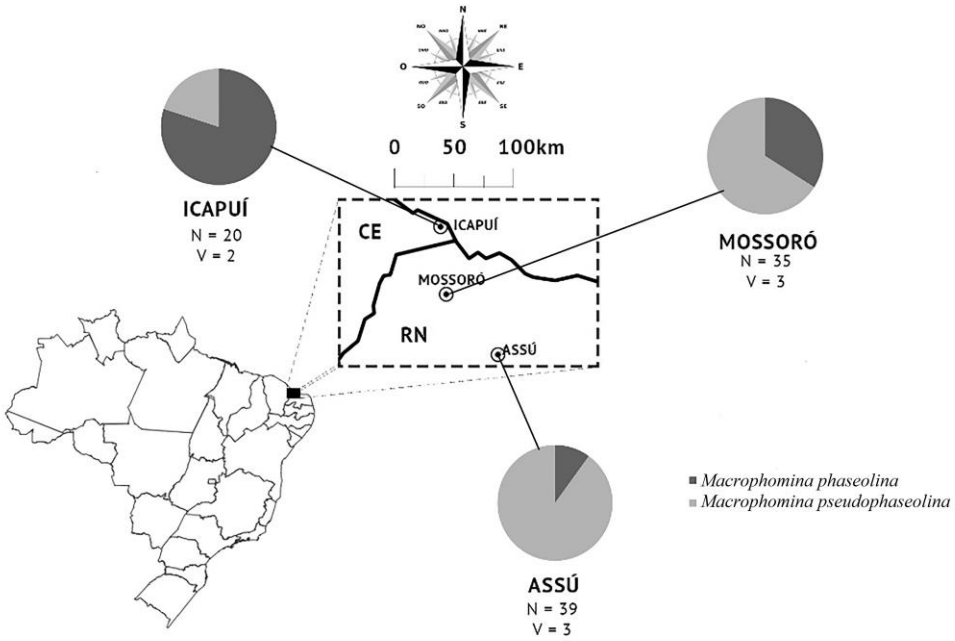

Figure 1 - Collection sites of *Macrophomina* species obtained from the weeds *Trianthema portulacastrum* and *Boerhavia diffusa* in the melon growing areas of Mossoró and Assú (Rio Grande do Norte state) and Icapuí (Ceará state), located in the Northeast Region of Brazil. Circles represent association frequency of each *Macrophomina* species in each agricultural area sampled, N is the number of isolates analyzed in each agricultural area, and V is the number of commercial crops areas sampled in each agricultural center. CE, Ceará; RN, Rio Grande do Norte.

For fungal isolation, roots of weeds were washed under running tap water, immersed for 1 min in 1.5% sodium hypochlorite solution, and washed twice with distilled water for 1 min. Subsequently, small pieces of roots (4–5 mm) were dried on sterilized paper towels, and plated in Petri plates with Potato Dextrose Agar (PDA; Merck KGaA, Darmstadt, Germany) supplemented with 0.5 g L^{-1} streptomycin sulphate (PDAS) (seven pieces per plate). Plates were incubated at 30±1°C in the dark for 3–4 days. Fungal colonies emerging from roots pieces, which were morphologically similar to *Macrophomina* (Sarr, Ndiaye, Groenewald, & Crous, 2014) were transferred to PDA plates and incubated at 30±1°C in the dark.

For the identification of the colonies at the genus level, slides were prepared for microscopy containing fungal structures (mycelium and sclerotia), stained with l*actophenol cotton blue*, observed under an optical microscope and compared to the typical morphological characteristics of the genus *Macrophomina* (Sarr, Ndiaye, Groenewald, & Crous, 2014). Based on this, a total of 94 isolates were tentatively identified as *Macrophomina*. All isolates

were hyphal-tipped and, then, they were stored on sandy-organic substrate and *Castellani's method* with distilled water *(*Alfenas & Mafia, 2016; Medeiros, Melo, Ambrósio, Nunes, & Costa, *2015).*

2.2 DNA isolation, PCR amplification and sequencing

Molecular analysis was used to identify 94 isolates of *Macrophomina* at the species level (Table 1). Total genomic DNA was extracted from mycelium and sclerotia of pure cultures grown on PDA for two weeks at 30±1°C in the dark, using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, USA) following the manufacturer's short protocol instructions with some modifications in the samples preparation step. Briefly, lysis buffer P1 (650 µl) was added to the mycelium and sclerotia in a 2 ml screw-capped conical tubes (Thermo Scientific) containing four metal 2.38 mm beads (Qiagen) and two tungsten carbide 3 mm beads (Qiagen) and homogenized twice at speed 5 m s^{-1} for 20 sec using FastPrep-24™5G homogenizer (MP Biomedicals, Santa Ana, CA, USA).

TABLE 1 - List of isolates used in phylogeny of *Macrophomina* species.

