Prevalent weeds collected from cucurbit fields in Northeastern Brazil reveal new species diversity in the genus *Monosporascus*

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Abstract

Fungal species belonging to the ascomycete 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 confirms that *Monosporascus* spp. can colonize roots of very diverse hosts, even without causing noticeable disease symptoms, and reveals that the diversity of species in the genus *Monosporascus* is potentially greater than previously expected.

Introduction

The ascomycete 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 76% 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).

Materials and Methods

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).

DNA extraction, PCR amplification and sequencing

Total fungal DNA was extracted using the E.Z.N.A. Plant DNA 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 mycelia 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 5 m/s 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 (Gardes & Bruns, 1993) and ITS4 (White *et al*., 1990), part of the translation elongation factor gene (*tef-1α*) using primers EF1-688F (forward) and EF1-1251R (reverse) (Alves *et al*., 2008), part of the β-tubulin gene (*tub*) using primers BtCadF and BtCadR (Travadon *et al*., 2015), part of the nuclear small subunit rDNA (SSU) using primers NS1 and NS4 (White *et al*., 1990), and part of the large subunit rDNA (LSU) using primers LROR and LR5 (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). 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).

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/).

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 (Table 2) 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. Four 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 (R2) to estimate the optimum growth temperature.

Results

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, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14 and S15) 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, S17, S18 and S19), therefore only the ML trees are presented with ML and MP bootstrap support values and BI posterior probability scores at the nodes.

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 (data not shown) and the two-loci phylogeny (Fig. 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 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 variable 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.

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 ten isolates, and the *M. nordestinus* clade, represented by three isolates, are both phylogeneticaly close to *M. cannonballus* and *M. eutypoides* (Fig. 2). The *M. semiaridus* clade with nine isolates and the *M. brasiliensis* clade, with eight isolates, formed a group closely related between them. The *M. caatinguensis* clade, with five 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 3.

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

Taxonomy

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

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

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. Growth rate of colonies on PDA at 30 and 35ºC was 89 and 96 mm per day, respectively. No growth was observed at 10 and 45ºC.

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. 3).

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. Growth rate of colonies on PDA at 30 and 35ºC was 53 and 45 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. 3).

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. Growth rate of colonies on PDA at 30 and 35ºC was 86 and 74 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 North East 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. Growth rate of colonies on PDA at 30 and 35ºC were 92 and 96 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).

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

Etymology: Name refers to the name refers to 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. Growth rate of colonies on PDA at 30 and 35ºC was 96 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).

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.

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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	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
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

Species	Strain number[a]	Host	Collected/isolated by/year	Location	Cordinates	GenBank Accession Numbers.				
						BT	EF	ITS	LSU	SSU
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	MG74875
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*	-	Brazil, Pau Branco, Rio Grande do Norte		JQ907303	JQ907318	JQ771917	MG748825	MG748784
M. cannonballus	CMM-2429	*C. melo*	-	Brazil, Rio Grande do Norte, Mossoró		JQ907311	JQ907315	JQ762366	MG748826	MG748785
M. cannonballus	MC0603	*C. melo*	-	Spain, Chilches, Castellón		JQ907307	JQ907314	JQ762364	MG748824	MG748783
M. cannonballus	MC1103	*C. melo*	-	Spain, Meliana, Valencia		JQ907302	JQ907317	JQ762369	MG748823	MG748782
M. eutypoides	**MT45**	*Citrullus lanatus*	-	Tunisia, Sidi, Bouzid		JQ973834	JQ958959	JQ958963	MG748827	MG748786
M. ibericus	**CBS 110550**	-	-	Spain, Los Alfaques, Tarragona		JQ973833	JQ958958	JQ973832	MG748828	MG748787
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

Species	Strain number[a]	Host	Collected/isolated by/year	Location	Cordinates	GenBank Accession Numbers.				
						BT	EF	ITS	LSU	SSU
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
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

Species	Strain number[a]	Host	Collected/isolated by/year	Location	Cordinates	GenBank Accession Numbers.				
						BT	EF	ITS	LSU	SSU
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.

Table 2 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

Table 3 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*				

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.

