ALEXANDRE REIS MACHADO

TAXONOMIA E FILOGENIA MOLECULAR DE BOTRYOSPHAERIALES FITOPATOGÊNICOS NO BRASIL, COM ÊNFASE NA AVALIAÇÃO DE REGIÕES GÊNICAS DE CÓPIA ÚNICA PARA ESTUDOS FILOGENÉTICOS

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

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BIOGRAFIA

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RESUMO

MACHADO, Alexandre Reis, D.Sc., Universidade Federal de Viçosa, outubro de 2015. **Taxonomia e filogenia molecular de Botryosphaeriales fitopatogênicos no Brasil, com ênfase na avaliação de regiões gênicas de cópia única para estudos filogenéticos**. Orientador: Olinto Liparini Pereira. Coorientador: Gleiber Quintão Furtado.

No Brasil, alguns estudos evolvendo Botryosphaeriales vêm sendo realizados. Mas, diante da enorme diversidade de plantas cultivadas e nativas que são potenciais hospedeiros desse grupo de fungos, torna-se evidente que esses estudos ainda são incipientes. A mandioca é uma cultura de grande importância econômica no Brasil e é afetada por uma doença conhecida como podridão negra das raízes da mandioca, que é um dos principais fatores limitantes a rentabilidade dessa cultura. Contudo, até recentemente nenhum estudo etiológico mais acurado envolvendo essa doença tinha sido realizado. No passado, a podridão negra das raízes da mandioca era associada à espécie *Scytalidium lignicola*. Entretanto, no primeiro capítulo desse trabalho, foram identificados com base em análises morfológicas, apoiada por análises filogenéticas multilocus, as espécies de Botryosphaeriaceae, *Lasiodiplodia euphorbicola*, *Lasiodiplodia pseudotheobromae* e *Neoscytalidium hyalinum* associados a essa doença. Após a reprodução dos sintomas nos testes de patogenicidade, concluiu-se que a podridão negra das raízes da mandioca no Brasil é causada por várias espécies de Botryosphaeriaceae. A podridão de carvão é uma conhecida doença causada por *Macrophomina* em vários hospedeiros, e essa tem causado sérios problemas para a cultura da mamoneira no Brasil. Assim diante da inexistência de estudos etiológicos mais acurados para essa doença, o segundo capítulo desse trabalho buscou identificar com auxílio de ferramentas moleculares, espécies de *Macrophomina* associadas a diferentes culturas oleaginosas no Brasil. Foram identificadas três espécies, *Macrophomina phaseolina*, *M. pseudophaseolina* e uma nova espécie filogenética a ser proposta nesse trabalho, associadas a diversas culturas. Assim, concluiu-se que a podridão de carvão no Brasil é causada por várias espécies de *Macrophomina*. Outro grupo de culturas de grande importância no Brasil pertence à família Annonaceae, sendo que as principais espécies cultivadas são *Annona muricata*, *Annona squamosa* e um híbrido conhecido como atemoya. Essas culturas são afetadas por uma doença que causa seca descendente e em casos mais severos, provoca a morte de plantas. Até o momento, apenas a espécie *Lasiodiplodia theobromae* tem sido associada a essa doença. Porém, diante da ausência de estudos etiológicos mais acurados para essa doença, o terceiro capítulo desse trabalho buscou identificar com auxílio de ferramentas moleculares, espécies de

Botryosphaeriaceae associadas a seca descendente em plantas da família Annonaceae no Brasil. Baseando-se em análises filogenéticas de ITS e TEF1-α combinados, dez espécies de Botryosphaeriaceae foram identificados, sendo uma destas proposta como uma nova espécie de *Lasiodiplodia*. Das dez espécies encontradas, oito foram patogênicas e reproduziram os sintomas de seca descendente. Assim, a seca descendente em Annonaceae no Brasil é causada por várias espécies de *Lasiodiplodia*. Nos últimos anos, vários trabalhos têm mostrado que a maioria das regiões gênicas comumente utilizadas em filogenia de fungos, não são apropriadas. Assim o quarto capítulo deste trabalho buscou avaliar o desempenho de regiões gênicas de cópia única para estudos filogenéticos de Botryosphaeriales, seu potencial em estudos taxonômicos e como possível marcador para reconhecimento de espécies. A região gênica TEF1-α, muito utilizada em estudos filogenéticos de Botryosphaeriales, juntamente com as regiões gênicas de cópia única RPB2 e MS204, tanto isoladas como concatenadas, obtiveram os melhores resultados, uma vez que discriminaram a maioria de espécies avaliadas. Porém, uma condição para que nós possamos determinar as regiões gênicas de cópia única como apropriadas para futuros estudos, será o desenvolvimento de banco de dados de referência com sequências de RPB2 e MS204 para todas as espécies possíveis de Botryosphaeriales.

ABSTRACT

MACHADO, Alexandre Reis, D.Sc., Universidade Federal de Viçosa, October, 2015. **Taxonomy and molecular phylogeny of phytopathogenic Botryosphaeriales in Brazil, with emphasis in the evaluation of single-copy gene regions for phylogenetic studies**. Adviser: Olinto Liparini Pereira. Co-adviser: Gleiber Quintão Furtado.

In Brazil, some few works involving Botryosphaeriales have been made. However, given the huge diversity of cultivated and native plants in Brazil that are potential hosts of this group of fungi, it becomes clear that these studies are still incipient. The cassava is a crop of great economic importance in Brazil and it is affected by a disease known as black root rot, which represents one of the main limiting factor for this crop rentability, however, until recently, no suitable etiologic studies involving this disease had been performed. In the past, this disease was associated with *Scytalidium lignicola*. However, in the first chapter of this work, it were identified based on morphological analysis allied to multilocus phylogenetic analysis, the species of Botryosphaeriaceae *Lasiodiplodia euphorbicola*, *L. pseudotheobromae* and *Neoscytalidium hyalinum* associated with this disease. Upon the pathogenicity tests and reproduction of the symptoms, we could conclude that the black root rot of cassava is caused by several species of Botryosphaeriaceae. The charcoal rot is a known disease caused by *Macrophomina* in several hosts, and it has caused serious problems in castor crop in Brazil. Thus, given the lack of suitable etiologic studies to this disease, the second chapter of this work aimed to identify with molecular tools, *Macrophomina* species associated with different oil crops in Brazil. It were identified three species, *Macrophomina phaseolina*, *M. pseudophaseolina*, and a new species to be proposed here, associated with several crops. Thus, the charcoal rot in Brazil is caused by several species of *Macrophomina*. Other group of crops of great economic importance in Brazil belong to Annonaceae family, and the main cultivated species are *Annona muricata*, *Annona squamosa* and a hybrid knows as atemoya. These crops are affected by a disease, which causes dieback and in more severe cases, causes the death of the plants. Until now, only the species *Lasiodiplodia theobromae* has been associated with this disease. However, given the lack of suitable etiologic studies to this disease, the third chapter of this study aimed to identify with molecular tools, Botryosphaeriaceae species associated with dieback of Annonaceae plants in Brazil. Based on phylogenetic analyses of ITS and TEF1-α combined, ten species of Botryosphaeriaceae were identified, once one of these is proposed in this work as a new species of *Lasiodiplodia*. From ten species found, eight were pathogenic and

reproduced the dieback symptoms observed in the field. Thus, the results showed that the dieback of Annonaceae in Brazil is caused by several species of *Lasiodiplodia*.

In the last years, several works have shown that most of gene regions commonly used in phylogenetic studies of fungi, are not appropriate. Thus, the fourth chapter of this work aimed to investigate the performance of single-copy gene regions in phylogenetic studies of Botryosphaeriales, its potential in taxonomic studies and as a possible marker for the species recognition. The gene region TEF1-α, commonly used in phylogenetic studies of Botryosphaeriales, in addition to the gene regions RPB2 and MS204, alone or in combination, obtained the better results, once resolved most of species evaluated. However, a condition for that we can determine the single copy gene regions as appropriate for future studies, would be the development of reference datasets with RPB2 and MS204 sequences for all species of Botryosphaeriales.

INTRODUÇÃO GERAL

Fungos da ordem Botryosphaeriales (Dothideomycetes, Ascomycota) são conhecidos pela sua importância fitopatológica, podendo causar uma grande variedade de doenças em uma ampla gama de hospedeiros, como podridões de frutos, manchas foliares, tombamento de plântulas, cancros, gomoses, podridões de colo e raízes, morte descendente dentre outros tipos de doenças, sendo essas responsáveis muitas vezes por grandes perdas em diversas culturas de importância econômica (Slippers & Wingfield 2007; Slippers et al. 2009; Alves et al. 2013; Jami et al. 2014).