Species	Strain number	Host	Collected by/year	Location	GenBank Accession Numbers
M. phaseolina	CMM 4733[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373464
M. phaseolina	CMM 4734	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373440
M. phaseolina	CMM 4735	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373441
M. phaseolina	CMM 4736	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373436
M. phaseolina	CMM 4737	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373442
M. phaseolina	CMM 4738	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373461
M. phaseolina	CMM 4739	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373457
M. phaseolina	CMM 4740	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373465
M. phaseolina	CMM 4741	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373443
M. phaseolina	CMM 4742[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373466
M. phaseolina	CMM 4743	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373453
M. phaseolina	CMM 4744	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Ceará, Icapuí	MH373458
M. phaseolina	CMM 4745	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Ceará, Icapuí	MH373467
M. phaseolina	CMM 4746	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Ceará, Icapuí	MH373462
M. phaseolina	CMM 4747	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373437
M. phaseolina	CMM 4748[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373438
M. phaseolina	CMM 4749	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373444
M. phaseolina	CMM 4750[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373445
M. phaseolina	CMM 4751	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373446
M. phaseolina	CMM 4752	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373454
M. phaseolina	CMM 4753	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373459
M. phaseolina	CMM 4754	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373447
M. phaseolina	CMM 4755[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373463
M. phaseolina	CMM 4756	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373460
M. phaseolina	CMM 4757	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373448
M. phaseolina	CMM 4758[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373450
M. phaseolina	CMM 4759	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373449
M. phaseolina	CMM 4760[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373439

Species	Strain number	Host	Collected by/year	Location	GenBank Accession Numbers
M. phaseolina	CMM 4761[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373452
M. phaseolina	CMM 4762[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373456
M. phaseolina	CMM 4763	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373451
M. phaseolina	CMM 4764[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373455
M. phaseolina	CDA 1100	*Ricinus communis*	-	Brazil, Bahia	KU058910
M. phaseolina	CBS 457.70	*Phaseolus aureus*	-	Denmark	KF952009
M. phaseolina	CBS 461.70	*Phaseolus vulgaris*	-	Denmark	KF952013
M. phaseolina	CBS 270.34	*Vigna sinensis*	-	USA, Missouri	KF952005
M. phaseolina	CBS 205.47	*Phaseolus vulgaris*	-	Italy	KF951997
M. phaseolina	CBS 224.33	*Sesamum indicum*	-	Uganda	KF951998
M. pseudophaseolina	CMM 4765[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373511
M. pseudophaseolina	CMM 4766	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Ceará, Icapuí	MH373507
M. pseudophaseolina	CMM 4767	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Ceará, Icapuí	MH373513
M. pseudophaseolina	CMM 4768	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Ceará, Icapuí	MH373468
M. pseudophaseolina	CMM 4769	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373469
M. pseudophaseolina	CMM 4770[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373470
M. pseudophaseolina	CMM 4771	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373471
M. pseudophaseolina	CMM 4772	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373514
M. pseudophaseolina	CMM 4773	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373472
M. pseudophaseolina	CMM 4774	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373512
M. pseudophaseolina	CMM 4775	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373473
M. pseudophaseolina	CMM 4776	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373508
M. pseudophaseolina	CMM 4777	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373474
M. pseudophaseolina	CMM 4778	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373509
M. pseudophaseolina	CMM 4779	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373475
M. pseudophaseolina	CMM 4780[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373515
M. pseudophaseolina	CMM 4781	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373476
M. pseudophaseolina	CMM 4782	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373477
M. pseudophaseolina	CMM 4783	*Trianthema portulacastrum*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373478
M. pseudophaseolina	CMM 4784	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373479

Species	Strain number	Host	Collected by/year	Location	GenBank Accession Numbers
M. pseudophaseolina	CMM 4785	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373480
M. pseudophaseolina	CMM 4786[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373481
M. pseudophaseolina	CMM 4787	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373482
M. pseudophaseolina	CMM 4788[a]	*Trianthema portulacastrum*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373483
M. pseudophaseolina	CMM 4789	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373484
M. pseudophaseolina	CMM 4790[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373485
M. pseudophaseolina	CMM 4791	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373486
M. pseudophaseolina	CMM 4792	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373487
M. pseudophaseolina	CMM 4793	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373488
M. pseudophaseolina	CMM 4794	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373489
M. pseudophaseolina	CMM 4795	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373490
M. pseudophaseolina	CMM 4796	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373491
M. pseudophaseolina	CMM 4797	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373492
M. pseudophaseolina	CMM 4798	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373493
M. pseudophaseolina	CMM 4799	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373494
M. pseudophaseolina	CMM 4800[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373516
M. pseudophaseolina	CMM 4801	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373517
M. pseudophaseolina	CMM 4802	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373495
M. pseudophaseolina	CMM 4803	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373496
M. pseudophaseolina	CMM 4804	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Assú	MH373527
M. pseudophaseolina	CMM 4805	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373497
M. pseudophaseolina	CMM 4806	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373498
M. pseudophaseolina	CMM 4807[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373518
M. pseudophaseolina	CMM 4808	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373499
M. pseudophaseolina	CMM 4809	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373519
M. pseudophaseolina	CMM 4810	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373520
M. pseudophaseolina	CMM 4811	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373501
M. pseudophaseolina	CMM 4812	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373521
M. pseudophaseolina	CMM 4813	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Assú	MH373510
M. pseudophaseolina	CMM 4814[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373500