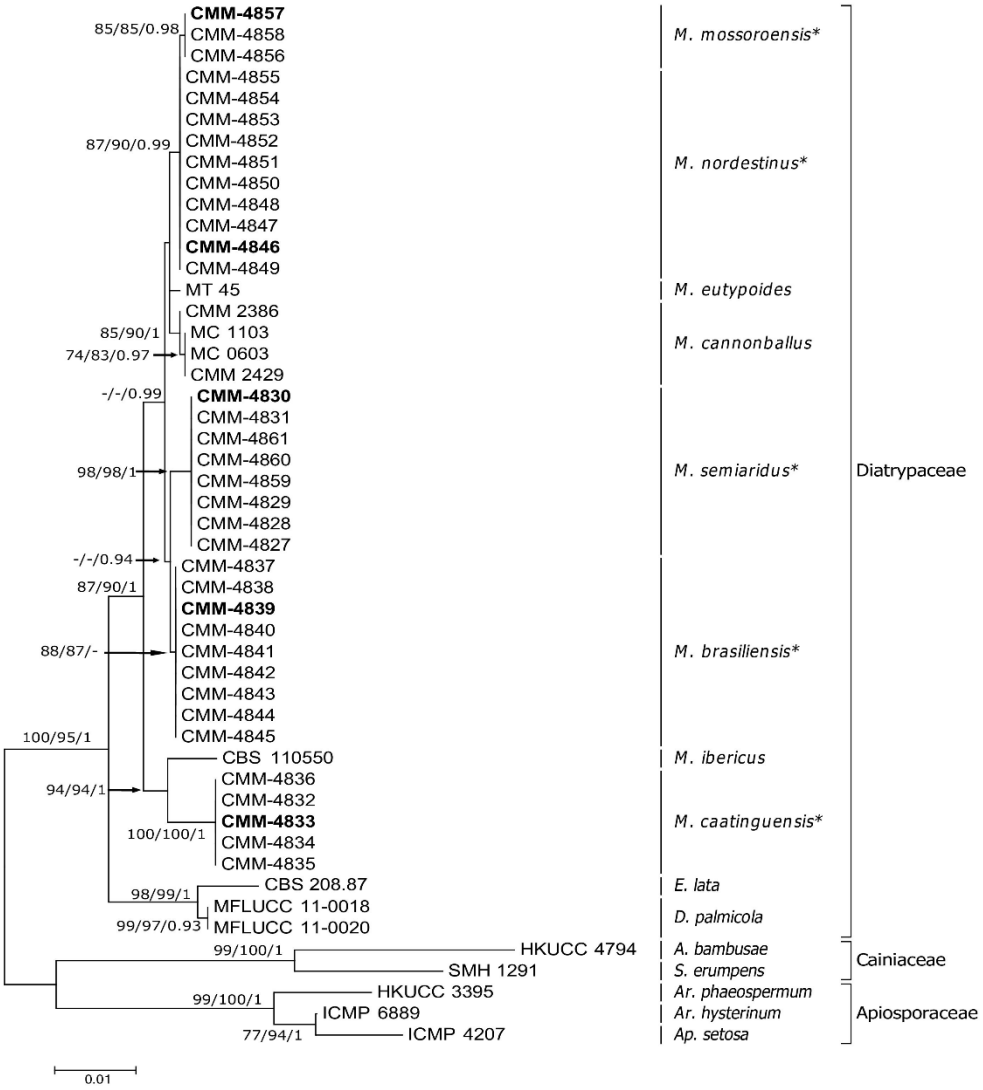
Figure 2 Maximum likelihood phylogeny inferred from the combined ITS, *tef1-α* and *tub* sequence alignments used to infer the relative position of species inside the *Monosporascus* genus. Support values (ML bootstrap / MP bootstrap / BI posterior probabilities) are given at the nodes. The tree was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

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.

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.