Esse grupo de fungos incluem espécies saprófitas, parasitas e endofíticos. Este último representa um desafio em termos quarentenários, pois espécies de Botryosphaeriales são conhecidas por eventualmente possuírem fase latente como endofíticos, manifestando-se como fitopatógenos especialmente quando seus hospedeiros são submetidos a algum tipo de estresse abiótico (Slippers & Wingfield 2007). Tal fato possibilita sua disseminação para outras regiões do mundo em material vegetal aparentemente sadio (Slippers & Wingfield 2007; Wikee et al. 2013).

Observa-se na literatura mundial um aumento significativo de estudos taxonômicos e etiológicos de Botryosphaeriales fitopatogênicos em espécies agronômicas e florestais nos últimos anos (Abreo et al. 2013; Al-Sadi et al. 2013; Alves et al. 2013, 2014; Phillips et al. 2013; Pitt et al. 2013, 2015; Slippers et al. 2013, 2014; Trakunyingcharoen et al. 2013, 2015; Twizeyimana et al. 2013; Úrbez-Torres et al. 2013; Wikee et al. 2013; Yan et al. 2013; Abdollahzadeh et al., 2013, 2014; Adesemoye et al. 2014; Chen et al. 2014; Jami et al., 2014; Sarr et al. 2014; Xu et al. 2014; Zhou et al. 2015). No Brasil, recentemente alguns trabalhos utilizando ferramentas moleculares combinadas aos estudos morfológicos vêm sendo conduzidos envolvendo esse grupo de fitopatógenos, revelando uma grande diversidade de novas espécies ou espécies já descritas, mas nunca antes relatadas no país, associadas às culturas do coqueiro, mandioca, mangueira, mamoeiro, morangueiro, ornamentais e pinhão manso (Marques et al. 2012, 2013a,b; Machado et al. 2012, 2014a,b; Silva et al. 2013; Lopes et al. 2014; Netto et al. 2014; Correia et al. 2015; Rosado et al. 2015). Porém, diante da enorme diversidade de plantas cultivadas e nativas no Brasil que são potenciais hospedeiros de Botryosphaeriales, torna-se evidente que esses estudos ainda são incipientes e que o

Brasil ainda carece de estudos etiológicos acurados de doenças causadas por Botryosphaeriales, especialmente envolvendo análise de sequências de DNA.

A taxonomia de Botryosphaeriales tem sido fonte de controvérsia entre diferentes grupos de pesquisa por vários fatores, especialmente pelo fato dos caracteres morfológicos (que são tipicamente utilizados para a identificação de espécies, como dimensões e forma de conídios ou ascósporos, septação e pigmentação, etc.) serem considerados pouco informativos. Além disso, os dados ecológicos e geográficos são muitas vezes são difíceis de interpretar (Abdollahzadeh et al. 2014; Alves et al. 2014; Jami et al. 2014; Slippers et al. 2014). Assim, a associação com hospedeiros que, em muitos casos foi utilizado para definição de espécies, pode conduzir a diversos erros. Por estas razões, a história taxonômica do grupo é considerada confusa. Entretanto, nos últimos anos, os avanços das técnicas moleculares e da utilização de sequencias de DNA em estudos filogenéticos de fungos têm contribuído substancialmente na resolução de dificuldades taxonômicas envolvendo a ordem Botryosphaeriales (Crous et al. 2006; Slippers & Wingfield 2007; Liu et al. 2012, Phillips et al. 2008, 2013; Slippers et al. 2009, 2013, 2014; Jami et al. 2014).

Um dos passos iniciais e mais importantes para estudos filogenéticos e moleculares de fungos é a definição de qual região gênica será utilizada nestas análises. A escolha das regiões gênicas tem-se baseado simplesmente na disponibilidade de sequências nos bancos de dados e muitas vezes em regiões tradicionalmente utilizadas por outros grupos de pesquisa, sem a preocupação se estas representam bem a história evolutiva das espécies (Sharma et al. 2015). Dessa forma, a utilização de genes não apropriados pode acarretar em uma subestimação ou superestimação da biodiversidade ou mesmo estabelecer um posicionamento filogenético inapropriado das espécies (Hyde et al. 2014).

Dentre as regiões gênicas utilizadas em filogenia de fungos, as mais comumente indicadas em estudos taxonômicos da ordem Botryosphaeriales são LSU (Região 28S do DNA ribossomal), SSU (Região 18S do DNA ribossomal), RPBII (RNA Polimerase Subunidade II), BT (β-tubulina), ITS (Espaçador Interno Transcrito do DNA ribossomal) e TEF1-α (Fator de Elongação da Tradução 1α), sendo estes três últimos os mais utilizados (Hyde et al. 2014). Entretanto vários trabalhos têm mostrado que a maioria destes genes não são apropriados para estudos filogenéticos (Aguileta et al. 2008; Schmitt et al. 2009; Schoch et al. 2012; Walker et al. 2012).

Apesar da região ITS ser a mais utilizada em estudos filogenéticos de fungos e ter sido escolhida recentemente como o marcador mais apropriado para o "DNA barcode" (Schoch et al. 2012), alguns trabalhos têm mostrado a ocorrência de diferentes cópias desse gene no genoma dos fungos, o que pode acarretar sérios problemas em análises filogenéticas, bem como na detecção de espécies como proposto pelo "DNA barcode" para fungos (O'Donnell & Cigelnik 1997; Ko & Jung 2002; Lindner & Banik 2011; Schoch et al. 2012; Harrington et al. 2014). Além da região ITS, alguns trabalhos também têm mostrado a existência de múltiplas cópias ou cópias parálogas de genes codificadores de proteínas em fungos, como β-tubulina (Ayliffe et al. 2001; Landvik et al. 2001; Corradi et al. 2004) e fator de elongação (James et al. 2006), tornando-os assim impróprios para estudos filogenéticos.

A partir dessa premissa, alguns trabalhos têm buscado testar novas regiões gênicas que poderiam representar melhor a história evolutiva das espécies fúngicas. Com esse objetivo, Aguileta et al. (2008) avaliaram a performance filogenética de genes codificadores de proteínas de cópia única no genoma que resultou em dois genes promissores, o MCM7 (fator de licenciamento de replicação necessária para a iniciação da replicação de DNA) e o Tsr1 (requerido para a acumulação do rRNA durante a biogênese dos ribossomos). Estes obtiveram topologias, poder de resolução e suporte semelhantes à árvore com 246 genes ortólogos concatenados e mostraram-se potenciais para estudos filogenéticos de fungos.

Posteriormente, Schmitt et al. (2009) desenvolveram oligonucleotídeos degenerados para a amplificação dessas regiões para uma ampla gama de espécies em Ascomycota e Basidiomycota. Outros pesquisadores confirmaram a utilidade filogenética do gene MCM7 tanto para o nível de espécies como para classes em Dothideomycetes, Eurotiomycetes, Geoglossomycetes, Lecanoromycetes, Leotiomycetes e Sordariomycetes (Raja et al. 2011). Walker et al. (2012) identificaram dois genes de cópia única, o FG1093 (Proteína Ribossomal L37 da subunidade 60S) e MS204 (Proteína de ligação do nucleotídeo guanina à subunidade semelhante a proteína Beta - "guanine nucleotide-binding protein subunit beta-like protein"), que se mostraram úteis na resolução de espécies filogenéticas em Sordariomycetes, e também forneceram evidências de suas potencialidades para estudos envolvendo outros grupos de fungos.

Assim, na atual impossibilidade de realização de estudos filogenéticos amplos utilizando genomas de fungos, tanto pela inexistência de ferramentas de bioinformática

que processem esse volume de dados, quanto pelos custos de sequenciamento, a busca por regiões gênicas que melhor representem o genoma é uma alternativa viável para estudos de taxonomia e sistemática filogenética de fungos. Além disso, diante dos problemas relacionados à utilização de regiões gênicas tradicionais em filogenias de fungos, esta abordagem possibilitará a definição de novas hipóteses a serem testadas em relação a história evolutiva dos mais diversos grupos de fungos.

Apesar dos diversos trabalhos citados anteriormente mostrarem a eficácia dos genes de cópia única no genoma ou genes ortólogos em estudos filogenéticos de diversos grupos de fungos, até o momento nenhum trabalho mais específico envolvendo importantes grupos de fitopatógenos, como Botryosphaeriales, foi realizado. Embora esse grupo tenha passado por um grande avanço na taxonomia e sistemática nos últimos anos, os estudos que fornecem o posicionamento da ordem, famílias, gêneros ou mesmo da proposição de espécies filogenéticas em Botryosphaeriales têm-se baseado em algumas poucas regiões gênicas que possivelmente não são adequadas. Além disso, a região ITS escolhida como o "barcoding" para fungos não possibilita a discriminação de espécies em Botryosphaeriales (Schoch et al. 2012; Machado et al. 2014a; Hyde et al. 2014).