Species	Strain number	Host	Collected by/year	Location	GenBank Accession Numbers
M. pseudophaseolina	CMM 4815	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373522
M. pseudophaseolina	CMM 4816	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373523
M. pseudophaseolina	CMM 4817	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373524
M. pseudophaseolina	CMM 4818	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373504
M. pseudophaseolina	CMM 4819	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373525
M. pseudophaseolina	CMM 4820	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373526
M. pseudophaseolina	CMM 4821[a]	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373502
M. pseudophaseolina	CMM 4822	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373503
M. pseudophaseolina	CMM 4823	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373505
M. pseudophaseolina	CMM 4824	*Boerhavia diffusa*	A.M.P. Negreiros, 2015	Brazil, Rio Grande do Norte, Mossoró	MH373506
M. pseudophaseolina	CMM 4825	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373528
M. pseudophaseolina	CMM 4826	*Boerhavia diffusa*	A.M.P. Negreiros, 2016	Brazil, Rio Grande do Norte, Mossoró	MH373529
M. pseudophaseolina	CPC 21394	*Vigna unguiculata*	-	Senegal, Thiès	KF952148
M. pseudophaseolina	CPC 21417	*Arachis hypogaea*	-	Senegal, Louga	KF952153
M. euphorbiicola	CMM4045	*Jatropha gossypifolia*	-	Brazil: Paraíba	KU058898
M. euphorbiicola	CMM4134	*Ricinus communis*	-	Brazil: Bahia	KU058906
M. euphorbiicola	CMM4145	*Ricinus communis*	-	Brazil: Bahia	KU058907
Botryosphaeria dothidea	CMW 8000	*Prunus* sp.	B. Slippers, 2000	Switzerland, Crocifisso	AY236898

[a] Isolates used in the pathogenicity test.

The translation elongation factor-1*alpha* (*tef-1α*) was used as the *Macrophomina* species marker (Machado, Pinho, Soares, Medeiros-Gomes, & Pereira, 2018; Sarr, Ndiaye, Groenewald, & Crous, 2014). Polymerase Chain Reaction (PCR) amplifications were performed using Horse-Power™ Taq DNA Polymerase (Canvax Biotech SL, Córdoba, Spain) and the primers EF728F and EF986R (Carbone & Kohn, 1999). The amplification program consisted of an initial step of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 45 sec. A final extension was performed at 72°C for 10 min. The PCR products were separated by electrophoresis in 1% agarose gel (agarose D-1 Low EEO, Conda, Madrid, Spain), stained with Realsafe (Real, Paterna Valencia, Spain), and visualized under UV light. Gene-ruler 100-bp DNA ladder plus was used as a molecular weight marker (Fermentas, St. Leon-Rot, Germany). The resulting products were sequenced by Macrogen Inc. (Madrid, Spain). Consensus sequences were assembled using Sequencher software package version 5.0 (Gene Codes Corp., Ann Arbor, MI).

2.3 Phylogenetic analyses

The DNA sequences generated in this study together with representative sequences for the genus *Macrophomina* (Machado, Pinho, Soares, Medeiros-Gomes, & Pereira, 2018; Sarr, Ndiaye, Groenewald, & Crous, 2014) from GenBank (Table 1) were aligned using the ClustalW (Thompson, Higgins, & Gibson, 1994) contained within MEGA7 software package (Kumar, Stecher, & Tamura, 2016). The alignments were inspected and manual adjustments were made when necessary. Incomplete portions at either end of the alignments were excluded prior to analyses. All sequences from this study were deposited on GenBank. The tree was rooted to *Botryosphaeria dothidea* CMW8000 (Table 1). Sequence alignments were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S23031).

The sequences of all isolates were analyzed through Bayesian inference (BI), Maximum Likelihood (ML) and Maximum Parsimony (MP) generating phylogenetic trees that enabled their identifications. For BI analysis, the optimal substitution model was determined using MrModeltest software v. 2.2. (Nylander, 2004), computed using MrBayes v3.2 (Ronquist et al., 2012) with four simultaneous *Markov Chain Monte Carlo* from random trees over 100 million generations with trees sampled every 1000th generation were run, resulting in 100,000 total trees. The first 25% of saved trees were discarded as the “burn-in” phase and posterior probabilities determined from the remaining. The ML analysis was

performed with RAxML-HPC2 on XSEDE v. 8.2.10 (Stamatakis, 2014) using a GTR+GAMMA substitution model with 1000 bootstrap iterations. Both BI and ML were run on the CIPRES Science Gateway portal (Miller, Pfeiffer, & Schwartz, 2012), and the trees were visualized by FigTree (http://tree.bio.ed.ac.uk/software/figtree/).The MP genealogies was estimated in MEGA7 software package (Kumar, Stecher, & Tamura, 2016), using the Tree-Bisection-Regrafting (TBR) algorithm, and the tree was visualized in the same software.

2.4 Pathogenicity and virulence on melon

Ten representative isolates of each *Macrophomina* species were used for this experiment (Table 1). In addition one isolate of *M. phaseolina* obtained from melon plants (CMM-1531) was included as positive control. Melon seeds of the cv. 'Gladial' were germinated in a 'Tropstrato HT®' commercial substrate previously autoclaved. The plants were irrigated daily to drainage with tap water and were not fertilized during the experiment.

The inoculation technique used was the toothpick method, because of the easy multiplication of inoculum and fast inoculation (Ambrósio et al., 2015; Medeiros, Melo, Ambrósio, Nunes, & Costa, 2015; Mir et al., 2018). Twelve mm long toothpicks were placed, with the sharpened end up, in holes made in a 90 mm diameter filter paper. The toothpicks were then placed in a Petri plate and autoclaved for 30 min, for 2 days with an interval of 24 h, at 121ºC. Twenty ml of melted PDA + streptomycin sulfate was added to each toothpick-containing Petri plate. Once solidified, the PDAS plates were inoculated with five mycelial plugs (8 mm in diameter) of from each isolate of *Macrophomina* and then were incubated at 28 ± 2ºC in the dark for 8 days.