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.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MT 45
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ICMP 4207
0.01
M. mossoroensis*
M. nordestinus*
M. eutypoides
M. cannonballus
M. semiaridus*
M. brasiliensis*
M. ibericus
M. caatinguensis*
E. lata
D. palmicola
A. bambusae
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Ar. phaeospermum
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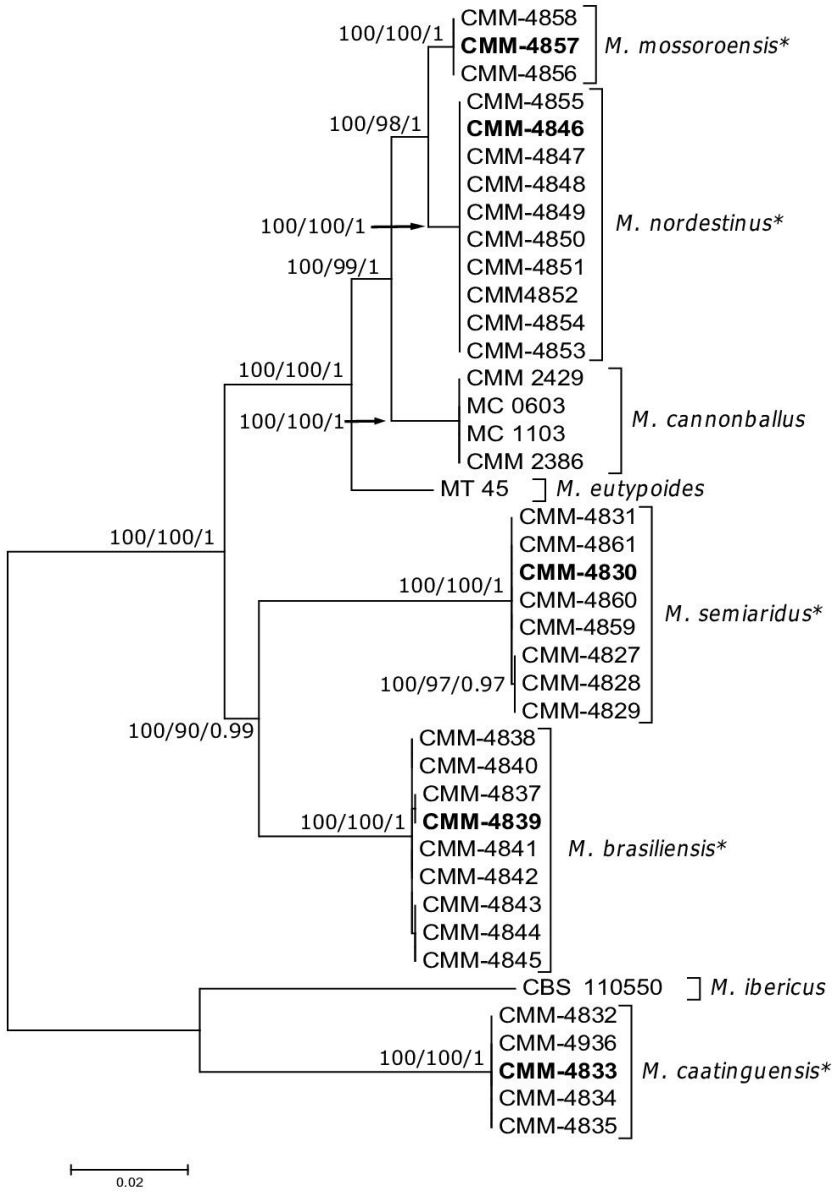
Ap. setosa
Diatrypaceae
Cainiaceae
Apiosporaceae

Figure 1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M. ibericus
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M. caatinguensis*
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Figure 2