Diante da importância fitopatológica de fungos da ordem Botryosphaeriales, da enorme diversidade de hospedeiros potenciais e da carência de estudos taxonômicos baseados em análise de sequencias de DNA envolvendo esse grupo de fungos no Brasil, são objetivos do presente trabalho: i) testar regiões gênicas de cópia única no genoma para estudos filogenéticos de Botryosphaeriales e como possível marcador para discriminação de espécies desse grupo; ii) caracterizar espécies de *Macrophomina* associadas a diferentes culturas oleaginosas no Brasil; iii) investigar a diversidade de espécies de Botryosphaeriaceae associados a morte descendente em espécies cultivadas e não cultivadas da família Annonaceae; iv) identificar através de estudos morfológicos e filogenéticos, os agentes etiológicos da podridão negra da mandioca.

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ARTIGO 1

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SHORT COMMUNICATION

New occurrences of Botryosphaeriaceae causing black root rot of cassava in Brazil

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ABSTRACT

Despite the occurrence of several diseases of cassava, the cassava black root rot (CBR) represents one of the main limiting factor for crop rentability in the world. However, the etiology of CBR is complex and it needs to be revised based on current molecular analysis. On this work, molecular and morphological studies allowed for the identification of three species of Botryosphaeriaceae causing black root rot disease of cassava in the states of Maranhão and Paraíba, Brazil, namely: *Lasiodiplodia euphorbicola*, *Lasiodiplodia pseudotheobromae* and *Neoscytalidium hyalinum*. This is the first report of these three fungal species as causal agents of CBR in the world.
Key words: *Lasiodiplodia* spp., *Manihot esculenta*, *Neoscytalidium hyalinum*, Botryosphaeriales, Dothideomycetes, soilborne fungi.

Cassava (*Manihot esculenta* Crantz) is an important food source. Its tuberous edible roots are high in calories and are a source of starch, the major form of carbohydrate consumed in the tropics for human and animal nutrition (Adeoti, 2010; CEPLAC, 2013). Furthermore, it tolerates adverse climatic and edaphic conditions and requires little care (Nweke et al., 2002). Due to these characteristics, cassava is an important activity for smallholders and it is widely cultivated in developing countries to reduce famine, providing a major source of nutrition for over 500 million people (FAO, 2013).

Currently, cassava productivity in Brazil, one of the probable centers of origin of cassava, is low. Cassava cultivation by family stallholders utilize low-level of technologies, including manivas (propagation materials) of poor physiological and phytosanitary quality (Silva et al., 2013). The high occurrence of disease transmitted by propagation material is one of the main factors that contribute to lower cassava productivity in Brazil (Cavalcante, 2001).

Some of these disease transmitted by propagative materials also occur in the postharvest phase. Among these is root rot, which is the main limiting factor for the production of cassava because it directly affects the marketable product. In Brazil numerous root rot fungi are listed as associated with cassava, namely: *Fusarium solani* (Mart.) Sacc., *Phytophthora capsici* Leonian, *P. drechsleri* Tucker, *P. nicotianae* var. *parasitica* (Dastur) G.M. Waterh., *P. richardiae* Buisman, *Scytalidium lignicola* Pesante, *Rhizoctonia solani* J.G. Kühn, *Rosellinia necatrix* Berl. ex Prill., and some Botryosphaeriaceae as *Diplodia manihotis* Sacc. and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Mendes and Urben, 2014). However, among these agents, only *Scytalidium lignicola* is considered to be the causal agent of cassava black root rot (CBR) (Laranjeira et al., 1994; Poltronieri et al., 1998; Muniz et al., 1999; Serra et al., 2009; Silva et al., 2013). It causes severe yield losses (Silva et al., 2013).

Nevertheless, the status of *Scytalidium lignicola* as the causal agent of CBR in Brazil needs to be revised based on molecular analysis. Recently, several species previously identified as *Scytalidium* were transferred to the genus *Neoscytalidium* (Seifert et al., 2011). *Neoscytalidium* is morphologically similar to *Scytalidium*, but under certain conditions this fungus forms synnanamorphs having pycnidia which contain *Fusicoccum*-like conidia. Moreover, these two genera belong to different orders of Ascomycota (Crous et al., 2006; Seifert et al., 2011; Phillips et al., 2013). Therefore, the identification of these fungi only by morphological characters can lead to errors. A morphological and molecular approach is required for a more accurate identification of the fungi associated with CBR (Hyde et al., 2010; Cai et al., 2011a; 2011b).

In 2011, six fungal isolates from cassava plants with symptoms of CBR collected in the states of Maranhão and Paraíba were initially identified as *Scytalidium* sp. and *Lasiodiplodia* sp. based upon morphological characteristics. Later, these isolates were provided by Embrapa Mandioca e Fruticultura to the Laboratório de Patologia de Sementes e de Pós-Colheita of the Universidade Federal de Viçosa for taxonomical and molecular studies. The aim of the present study was to identify these isolates based on morphological characters and molecular analysis and to verify their pathogenicity.

The isolates were grown on Petri dishes containing 2% water-agar (WA) overlaid with double-sterilized twigs of *Pinus* and incubated at 25°C with a photoperiod of 12 h to induce the formation of fruiting bodies and sporulation. The single-spore derived cultures were deposited in the Coleção de Culturas de Fungos Fitopatogênicos "Prof. Maria Menezes" (CMM) at the Universidade Federal Rural de Pernambuco, Brazil. Sections of the fruiting bodies were prepared and mounted in lactophenol. Thirty measurements of conidia, paraphyses and conidiogenous cells were made with an Olympus CX31 light microscope. Images were obtained with an Olympus BX 51 light microscope fitted with a digital camera (Olympus EVOLT330).

Genomic DNA was extracted from colonies grown on PDA at 25°C for one week. Approximately 40 mg of mycelia were collected. Extraction was carried out through a process involving freezing the samples with liquid nitrogen and grinding them into a fine powder using a microcentrifuge tube pestle. The crushing was resumed after adding 100 µL of Nuclei Lysis Solution of the Wizard Genomic DNA Purification kit (Promega). Extraction continued as described by Pinho et al. (2012). PCR reagents, primers and conditions were as described by Machado et al. (2014). PCR products were directly sequenced at Macrogen (South Korea).

Nucleotide sequences were edited with the BioEdit software (Hall, 2012). All sequences were checked manually and positions with ambiguous nucleotides were clarified using sequences from both DNA strands. New sequences were deposited in GenBank (see Table 1 for accession numbers). Sequences of internal transcribed spacer regions 1 and 2 including the 5.8S rRNA gene (ITS), translation elongation factor 1-α (TEF1-α) and β-tubulin (βt) of additional species were retrieved from GenBank (Table 1). Consensus sequences were compared against GenBank's database using the MegaBLAST algorithm. The closest hit sequences were aligned using MUSCLE (Edgar, 2004) implemented in MEGA v. 5 (Tamura et al., 2011). Alignments were checked visually, and manual adjustments were made when necessary. Ambiguously aligned sequences within the dataset were excluded from the analysis. The resulting alignment was deposited into TreeBASE (www.treebase.org) under accession number S15379. Phylogenetic analyses were conducted as described by Machado et al. (2014). The models of evolution selected according to the Akaike Information Criterion (AIC) were GTR+I for ITS, HKY+G for TEF and GTR+G for βt and the tree was rooted to *Spencermartinsia viticola* CBS117009.

Pathogenicity one representative isolate of each species was tested. Each selected isolate was grown in a Petri dish with PDA for 7 days at 25°C. Roots that were approximately 20 cm x 7 cm wide had their bark wounded superficially with a scalpel on the inoculation site. Six mm diam culture disks obtained from the margins of the growing culture were placed on the wounds. Wounded roots on which PDA plugs were deposited served as controls. Five roots were inoculated with each isolate and placed in plastic boxes that contained a portion of moistened cotton wool and were maintained in a moist chamber at approximately 25°C for two weeks.

Phylogenetic analysis (Figure 1) and morphological comparisons (Table 2) revealed three distinct species of Botryosphaeriaceae in association with CBR among the six fungal isolates: *Lasiodiplodia euphorbicola* A.R. Machado & O.L. Pereira (Figure 2H-K), *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous (Figure 2L-O) and *Neoscytalidium hyalinum* (C.K. Campb. & J.L. Mulder) A.J.L. Phillips, Groenewald & Crous (Figure 2C-G).