Melon seedlings were inoculated 10 days after sowing (DAS) by inserting the toothpicks colonized with mycelia and microsclerotia of the corresponding isolate in each hypocotyl, 1 cm above the soil. Non colonized toothpicks were used as negative controls. The inoculated plants were maintained in a greenhouse at an average temperature of 35°C for 30 days, under natural daylight conditions.

Thirty days after inoculation, the virulence of the isolates was assessed as disease severity using a modified version of the rating scale described by Ambrósio et al. (2015), where, 0 = symptomless, 1 = less than 3% of shoot tissues infected, 2 = 3–10% of shoot tissues infected, 3 = 11–25% of shoot tissues infected, 4 = 26–50% of shoot tissues infected and 5 = more than 50% of shoot tissues infected. Disease incidence was determined as the

total number of infected plants from each *Macrophomina* species and expressed as percentage.

Seven small fragments (0.2 to 0.5 cm) of necrotic lesions from each symptomatic plant were cut and placed on PDAS in an attempt to recover the inoculated fungi and complete Koch's postulates. *Macrophomina* spp. were identified as described above.

The experiment was arranged in a completely randomized design with five replicates per treatment (isolate) and one plant per replicate. The experiment was conducted twice. For each species of *Macrophomina*, a preliminary ANOVA was performed to determine if there were significant differences between the two repetitions of the experiments, and if the data could be combined. Disease severity and incidence results by isolates of *M. phaseolina* and *M. pseudophaseolina* were analysed with the nonparametric Kruskal-Wallis test at the probability level of 5% ($p < .05$) using the software Assistat, version 7.7 (Silva & Azevedo, 2016). Differences in disease severity and incidence caused by *Macrophomina* species were determined using Mann-Whitney test at the 5% significance level using STATISTIX v. 9.0 (Analytical Software).

3 RESULTS

3.1 PCR, sequencing, and *tef-1α* phylogeny

All the isolates were identified based on the phylogenetic analysis of the *EF1-α* gene, which was amplified with the primers EF728F and EF986R. A PCR fragment ranging from 217–221 bp was obtained for them. The first approximation to the identification of the 94 isolates, putative belonging to *Macrophomina* genus, was based on the BLAST analysis of their *EF1-α* sequence.

Phylogenetic analysis on the *tef-1α* locus alignment contained a total of 106 taxa, from which 94 were of the studied isolates, six of *M. phaseolina*, two of *M. pseudophaseolina*, three of *M. euphorbiicola*, and *Botryosphaeria dothidea* CMW8000, which was used as outgroup, resulting in a dataset of 227 characters, including alignment gaps, of which 162 were constant, 22 parsimony-informative, and 43 parsimony-uninformative. Sequences of *M. phaseolina*, *M. pseudophaseolina*, *M. euphorbiicola* and *B. dothidea* were obtained from GenBank (Table 1).

The topology of the tree identified by MP analysis were similar to those obtained by the BI and ML analyses, therefore only the MP tree is presented, with ML and MP bootstrap support values and BI posterior probability scores at the nodes.

The *Macrophomina* group was divided into three well-supported clades (Figure 2). Each clade corresponded to previously described species. One clade (62 isolates) clustered together with the species *M. pseudophaseolina* (KF952153, KF952148), strongly supported by bootstrap values (ML/MP/BI: 99/100/1). The remaining 32 isolates clustered together with *M. phaseolina* (KF951997, KU058910, KF952009, KF952013, KF952005, KF951998), with high bootstrap support for ML and MP (ML/MP/BI: 98/94/0.99). These isolates were subdivided into three sub-clades, with low support. None of our isolates clustered with *M. euphorbiicola*.