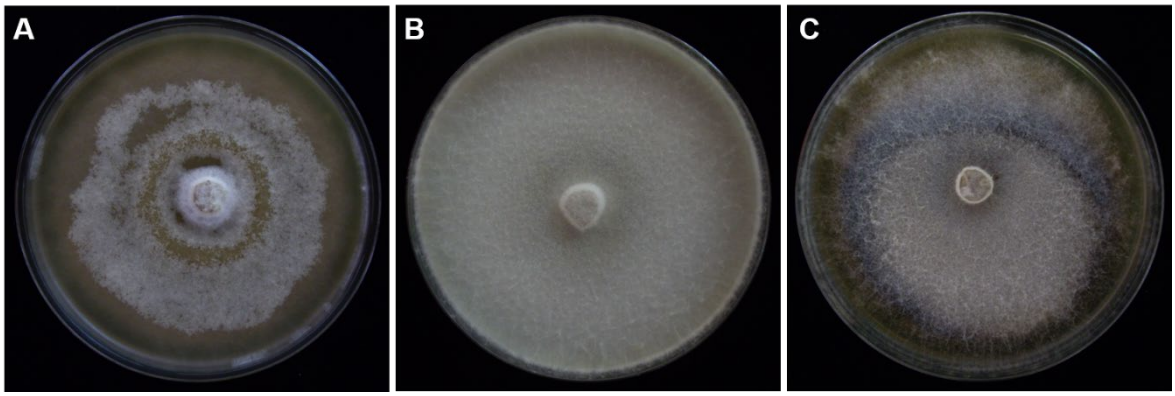

Figure 3

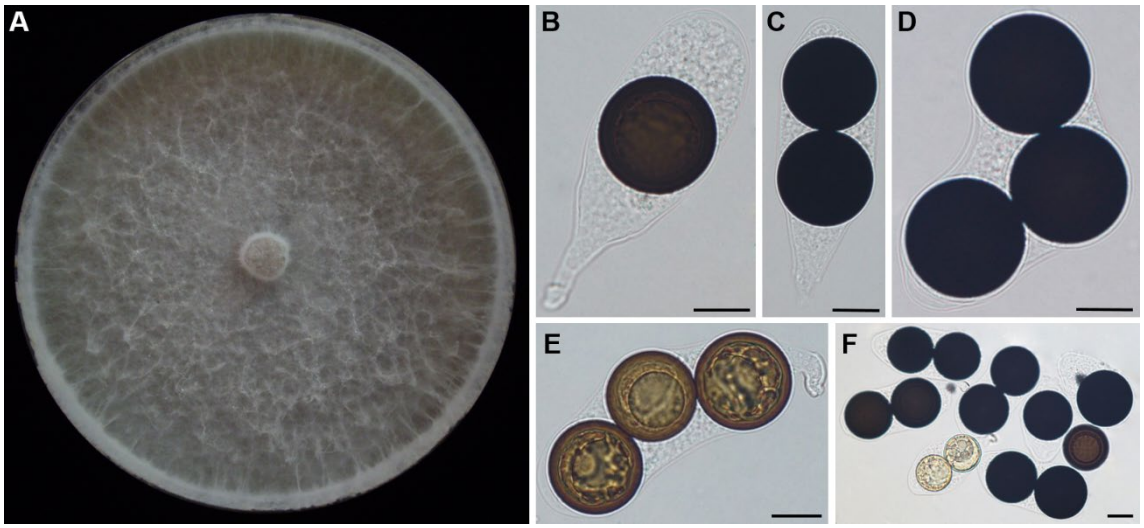

Figure 4

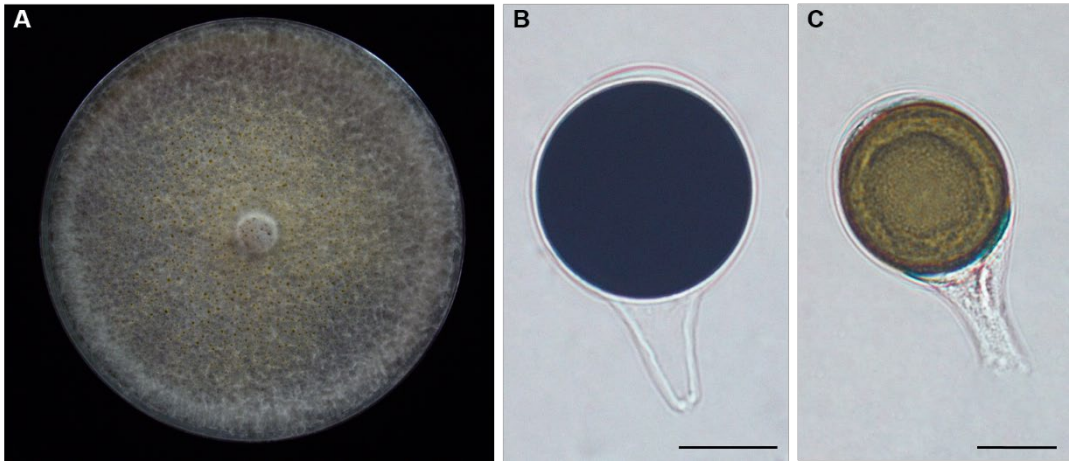

Figure 5