In recent years, morphological and molecular analyses have revealed a great diversity of species within plant pathogenic Botryosphaeriaceae (Begoude et al., 2010; Mehl et al., 2011; Ismail et al., 2012; Urbez-Torres et al., 2012; Marques et al., 2013a; 2013b; Machado et al., 2014). Despite the usefulness of morphological characters, molecular analysis became essential for recognizing taxa that are included in species complexes, such as *Lasiodiplodia* (Alves et al., 2008; Abdollahzadeh et al., 2010; Ismail et al., 2012; Urbez-Torres et al., 2012; Marques et al., 2013a). Molecular analysis can also distinguish taxa that show similar morphologies but are phylogenetically distant, such as the genera *Neocytalidium* and *Scytalidium*, which belong to Botryosphaeriaceae and Helotiaceae, respectively (Crous et al., 2006; Seifert et al., 2011; Phillips et al., 2013). Similarly, molecular analysis in this study revealed that two species of *Lasiodiplodia* are associated with CBR, and demonstrated that the *Scytalidium*-like fungus that causes this disease belongs to *Neoscytalidium*. Thus, it is possible that previous reports of fungi causing CBR in Brazil (Laranjeira et al., 1994; Poltronieri et al., 1998; Muniz et al., 1999; Serra et al., 2009; Silva et al., 2013) were misidentified as *Scytalidium lignicola*.

The species *Neoscytalidium hyalinum* (=*N. dimidiatum*) is a botryosphaeriaceous fungus that, under special conditions, forms *Scytalidium*-like and *Fusicoccum*-like synnanamorphs (Crous et al., 2006; Phillips et al., 2013). This is probably the main reason for the misidentification of this pathogen, which is often confused with the hyphomycete fungus *Scytalidium*. Therefore, it is clear that the identification of the etiologic agent of CBR requires a careful polyphasic approach.

Pathogenicity of the isolates representing the three species was confirmed two weeks after inoculation. All inoculated roots showed symptoms that were similar to those observed in the field, with the subsequent emergence of fungal structures occurring externally on the bark (Figure 2A). From the lesions, it was possible retrieve each of the inoculated fungi. Symptoms were not observed in control roots (Figure 2B).

Diseases caused by *Neoscytalidium hyalinum* tend to be more common in tropical countries (Phillips et al., 2013). In Brazil, this species was previously reported on *Jatropha curcas* L. and *Mangifera indica* L. (Machado

TABLE 1 - Genbank accession numbers of DNA sequences of Botryosphaeriaceae used in phylogenetic analysis. The specimens obtained in this study are highlighted in bold.

Species	Isolates	Host/Substrate	Genbank accession no.		
			ITS	EF1-α	βt
Neoscytalidium hyalinum	CBS 499.66	*Mangifera indica*	AY819727	EU144063	FM2111671
N. hyalinum	PD104	*Ficus carica*	GU251107	GU251239	GU251767
N. hyalinum	**CMM4022**	*Manihot esculenta*	KF369269	KF553902	KF720790
N. hyalinum	**CMM3895**	*M. esculenta*	KF369265	KF553898	KF720786
N. novaehollandiae	CBS122072	*Adansonia gibbosa*	EF585535	EF585581	-
N. novaehollandiae	CBS122610	*Acacia synchronicia*	EF585536	EF585578	-
Lasiodiplodia venezuelensis	WAC12539	*Acacia mangium*	DQ103547	DQ103568	-
L. venezuelensis	CMW13513	*Acacia mangium*	DQ103549	DQ103570	-
L. rubropurpurea	WAC12536	*Eucalyptus grandis*	DQ103554	DQ103572	-
L. gonubiensis	CBS115812	*Syzygium cordatum*	DQ458892	DQ458877	DQ458860
L. crassispora	CBS110492	Unknown	EF622086	EF622066	EU673134
L. crassispora	CMW22653	*Pterocarpus angolensis*	FJ888465	FJ888452	-
L. margaritacea	CBS122519	*Adansonia gibbosa*	EU144050	EU144065	-
L. pseudotheobromae	CBS116459	*Gmelina arborea*	EF622077	EF622057	EU673111
L. pseudotheobromae	CMM3887	*Jatropha curcas*	KF234559	KF226722	KF254943
L. pseudotheobromae	**CMM3894**	*M. esculenta*	KF369264	KJ452244	-
L. parva	CBS456.78	Cassava-field soil	EF622083	EF622063	-
L. parva	CBS495.78	Cassava-field soil	EF622085	EF622065	-
L. euphorbicola	CMM3651	*Jatropha curcas*	KF234553	KF226711	KF254937
L. euphorbicola	CMM3652	*Jatropha curcas*	KF234554	KF226715	KF254938
L. euphorbicola	CMM3609	*Jatropha curcas*	KF234543	KF226689	KF254926
L. euphorbicola	**CMM4018**	*Manihot esculenta*	KF369268	KF553901	KF720789
L. euphorbicola	**CMM3973**	*Manihot esculenta*	KF369267	KF553900	KF720788
L. euphorbicola	**CMM3897**	*Manihot esculenta*	KF369266	KF553899	KF720787
L. citricola	IRAN1521C	*Citrus* sp.	GU945353	GU945339	-
L. citricola	IRAN1522C	*Citrus* sp.	GU945354	GU945340	-
L. egyptiacae	CBS130992	*Mangifera indica*	JN814397	JN814424	-
L. egyptiacae	BOT-29	*Mangifera indica*	JN814401	JN814428	-
L. hormozganensis	IRAN1500C	*Olea* sp.	GU945355	GU945343	-
L. hormozganensis	IRAN1498C	*Mangifera indica*	GU945356	GU945344	-
L. subglobosa	CMM3872	*Jatropha curcas*	KF234558	KF226721	KF254942
L. subglobosa	CMM4046	*Jatropha curcas*	KF234560	KF226723	KF254944
L. macrospora	CMM3833	*Jatropha curcas*	KF234557	KF226718	KF254941
L. plurivora	STE-U5803	*Vitis vinifera*	EF445362	EF445395	-
L. gilanensis	IRAN1523C	Unknown	GU945351	GU945342	-
L. gilanensis	IRAN1501C	Unknown	GU945352	GU945341	-
L. iraniensis	IRAN1517C	*Citrus* sp.	GU945349	GU945337	-
L. iraniensis	IRAN1519C	*Mangifera indica*	GU945350	GU945338	-
L. brasiliense	CMM4015	*Mangifera indica*	JX464063	JX464049	-
L. brasiliense	CMM2186	*Carica papaya*	KC484812	KC481542	-
L. brasiliense	CMM2255	*Carica papaya*	KC484792	KC481523	-
L. brasiliense	CMM2313	*Carica papaya*	KC484793	KC481524	-
L. jatrophicola	CMM3610	*Jatropha curcas*	KF234544	KF226690	KF254927
L. mahajangana	CMW27801	*Terminalia catappa*	FJ900595	FJ900641	FJ900630
L. mahajangana	CMW27820	*Terminalia catappa*	FJ900597	FJ900643	FJ900632
L. theobromae	CMW28571	*Terminalia ivorensis*	GQ469924	GQ469897	-
Botryosphaeria rhodina	CBS164.96	Unknown	AY640255	AY640258	EU673110
B. rhodina	CBS124.13	Unknown	DQ458890	DQ458875	DQ458858
L. theobromae	CBS111530	Unknown	EF622074	EF622054	-
B. rhodina	CMW9074	*Pinus* sp.	AY236952	AY236901	AY236930
L. viticola	UCD2553AR	*Vitis vinifera*	HQ288227	HQ288269	HQ288306
L. viticola	UCD2604MO	*Vitis vinifera*	HQ288228	HQ288270	HQ288307
L. missouriana	UCD2193MO	*Vitis vinifera*	HQ288225	HQ288267	HQ288304
L. missouriana	UCD2199MO	*Vitis vinifera*	HQ288226	HQ288268	HQ288305
Spencermartinsia viticola	CBS117009	*Vitis vinifera*	AY905554	AY905559	EU673104