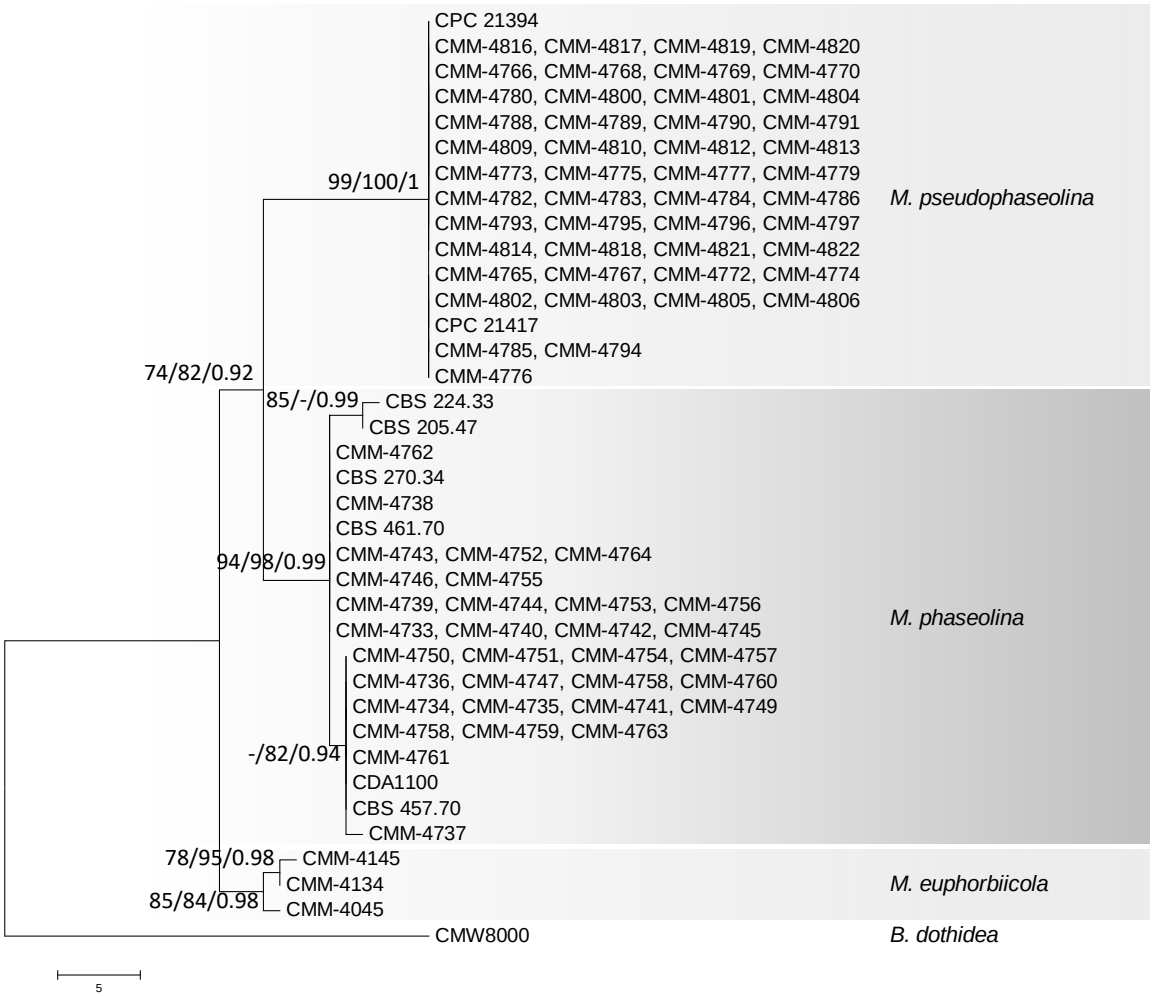

Figure 2 - Phylogenetic relationships within the genus *Macrophomina*. Maximum parsimony (MP) phylogeny based on *tef-1α* sequence alignment. Nodes receiving Maximum Likelihood and MP bootstrap > 70% and Bayesian posterior probabilities > 0.9 are considered as supported. The tree was rooted to *Botryosphaeria dothidea* CMW8000.

3.2 Pathogenicity and virulence on melon

All *M. phaseolina* isolates inoculated were pathogenic to melon, while only three *M. pseudophaseolina* isolates (CMM-4780, CMM-4788 and CMM-4807) were able to infect melon seedlings. Percent recovery of the inoculated isolates from the necrotic tissues of symptomatic plants was higher than 95% and reisolated species were confirmed to be the same inoculated previously. No isolates were obtained from the negative controls.

Disease severity and disease incidence presented significant differences ($P \leq 0.05$) between *M. phaseolina* and *M. pseudophaseolina* isolates (Table 2). Amaral et al. (2016) evaluating the pathogenicity of 22 *M. phaseolina* isolates in cowpea, observed that all isolates were pathogenic to this crop, with levels of disease severity varying from 8.8 to 78.4 %. The presence of pathogenic variability among of *Macrophomina* species characterized by variations in disease intensity should be considered when searching for sources of resistance to these pathogens. When results from all isolates of each species were combined, disease severity was higher for *M. phaseolina* (3.84) than *M. pseudophaseolina* (0.22) (Figure 3A). Disease incidence caused by *M. phaseolina* on melon seedlings was also higher (86%) than *M. pseudophaseolina* (10%) (Figure 3B).

TABLE 2 - Reaction of *Cucumis melo* seedlings cv. Gladial to isolates of *Macrophomina phaseolina* and *M. pseudophaseolina*.

Isolates	***Macrophomina phaseolina***				**Isolates**	***Macrophomina pseudophaseolina***			
	Disease Severity		Disease Incidence			Disease Severity		Disease Incidence	
	Rank	Mean	Rank	Mean (%)		Rank	Mean	Rank	Mean (%)
CMM-4733	34.9 ab	4.0	30.5 ab	80.0	**CMM-4765**	25.5 a	0.0	25.5 a	0.0
CMM-4742	42.0 b	5.0	36.5 b	100.0	**CMM-4770**	25.5 a	0.0	25.5 a	0.0
CMM-4748	19.7 ab	3.4	36.5 b	100.0	**CMM-4780**	36.5 ab	0.8	37.5 ab	40.0
CMM-4750	8.8 a	0.6	12.5 ab	20.0	**CMM-4786**	25.5 a	0.0	25.5 a	0.0
CMM-4755	42.0 b	5.0	36.5 b	100.0	**CMM-4788**	36.9 ab	1.2	37.5 ab	40.0
CMM-4761	24.6 ab	3.0	30.5 ab	80.0	**CMM-4790**	25.5 a	0.0	25.5 a	0.0
CMM-4762	42.0 b	5.0	36.5 b	100.0	**CMM-4800**	25.5 a	0.0	25.5 a	0.0
CMM-4758	24.3 ab	2.8	30.5 ab	80.0	**CMM-4807**	30.6 a	0.2	31.5 ab	20.0
CMM-4760	37.2 ab	4.6	36.5 b	100.0	**CMM-4814**	25.5 a	0.0	25.5 a	0.0
CMM-4764	42.0 b	5.0	36.5 b	100.0	**CMM-4821**	25.5 a	0.0	25.5 a	0.0
CMM-1531	42.0 b	5.0	36.5 b	100.0	**CMM-1531**	58.0 b	5.0	55.5 b	100.0
CONTROL	6.5 a	0.0	6.5 a	0.0	**CONTROL**	25.5 a	0.0	25.5 a	0.0
$c\chi^2$	42.49		39.33			40.26			

$c\chi^2$, chi-squared value significant at 5% by Kruskal–Wallis test. Letters are for comparison of means in the same column.

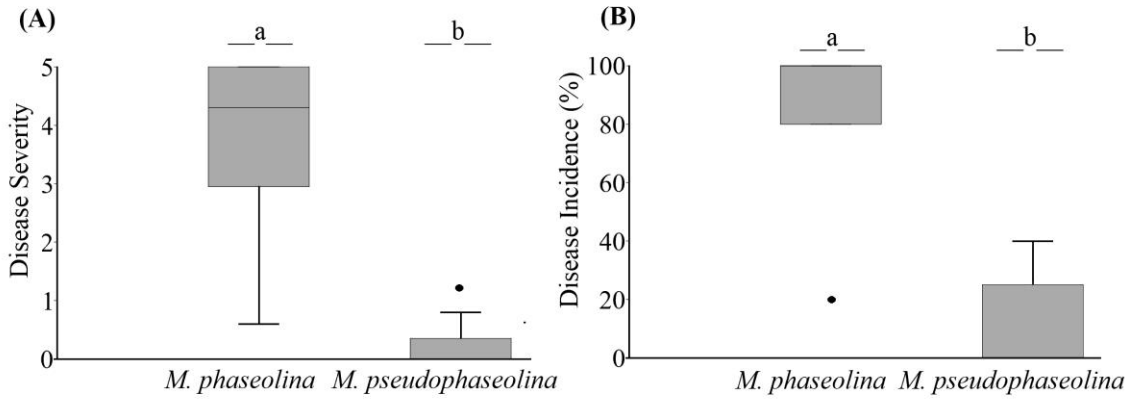

Figure 3 - Boxplots showing (A) Disease Severity and (B) Incidence of the *Macrophomina* species in melon plants. The boxes show the first and third quartiles. Bold horizontal line represents median of group. Lower and upper whiskers extend from the boxes to the extreme values, or outlying values are indicated by black dots. Different lowercase letters indicate significant differences according to Mann-Whitney test ($p \leq 0.05$).

4 DISCUSSION

The characterization of a wide collection of *Macrophomina* isolates obtained from asymptomatic *T. portulacastrum* and *B. diffusa* plants collected in melon growing fields in Northeastern Brazil, confirmed the identification of two *Macrophomina* species, *M. phaseolina* and *M. pseudophaseolina*, associated with the roots of both species. Moreover, *T. portulacastrum* and *B. diffusa* are reported for the first time as new hosts for *M. pseudophaseolina*.

Phylogenetic analyses confirmed the identification of *M. phaseolina* and *M. pseudophaseolina*. It was possible to distinguish both species using the gene *tef-1α*. In recent studies, this gene demonstrated to have potential for use as a tool to identify known species of *Macrophomina* and other Botryosphaeriaceae spp. in diagnostic studies (Machado, Pinho, & Pereira, 2014; Machado, Pinho, Soares, Medeiros-Gomes, & Pereira, 2018; Sarr, Ndiaye, Groenewald, & Crous, 2014).

In our research, *M. pseudophaseolina* was the most frequent species found among the 94 *Macrophomina* spp. isolates collected from *T. portulacastrum* and *B. diffusa* weeds in melon production fields located in Northeastern Brazil. Nevertheless, Sarr, Ndiaye, Groenewald, & Crous (2014) reported different results when determining the genetic vatiation of a global set of 189 isolates of *Macrophomina* spp. obtained from 23 hosts and 30 soil samples in 15 countries, in which only 18 isolates were identified as *M. pseudophaseolina* and 171 isolates were *M. phaseolina*. Recently, Machado, Pinho, Soares, Medeiros-Gomes, & Pereira (2018) determined the identity of 35 *Macrophomina* spp. isolates obtained from

diverse oilseed crops in Brazil using phylogenetic analysis and morphological characteristics, from which only 11 were confirmed as *M. pseudophaseolina*.

Results of the pathogenicity test to melon seedlings with *M. phaseolina* and *M. pseudophaseolina* conducted under greenhouse conditions revealed that both *Macrophomina* species are able to infect this crop, but *M. phaseolina* presented higher disease incidence and severity than *M. pseudophaseolina*. Similar differences in virulence of *Macrophomina* species were also observed by Ndiaye, Sarr, Cisse, & Ndoye (2015), where the isolates of *M. phaseolina* presented the highest values of incidence of charcoal rot when compared with *M. pseudophaseolina* after inoculation of bean cultivars.