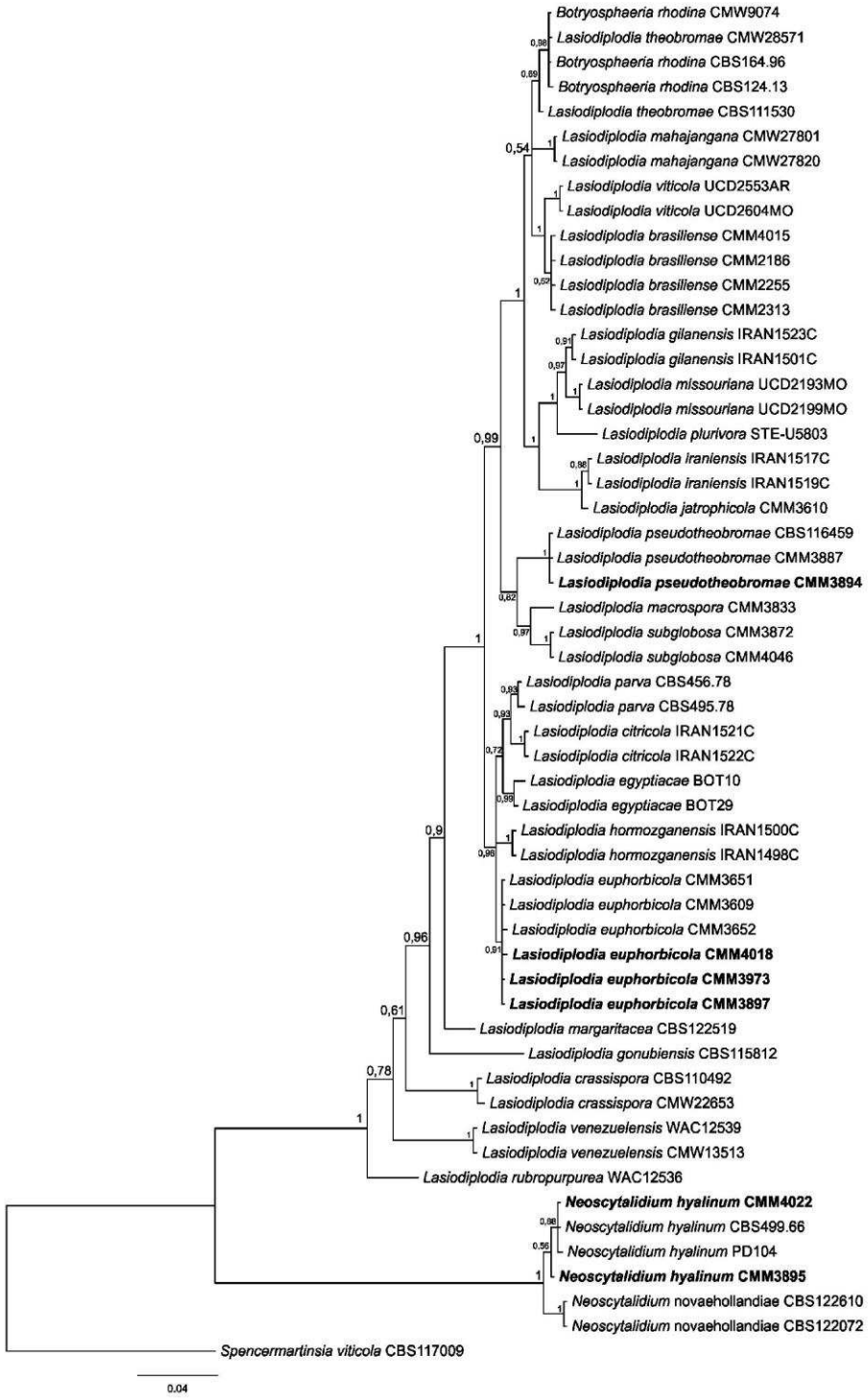

FIGURE 1 - Multilocus phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the ITS, TEF-1α and βt genes. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Spencermartinsia viticola* CBS117009. Isolates characterized in this study are highlighted in bold.

TABLE 2 - Biometric data of *Lasiodiplodia* spp. and *Neoscytalidium* spp. examined in this and in previous studies.

Species	Conidia (µm)	Paraphyses (µm)	Conidiogenous cells (µm)	Reference
L. theobromae	21–31 × 13–15.5	55 × 3–4	-	Alves et al., 2008
L. pseudotheobromae	23.5–32 ×14–18	58 × 3–4	-	Alves et al., 2008
	16–26 × 10–12	75 × 3–4	7–10 × 3–4	This study
L. euphorbicola	15–23 × 9–12	76 × 2–4	5–15 × 3–4	Machado et al., 2014
	17–24 × 10–12	40 × 2–3	5–12 × 2–3	This study
Species	**Conidia (µm)**	**Arthroconidia (µm)**	**Conidiogenous cells (µm)**	**Reference**
N. hyalinum	10–16(–21) × 3.5–6.5	4–16.5 × 8.5	6.5–14 × 2.5–4	Phillips et al., 2013
	8–12 × 4–5	4–12 × 2.5–8	6–10 × 1.5–2.5	Machado et al., 2014
	5–12 × 3–5	6–12 × 3–6	7–10 × 2–3	This study
N. novaehollandiae	10.5–12.5 × 4–5	5.5–7.5 × 3.5–4.5	7–10 × 2–3	Pavlic et al., 2008

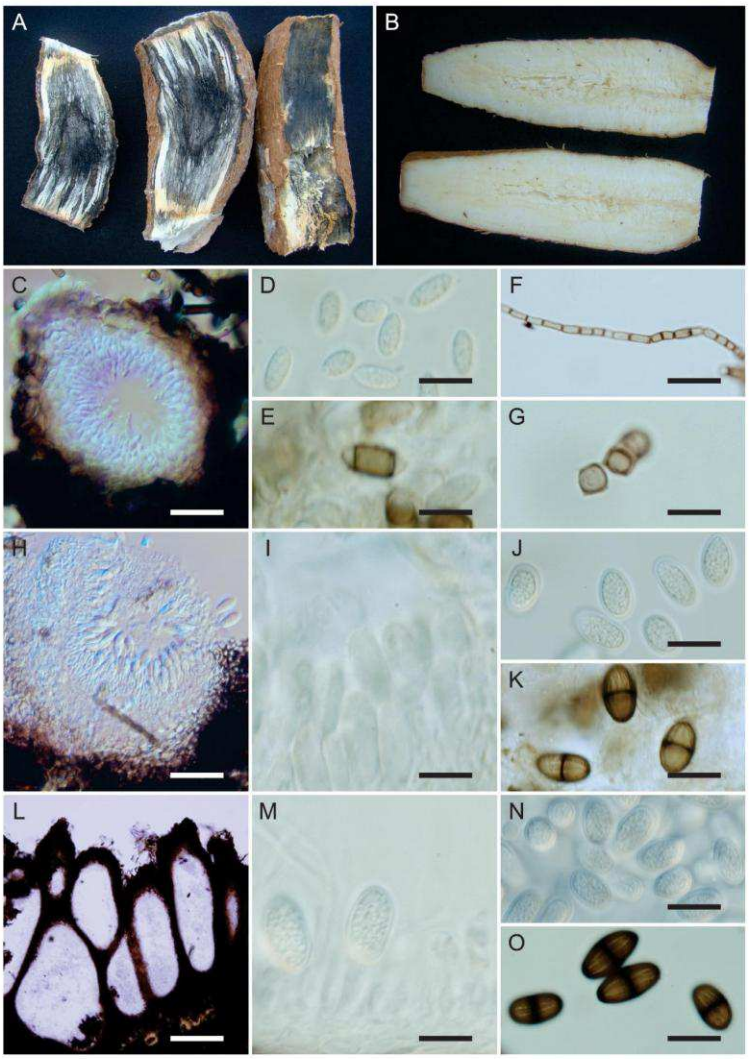

FIGURE 2 - Botryosphaeriaceae species causing black root rot of cassava. **A.** Symptoms of black root rot produced for Botryosphaeriaceae species in pathogenicity tests; **B.** Asymptomatic root used as control in pathogenicity tests; **C-G.** *Neoscytalidium hyalinum*. **C.** Conidiomata; **D, E.** Hyaline and septate mature conidia; **F, G.** Arthroconidia; **H-K.** *Lasiodiplodia euphorbicola*. **H.** Section of a conidiomata formed on *Pinus* twigs; **I.** Conidiogenous cells; **J, K.** Immature and mature pigmented conidia with longitudinal striations; **L-O.** *Lasiodiplodia pseudotheobromae*. **L.** Section of multilocular conidiomata formed on the host surface; **M.** Conidiogenous cells; **N, O**. Immature and mature pigmented conidia with longitudinal striations. Scale bars: C, H, L = 100 µm; I, J, K, M = 15 µm; N, O = 20 µm.

et al., 2012; 2014; Marques et al., 2013b). *Lasiodiplodia pseudotheobromae* has been described in *Carica papaya* L., *Jatropha curcas* and *Mangifera indica* (Marques et al., 2013a; Machado et al., 2014; Netto et al., 2014), whereas *L. euphorbicola* was reported only on *Jatropha curcas* and *Carica papaya* (Machado et al., 2014; Netto et al., 2014). This is the first report of the occurrence of *L. euphorbicola*, *L. pseudotheobromae* and *N. hyalinum* on cassava.

Since black rot is a major limiting factor for cassava production in Brazil, the correct identification of the associated pathogen(s) is essential for future studies of disease management and for the selection of resistant varieties, and provides new and relevant information for quarantine programs.