It is well known that *M. phaseolina* can be isolated from symptomless weed species (Fuhlbohm, Ryley, & Aitken, 2012; Rodrigues, 2013; Sales Júnior et al., 2012), which can serve as alternative hosts for the pathogen. This fact, together with the longevity of its microsclerotia in soil, enable *M. phaseolina* to survive for many years in the absence of a host crop (Short, Wyllie, & Bristow, 1980). Although the information about the host range of *M. pseudophaseolina* is limited due to its recent description (Machado, Pinho, & Pereira, 2014; Machado, Pinho, Soares, Medeiros-Gomes, & Pereira, 2018; Sarr, Ndiaye, Groenewald, & Crous, 2014), our results demonstrate that *T. portulacastrum* and *B. diffusa* can also be considered sources of inoculum for this fungus in cucurbits fields. Nevertheless, to date only *M. phaseolina* has been reported as causal agent of RRVD of melon in Northeastern Brazil (Andrade et al., 2005; Rodrigues, 2013).

This work reports for the first time the association of *M. pseudophaseolina* with asymptomatic roots of *T. portulacastrum* and *B. diffusa* weeds, which are common in the main Brazilian producing and exporting regions of melon. Although *M. pseudophaseolina* was the most frequent species and the pathogenicity tests showed that some isolates are able to infect melon seedlings, further research is needed for a better understanding of this fungus as a potentially emerging pathogen of melon and other crops (Machado, Pinho, Soares, Medeiros-Gomes, & Pereira, 2018; Sarr, Ndiaye, Groenewald, & Crous, 2014).

ACKNOWLEDGMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – Finance Code 001 and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

Agustí-Brisach, C., Gramaje, D., León, M., García-Jiménez, J., & Armengol, J. (2011). Evaluation of vineyard weeds as potential hosts of black-foot and Petri disease pathogens. *Plant Disease*, **95**, 803-810.

Alfenas, A. C., & Mafia, R. G. (2016). Métodos em fitopatologia (p. 516). Viçosa, Brasil: Ed. UFV, Universidade Federal de Viçosa.

Amaral, A. G. G.; Noronha, M. A. N; Damasceno-Silva, K. J. Patogenicidade de isolados de Macrophomina phaseolina em feijão-caupi. IV Congresso Nacional de Feijão-Caupi, **93**.

Ambrósio, M. M. Q., Dantas, A. C. A., Martínez-Perez, E., Medeiros, A. C., Nunes, G. H. S., & Picó, M. B. (2015). Screening a variable germplasm collection of *Cucumis melo* L. for seedling resistance to *Macrophomina phaseolina*. *Euphytica*, **206**, 287-300.

Andrade, D. E. G. T., Michereff, S. J., Biondi, C. M., Nascimento, C. W. A., & Sales Júnior, R. (2005). Frequência de fungos associados ao colapso do meloeiro e relação com características físicas, químicas e microbiológicas dos solos. *Summa Phytopathologica*, **31**, 327-333.

Anuário - Anuário Brasileiro da Fruticultura 2018 (p. 88). (2018). Santa Cruz do Sul: Ed. Gazeta Santa Cruz.

Baird, R. E., & Brock, J. H. (1999). First report of *Macrophomina phaseolina* on cotton (*Gossypium hirsutum*) in Georgia. *Plant Disease*, **83**, 487-487.

Baird, R. E., Watson, C. E., & Scruggs, M. (2003). Relative longevity of *Macrophomina phaseolina* and associated mycobiota on residual soybean roots in soil. *Plant Disease*, **87**, 563-566.

Carbone, I., & Kohn, L. M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia*, **91**, 553-556.

Chaves, A. L. R., Braun, M. R., Eiras, M., Colariccio, A., & Galleti, S. R. (2003). *Erigon bonariensis*: hospedeira alternativa do *Lettuce mosaic vírus* no Brasil. *Fitopatologia Brasileira*, **28**, 307-311.

Claudino, M. R., & Soares, D. J. (2014). Pathogenicity and aggressiveness of *Macrophomina phaseolina* isolates to castor (*Ricinus communis*). *Tropical Plant Pathology*, **39**, 453-456.

Cohen, R., Omari, N., Porat, A., & Edelstein, M. (2012). Management of *Macrophomina* wilt in melons using grafting or fungicide soil application: pathological, horticultural and economical aspects. *Crop Protection*, **35**, 58-63.

Cruciol, G. C. D., & Costa, M. L. N. (2017). Influência de metodologias de inoculação de *Macrophomina phaseolina* no desempenho de cultivares de soja. *Summa phytopathologica*, **43**, 337-343.

El-Kolaly G. A. A., & Abdel-Sattar M. A. (2013). The etiology of sudden wilt disease syndrome on melon in Egypt. *Nature and Science*, **11**, 79-87.

FAOSTAT (2018). FAO Statistical Databases, Food and Agriculture Organization of the United Nations. http://www.fao.org/faostat/en/#home.

Farr, D. F., & Rossman, A. Y. (2018). Fungal Databases, U.S. National Fungus Collections, ARS, USDA. https://nt.ars-grin.gov/fungaldatabases/.

Fuhlbohm, M. J., Ryley, M. J., & Aitken, E. A. B. (2012). New weed hosts of *Macrophomina phaseolina* in Australia. *Australasian Plant Disease Notes*, **7**, 193-195.

Funnell-Harris, D. L., O'neill, P. M., Sattler, S. E., & Yerka, M. K. (2016). Response of sweet sorghum lines to stalk pathogens *Fusarium thapsinum* and *Macrophomina phaseolina*. *Plant Disease*, **100**, 896-903.

IBGE (2018). Instituto Brasileiro de Geografia e Estatística. https://sidra.ibge.gov.br/home/pms/brasil.

Jacob, C. J., Krarup, C., Díaz, G. A., & Latorre, B. A. (2013). A severe outbreak of charcoal rot in cantaloupe melon caused by *Macrophomina phaseolina* in Chile. *Plant Disease,* **97**, 141.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology Evolution,* **33**, 1870-1874.

Machado, A. R., Pinho, D. B., & Pereira, O. L. (2014). Phylogeny, identification and pathogenicity of the Botryosphaeriaceae associated with collar and root rot of the biofuel plant *Jatropha curcas* in Brazil, with a description of new species of *Lasiodiplodia*. *Fungal Diversity*, **67**, 231-247.

Machado, A. R., Pinho, D. B., Soares, D. J., Medeiros-Gomes, A. A., & Pereira, O. L. (2018). Bayesian analyses of five gene regions reveal a new phylogenetic species of *Macrophomina* associated with charcoal rot on oilseed crops in Brazil. *European Journal of Plant Pathology* (in press). https://doi.org/10.1007/s10658-018-1545-1

Medeiros, A. C., Melo, D. R. M., Ambrósio, M. M. Q., Nunes, G. H. S., & Costa, J. M. (2015). Methods of inoculation of *Rhizoctonia solani* and *Macrophomina phaseolina* in melon (*Cucumis melo*). *Summa Phytopathologica*, **41**, 281-286.

Miller, M. A., Pfeiffer, W., & Schwartz, T. (2012). The CIPRES science gateway: enabling high-impact science for phylogenetics researchers with limited resources. In: *Proceedings of*

the 1st Conference of the Extreme Science and Engineering Discovery Environment: Bridging from the extreme to the campus and beyond (pp. 39). Chicago, Illinois, USA.

Mir, Z. R., Singh, P. K., Zaidi, P. H., Vinayan, M. T., Sharma, S. S., Krishna, M. K., … Nair, S. K. (2018). Genetic analysis of resistance to post flowering stalk rot in tropical germplasm of maize (*Zea mays* L.). *Crop Protection*, **106**, 42-49.

Ndiaye, M., Sarr, M. P., Cisse, N., & Ndoye, I. (2015). Is the recently described *Macrophomina pseudophaseolina* pathogenically different from *Macrophomina phaseolina*? *African Journal of Microbiology Research*, **9**, 2232-2238.

Nylander, J. A. A. (2004). MrModeltest V2. Program Distributed by the Author. Evolutionary Biology Centre, Uppsala University, Sweden.

Reuveni, R., Krikun, J., Nachmias, A., & Schlevin, E. (1982). The role of *Macrophomina phaseolina* in a collapse of melon plants in Israel. *Phytoparasitica*, **10**, 51-56.

Rodrigues, A. P. M. S. (2013). Ocorrência de plantas daninhas como hospedeiras alternativas de fitopatógenos radiculares e avaliação da patogenicidade sobre as culturas do melão e da melancia. Mossoró, Rio Grande do Norte, Brasil, Universidade Federal Rural do Semiárido, Master's Dissertation.

Ronquist, F., Teslenko, M., Van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, **61**, 539-542.

Rusuku, G., Buruchara, R. A., Gatabazi, M., & Pastor-Corrales, M. A. (1997). Occurrence and distribution in Rwanda of soilborne fungi pathogenic to the common bean. *Plant disease*, **81**, 445-449.

Salari, M., Panjehkeh, N., Nasirpoor, Z., & Abkhoo, J. (2012). Reaction of melon (*Cucumis melo* L.) cultivars to soil-borne plant pathogenic fungi in Iran. *African Journal of Biotecnology*, **11**, 15324-15329.

Sales Júnior, R., Oliveira, O. F., Medeiros, E. V., Guimarães, I. M., Correia, K. C., & Michereff, S. J. (2012). Ervas daninhas como hospedeiras alternativas de patógenos causadores do colapso do meloeiro. *Revista Ciência Agronômica*, **43**, 195-198.

Sarr, M. P., Ndiaye, M., Groenewald, J. Z., & Crous, P. W. (2014). Genetic diversity in *Macrophomina phaseolina*, the causal agent of charcoal rot. *Phytopathologia Mediterranea*, **53**, 250-268.

Short, G. E., Wyllie, T. D., & Bristow, P. R. (1980). Survival of *Macrophomina phaseolina* in soil and in residue of soybean. *Phytopathology*, **70**, 13-17.

Silva, F. A. Z., & Azevedo, C. A. V. (2016). The Assistat Software Version 7.7 and its use in the analysis of experimental data. *African Journal Agricultural Research*, **11**, 3733–3740.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, **30**, 1312-1313.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position- specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673-4680.

Wrather, J. A., Anderson, T. R., Arsyad, D. M., Gai, J., Ploper, L. D., Porta-Puglia, A., … Yorinori, J. T. (1997). Soybean disease loss estimates for the top 10 soybean producing countries in 1994. *Plant Disease*, **81**, 107-110.

Wrather, J. A., Anderson, T. R., Arsyad, D. M., Tan, Y., Ploper, L. D., Porta-Puglia, A., … Yorinori, J. T. (2001). Soybean disease loss estimates for the top 10 soybean producing countries in 1998. *Canadian Journal of Plant Pathology*, **23**, 115-121.