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ARTIGO 2

According to the guidelines of Tropical Plant Pathology

Taxonomy and phylogeny of *Macrophomina* species associated with oil crops in Brazil

Taxonomy and phylogeny of *Macrophomina* species associated with oil crops in Brazil

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Abstract

The genus *Macrophomina* comprises well-known necrotrophic pathogens related to more than 500 plant hosts in several regions of the world. Historically, only four species were accepted for the genus, but after epityfication of *M. phaseolina* using a morphological and molecular approach, a widely overlooked species was described in several hosts of agricultural importance. This discovery showed a paucity of phylogenetic studies involving this important group of plant pathogens. Previous studies revealed different levels of aggressiveness of *Macrophomina* isolates causing charcoal rot in castor plants in Brazil and shown of a possibility of the occurrence of different cryptic species among isolates. Thus, in present study, through phylogenetic studies with ITS rDNA and the Translation Elongation Factor 1-α regions and observations of the morphological characteristics, it was verified that these isolates of *Macrophomina* represent different species. Thus, based on the comparison of the morphological and molecular analyses it was possible to identify three different *Macrophomina* species: *Macrophomina phaseolina*, *M. pseudophaseolina* and a new phylogenetic species to be proposed here. This is first report of *Macrophomina pseudophaseolina* in Brazil, associated with *Arachis hypogaea*, *Gossypium hirsutum*, *Jatropha curcas* and *Ricinus communis*. In addition, the new species *Macrophomina* sp. to be proposed here is associated with *Ricinus communis* and *Jatropha gossypifolia*.

Key words: Botryosphaeriales, cryptic species, phylogeny, soil borne fungi

INTRODUCTION

The genus *Macrophomina* comprises well-known necrotrophic pathogens related to more than 500 plant hosts. The incidence of this fungus has steadily increased in diverse crop species worldwide (Far and Rossman 2015). Furthermore, this fungus can survive for long periods in soil and in crop debris, generally through microsclerotia, and it can also survive as mycelium in asymptomatic seeds and microsclerotia in symptomatic seeds (Dhingra and Sinclair 1978; Short et al. 1980; Singh and Singh 1982; Songa and Hillocks 1998; Gupta et al. 2012).

The genus *Macrophomina* belongs to the Botryosphaeriaceae and it is characterized by brown and septate mycelium with abundant production of black microsclerotia, pycnidial conidiomata and hyaline and aseptate conidia, with two apical mucoid appendages (Sutton 1980; Crous et al. 2006; Phillips et al. 2013).

Currently, there are five accepted species for the genus: *Macrophomina limbalis* Syd., *M. philippinensis* Petr., *M. pseudeverniae* Etayo & Diederich, *M. pseudophaseolina* Crous, Sarr & Ndiaye and the more common and important *M. phaseolina* (Tassi) Goid. Although several new species of Botryosphaeriaceae have been proposed based on molecular analyses over the last years (Phillips et al. 2005; Crous et al. 2006; Alves et al. 2008; Pavlic et al. 2008; Phillips et al. 2008; Pavlic et al. 2009; Begoude et al. 2010; Mehl et al. 2011; Liu et al. 2012; Marques et al. 2013a,b; Machado et al. 2014; Netto et al. 2014; Pitt et al. 2015; Trakunyingcharoen et al. 2015), until now, only one species of *Macrophomina* was proposed based on a molecular and morphological approach (Sarr et al. 2014). Perhaps the difficulty to obtain sporulation is a fact that has discouraged taxonomic works with these fungi.

Several studies have shown a high genetic diversity between isolates of *M. phaseolina* based on different molecular tools (Baird et al. 2010; Saleh et al. 2010; Gupta et al. 2012; Sarr et al. 2014) indicating the possibility of cryptic species within *Macrophomina phaseolina*. In addition, despite of the great diversity of Botryosphaeriaceae hosts in Brazil and its phytopathological importance, there is a paucity of studies involving this group. Recently some few works revealed several new species of Botryosphaeriaceae in Brazil (Marques et al. 2013a,b; Machado et al. 2014; Netto et al. 2014).

A recent work revealed different levels of aggressiveness of *Macrophomina*

isolates causing charcoal rot in castor plants in Brazil (Claudino & Soares, 2014). Based on the results, the authors deduced the possible existence of different species among isolates, since recently Sarr et al. (2014) showed the existence of cryptic species in the isolates previously identified as *Macrophomina phaseolina*. In present study, through phylogenetic studies with ITS rDNA and the Translation Elongation Factor 1-α regions and observations of the morphological characteristics, it was verified that these strains of *Macrophomina* represents different species. Thus, the objective of this study was to characterize the *Macrophomina* species associated with different oil crops in Brazil.

MATERIALS AND METHODS

Isolation

Initially, thirty isolates of *Macrophomina* obtained from *Ricinus communis* L., *Arachis hypogaea* L., *Glycine max* Merr.., *Sesamum indicum* L., *Helianthus annuus* L., *Gossypium hirsutum* L. and *Jatropha gossypifolia* L. by Claudino & Soares (2014) were provided by Embrapa Algodão to the Laboratório de Micologia e Etiologia de Doenças Fúngicas de Plantas of the Universidade Federal de Viçosa for taxonomical and molecular studies. In addition, standard blotter test were performed (Dhingra and Sinclair 1995) during works of fungal surveys on physic nut (*Jatropha curcas* L.) seeds from Jaíba, in the state of Minas Gerais, and Colatina, in the state of Espírito Santo, Brazil. After 7 days, the seeds were examined for the possible presence of fungal structures. After confirmation of the presence of *Macrophomina* on seeds, the microsclerotia were used for isolation in Petri dishes with potato dextrose agar (PDA, Himedia, Mumbai, India) and incubated at 25°C. The pure cultures obtained were stored in tubes with PDA at 10°C.

Morphological studies

The isolates with a dark mycelium, lacking sporulation and presence of black small sclerotia, characters typical of *Macrophomina*, were grown in Petri dishes with 2% water agar (WA - Agar Agar, type I, Himedia, Mumbai, India) overlaid with triple-sterilized physic nut seeds, needles or twigs of *Pinus* and incubated at 25°C with a 12 hour light-dark regime for 4-8 weeks to induce sporulation. The fruiting bodies were mounted in lactophenol. Thirty measurements of all relevant morphological characters (conidia and conidiogenous cells) were made using an OLYMPUS CX31 light microscope for the identification of the species. The images were obtained with an OLYMPUS BX 51 light microscope fitted with a digital camera (OLYMPUS

EVOLT330). A representative specimen was deposited in the local herbarium at the Universidade Federal de Viçosa (Herbarium VIC), and the isolates obtained in this study were deposited in the culture collection "Coleção de Culturas de Fungos Fitopatogênicos Prof. Maria Menezes" (CMM) at the Universidade Federal Rural de Pernambuco (Recife, Brazil). The isolates provided by Embrapa Algodão were maintained in the culture collection "Coleção de Culturas de Microrganismos Fitopatogênicos da Embrapa Algodão (CCMF-CNPA).

DNA extraction, sequencing and phylogenetic studies

The isolates were grown on PDA at 25 °C for 1 week. Approximately 40 mg of mycelia were scraped from the agar surface and placed in a sterile 1.5 mL microcentrifuge tube. The extraction was processed by freezing with liquid nitrogen and grinding into a fine powder using a microcentrifuge tube pestle. The crushing continued after the addition of 100 µL of Nuclei Lysis Solution from the Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.). Subsequently, an additional 500 µL of the previous solution was added. The extraction continued as described by Pinho et al. (2012).

PCR reactions included the following ingredients for each 25 µL reaction: 12.5 µL of Dream Taq TM PCR Master Mix 2X (MBI Fermentas, Vilnius, Lithuania), 1 µL of 10 µM of each forward and reverse primer synthesized by Invitrogen (Carlsbad, U.S.A), 1 µL of dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO, U.S.A.), 5 µL of 100× (10 mg/mL) bovine serum albumin (BSA, Sigma–Aldrich, St. Louis, MO, U.S.A.), 2 µL of genomic DNA (25 ng/µl) and 2.5 µL of nuclease-free water.

Target sequences of the Internal Transcribed Spacer regions 1 and 2 including the 5.8S rRNA gene (ITS) and Translation Elongation Factor 1-α (TEF1-α) were amplified using primers ITS1/ITS4 for ITS (White et al. 1990); EF1F/EF2R (Jacobs et al. 2004) and EF1-728F (Carbone and Kohn 1999)/EF2R (Jacobs et al. 2004) for partial TEF1-α.

The thermal cycle consisted of 95 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min (denaturation), 55 ºC for 1 min (for TEF1-α) or 52 ºC for 1 min (for ITS) (annealing), 72 ºC for 2 min (elongation), and 72 ºC for 10 min (final extension). PCR products were analyzed with 2% agarose electrophoresis gels stained with GelRed™ (Biotium Inc., Hayward, CA, U.S.A.) in a 1× TAE buffer and visualized under UV light to check for amplification size and purity. PCR products were purified and sequenced by

Macrogen Inc., South Korea (http://www.macrogen.com). The nucleotide sequences were edited with BioEdit software (Hall 2012). All sequences were checked manually, and nucleotides with ambiguous positions were clarified using both primer direction sequences. New sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov). Sequences of ITS and TEF1-α for additional species were retrieved from GenBank (Table 1).

Consensus sequences were compared against GenBank's database using their Mega BLAST program. The closest hit sequences were then downloaded in FASTA format and aligned using the multiple sequence alignment program MUSCLE® (Edgar 2004), built in MEGA v. 5 software (Tamura et al. 2011). Alignments were checked, and manual adjustments were made if necessary. The resulting alignment was deposited into TreeBASE (http://www.treebase.org/).

Bayesian inference concatenated (BI) analyses employing a Markov Chain Monte Carlo method were performed with all sequences, first with each gene/locus separately and then with the concatenated sequences (ITS and TEF1-α). Before launching the BI, the best nucleotide substitution model was determined for each gene with MrMODELTEST 2.3 (Posada and Buckley 2004). After the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). SYM model of evolution was used for ITS, and HKY+G was used for TEF1-α. The phylogenetic analysis of the concatenated alignment was performed with the CIPRES web portal (Miller et al. 2010) using MrBayes v.3.1.1 (Ronquist and Huelsenbeck 2003). In MrBayes, the data were partitioned by locus, and the parameters of the nucleotide substitution models for each partition were set as described above. Four MCMC chains were run simultaneously, starting from random trees, for 10,000,000 generations. Trees were sampled every 1000th generation for a total of 10,000 trees. The first 2500 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a majority-rule consensus tree generated with the remaining 7500 trees. Trees were visualized in FigTree v1.3.1 (Rambaut 2009) and exported to graphics programs. The tree was rooted to *Cophinforma eucalypti* MFLUCC110425 and MFLUCC110655.

RESULTS

Isolation

Thirty isolates *Macrophomina* obtained from castor and other oil plants were provided by Embrapa Algodão. Additionally, five isolates were obtained from physic nut seeds.

Phylogeny

PCR reactions were conducted successfully for the regions ITS and TEF1-α. The PCR fragments contained approximately 500 bp and 700 bp for ITS and TEF1-α, respectively.

The combined analysis of the ITS and TEF1-α regions included 50 taxa and contained 714 characters, of which 122 were parsimony informative, 126 were variable and 586 were conserved.

Based on the comparison of the morphological (Table 2) and molecular analyses (Fig. 1), it was possible to identify three different *Macrophomina* species: *Macrophomina phaseolina* (Tassi) Goid., *M. pseudophaseolina* Crous, Sarr & Ndiaye (Fig. 2) and a new phylogenetic species to be proposed here.

TAXONOMY

***Macrophomina* sp. (To be proposed as new species)** (Fig. 1)

MycoBank no.: XXXX

Etymology: XXXX.

Description: Cultures sterile. Aerial mycelia white on MEA (Malt Extract Agar 2% - Himedia) after 7 days at 25°C in dark. *Macrophomina* sp. differs from *M. phaseolina* by nucleotide polymorphisms in two loci (TEF and ITS) based on separate alignments of species types: TEF positions 3(T), 12 (T), 13(G), 21(A), 22(T), 30(C), 32(C), 55(T), 64(A), 95(A), 133(A), 138(T), 139(T), 140(T), 152(A), 174(C), 182(T), 200(A), 206(GAP), 207 (GAP), 210(T) and 239(C); ITS positions 31 (T). *Macrophomina* sp. differs from *M. pseudophaseolina* on TEF positions 3(T), 12(T), 16(C), 20(C), 22(T), 32(C), 37(G), 74(T), 90(G), 92(C), 95(A), 131(A), 134(T), 138(T), 139(T), 140(T), 174(C), 178(C), 182(T), 189(T), 206(C), 208(T), 209(T), 210(GAP), 211(GAP) and

219(T). Not shown variation on ITS sequences.

Type: **Brazil**: Bahia: Irecê, isolated from charcoal rot on *Ricinus communis* L., 2014, M. R. Claudino & D. J. Soares (VICXXXX – holotype; CCMF-CNPA288 ex-type culture); Additional cultures examined: CCMF-CNPA278 and CCMF-CNPA289.

DISCUSSION

In the last years, phylogenetic analyzes combined with morphological studies has been broadly used in the delimitation of cryptic species in Botryosphaeriales, as *Botryosphaeria* spp., *Diplodia* spp., *Lasiodiplodia* spp., *Neofusicoccum* spp., *Pseudofusicoccum* spp., *Phyllosticta* spp. and more recently *Macrophomina* spp., in which, based only on morphological data it would be not possible (Alves et al. 2008; Phillips et al. 2008, 2013; Pavlic et al. 2009; Begoude et al. 2010; Liu et al. 2012; Marques et al. 2013a,b; Slippers et al. 2013; Wikee et al. 2013; Alves et al. 2014; Abdollahzadeh et al., 2014; Machado et al. 2014; Netto et al. 2014; Sarr et al., 2014; Pitt et al. 2015; Trakunyingcharoen et al. 2015). Using this approach allowed us to find three *Macrophomina* species: *M. phaseolina*, *M. pseudophaseolina* and a new species associated with different hosts from Brazil.

Utilizing only the TEF1-α gene (data not shown), it was possible to distinguish the *Macrophomina* species, however, in taxonomic studies and for the characterization of new species, it is important to use other gene regions (Marques et al. 2013a; Machado et al. 2014; Hyde et al. 2014). For this reason, we used DNA sequences of the ITS and TEF1-α regions to provide sufficient support for the recognition of *Macrophomina* spp. Utilizing only the ITS sequences (data not shown), it was not possible to distinguish the *Macrophomina* species.

Macrophomina sp. is phylogenetically distinct from *M. phaseolina* and *M. pseudophaseolina*, but the sporulation inability of the new species strains became not possible to carry out morphological analysis of reproductive structures. Thus our description was based only in molecular data. The difficulty to obtain sporulation is a common fact in Botryosphaeriales and frequently it has discouraged taxonomic works with these fungi. Recently Marques et al. (2013b) proposed a new species of *Neofusicoccum* on *Mangifera indica* in Brazil based only on molecular data, due the lack of sporulation in culture.

In previous studies, Sarr et al. (2014) not shown clear morphological differences

between *M. phaseolina* and *M. pseudophaseolina*. However, in present study, *M. pseudophaseolina* has smaller conidia, larger conidiogenous cells and a rare small apical mucoid appendages on conidia in comparison with *Macrophomina phaseolina*. This is an evidence of morphological variation between isolates of the same species obtained from different hosts and geographical origin. This fact shows the importance of appropriate use of a polyphasic approach for species discrimination.

Macrophomina comprises species of great phytopathological importance due to its wide host range and cosmopolitan distribution (Far and Rossman 2015). In Brazil, this fungus was reported on *Abelmoschus esculentus* Moench, *Allium sativum* L., *Arachis hypogaea* L., *Brassica* sp., *Capsicum pendulum* Willd., *Citrus* sp., *Cocos nucifera* L., *Coffea arabica* L., *Corchorus capsularis* L., *Crotalaria juncea* L., *Cucumis melo* L., *Cucurbita* sp., *Cyamopsis tetragonolobus* Taub., *Daucus carota* L., *Dimorphandra gardneriana* Tul., *Dimorphandra mollis* Benth., *Eucalyptus deglupta* Blume, *Eucalyptus urophylla* S.T. Blake, *Fragaria* sp. L., *Glycine max* Merr., *Gossypium hirsutum* L., *Helianthus annuus* L., *Hibiscus esculentus* L., *Jatropha curcas* L., *Medicago sativa* L., *Opuntia ficus-indica* Mill., *Phaseolus aureus* Roxb., *Phaseolus vulgaris* L., *Pisum sativum* L., *Prosopis juliflora* (Swartz) DC., *Ricinus communis* L., *Sesamum indicum* L., *Sida* sp., *Solanum tuberosum* L., *Sorghum bicolor* (L.) Moench, *Triticum aestivum* L., *Vigna sesquipedalis* (L.) Fruw., *Vigna unguiculata* (L.) Walp., *Zea mays* L. and *Ziziphus* sp. (Mendes and Urben 2015).

This is first report of *Macrophomina pseudophaseolina* in Brazil, associated with *Arachis hypogaea*, *Gossypium hirsutum*, *Jatropha curcas* and *Ricinus communis*. In addition, the new species *Macrophomina* sp. is associated with *Ricinus communis* and *Jatropha gossypifolia*.

Based on results of this work and on the wide host range of *Macrophomina* spp. in Brazil, it is clear the need to conduct broader studies for the evaluation of diversity of species based on molecular and morphological data, since most reports do not provide sufficient data to identify the species level.

Thus, the utilization of DNA sequences and the discovery of cryptic species within *Macrophomina* are crucial for conducting studies of the genetic variability of this pathogen, formulating trade quarantine policies that prevent the dissemination of pathogens to other regions of the world and defining effective management strategies.

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Table 1. GenBank accession numbers of DNA sequences of Botryosphaeriaceae used in phylogenetic analyses.

Species	Isolates	Host	Genbank acession n°	
			ITS	EF
Botryosphaeria dothidea	CMW8000	*Prunus sp.*	AY236949	AY236898
B. dothidea	CBS110302	*Vitis vinifera*	AY259092	AY573218
Cophinforma eucalypti	MFLUCC110425	*Eucalyptus* sp.	JX646800	JX646865
C. eucalypti	MFLUCC110655	*Eucalyptus* sp.	JX646801	JX646866
Macrophomina phaseolina	CBS162.25	*Eucalyptus* sp.	KF531826	KF531803
M. phaseolina	CBS205.47	*Phaseolus vulgaris*	KF951622	KF951997
M. phaseolina	CBS227.33	*Zea mays*	KF531825	KF531804
M. phaseolina	CMM3615	*Jatropha curcas*	KF234547	KF226693
M. phaseolina	CMM3650	*Jatropha curcas*	KF234552	KF226710
M. phaseolina	**CMM3875**	*Jatropha curcas*	KF369263	KF553897
M. phaseolina	**CCMF-CNPA 617**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 281**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 283**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 651**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 707**	*Glycine max*	-	-
M. phaseolina	**CCMF-CNPA 274**	*Sesamum indicum*	-	-
M. phaseolina	**CCMF-CNPA 277**	*Helianthus annuus*	-	-
M. phaseolina	**CCMF-CNPA 285**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 286**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 290**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 292**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 279**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 280**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 282**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 284**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 295**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 296**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 654**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 652**	*Ricinus communis*	-	-
M. phaseolina	**CCMF-CNPA 653**	*Ricinus communis*	-	-
M. pseudophaseolina	**CMM3653**	*Jatropha curcas*	KF369262	KF553906
M. pseudophaseolina	**CMM4029**	*Jatropha curcas*	KF369270	KF553903
M. pseudophaseolina	**CMM4030**	*Jatropha curcas*	KF369271	KF553904
M. pseudophaseolina	**CMM4032**	*Jatropha curcas*	KF369272	KF553905
M. pseudophaseolina	**CCMF-CNPA 287**	*Ricinus communis*	-	-
M. pseudophaseolina	**CCMF-CNPA 291**	*Ricinus communis*	-	-
M. pseudophaseolina	**CCMF-CNPA 293**	*Gossypium hirsutum*	-	-

M. pseudophaseolina	**CCMF-CNPA 294**	*Gossypium hirsutum*	-	-
M. pseudophaseolina	**CCMF-CNPA 668**	*Arachis hypogaea*	-	-
M. pseudophaseolina	**CCMF-CNPA 667**	*Arachis hypogaea*	-	-
M. pseudophaseolina	**CCMF-CNPA 699**	*Arachis hypogaea*	-	-
M. pseudophaseolina	CPC21400	*Arachis hypogaea*	-	-
M. pseudophaseolina	CPC21417	*Arachis hypogaea*	-	-
M. pseudophaseolina	CPC21502	*Hibiscus sabdarifa*	-	-
M. pseudophaseolina	CPC21524	*Hibiscus sabdarifa*	-	-
M. pseudophaseolina	CPC21527	*Hibiscus sabdarifa*	-	-
M. pseudophaseolina	CPC21528	*Hibiscus sabdarifa*	-	-
Macrophomina sp.	**CCMF-CNPA 278**	*Jatropha gossypifolia*	-	-
Macrophomina sp.	**CCMF-CNPA 288***	*Ricinus communis*	-	-
Macrophomina sp.	**CCMF-CNPA 289**	*Ricinus communis*	-	-

The specimens obtained in this study are highlighted in bold (* = ex-type).

Table 2. Principal morphological characteristics of *Macrophomina* spp.

Species	Conidial dimensions (µm)	Conidiogenous Cells (µm)	Reference
Macrophomina limbalis	22–28 × 8–10	10–20 × 1–2	Sydow (1924)
M. philippinensis	16–24 × 6–7.5	6–12 × 1.5	Petrak (1923)
M. pseudeverniae	16–22 × 6–9	7–10 × 6–7	Etayo and Diederich (1996)
M. phaseolina	19–30 × 6–9	6–12 ×4–6	Sarr et al. (2014)
M. pseudophaseolina	19–27 × 7.5–9	8–15 × 3–4	Sarr et al. (2014)
M. pseudophaseolina	15–22 × 5,5–8	10–17 × 4–5	This study

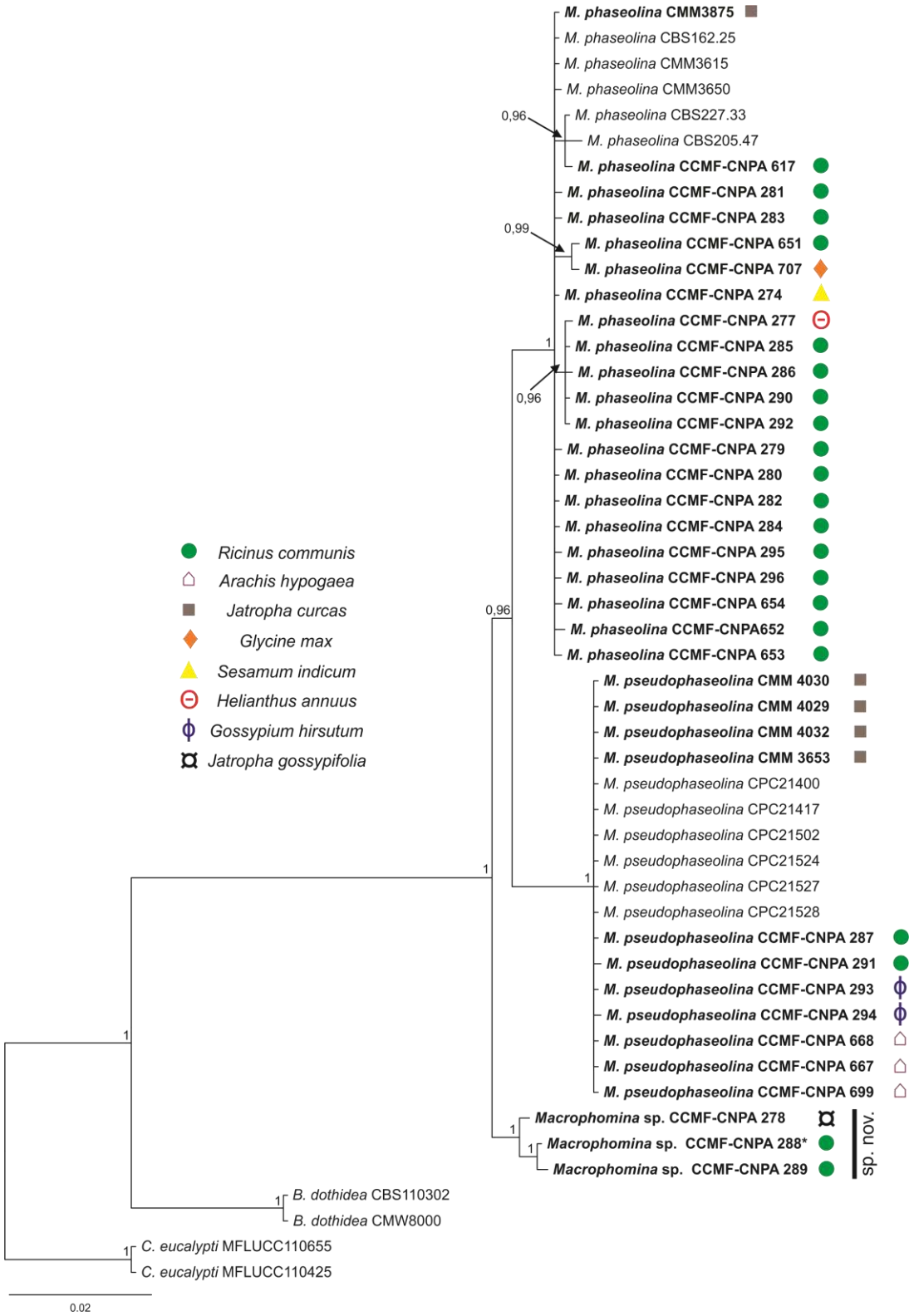

Fig. 1. Multilocus phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the ITS and TEF-1α. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Cophinforma eucalypti* MFLUCC110425 and MFLUCC110655. The species obtained in this study are highlighted in bold. *Indicates the ex-type culture. The colored symbols represent the host of each isolate.

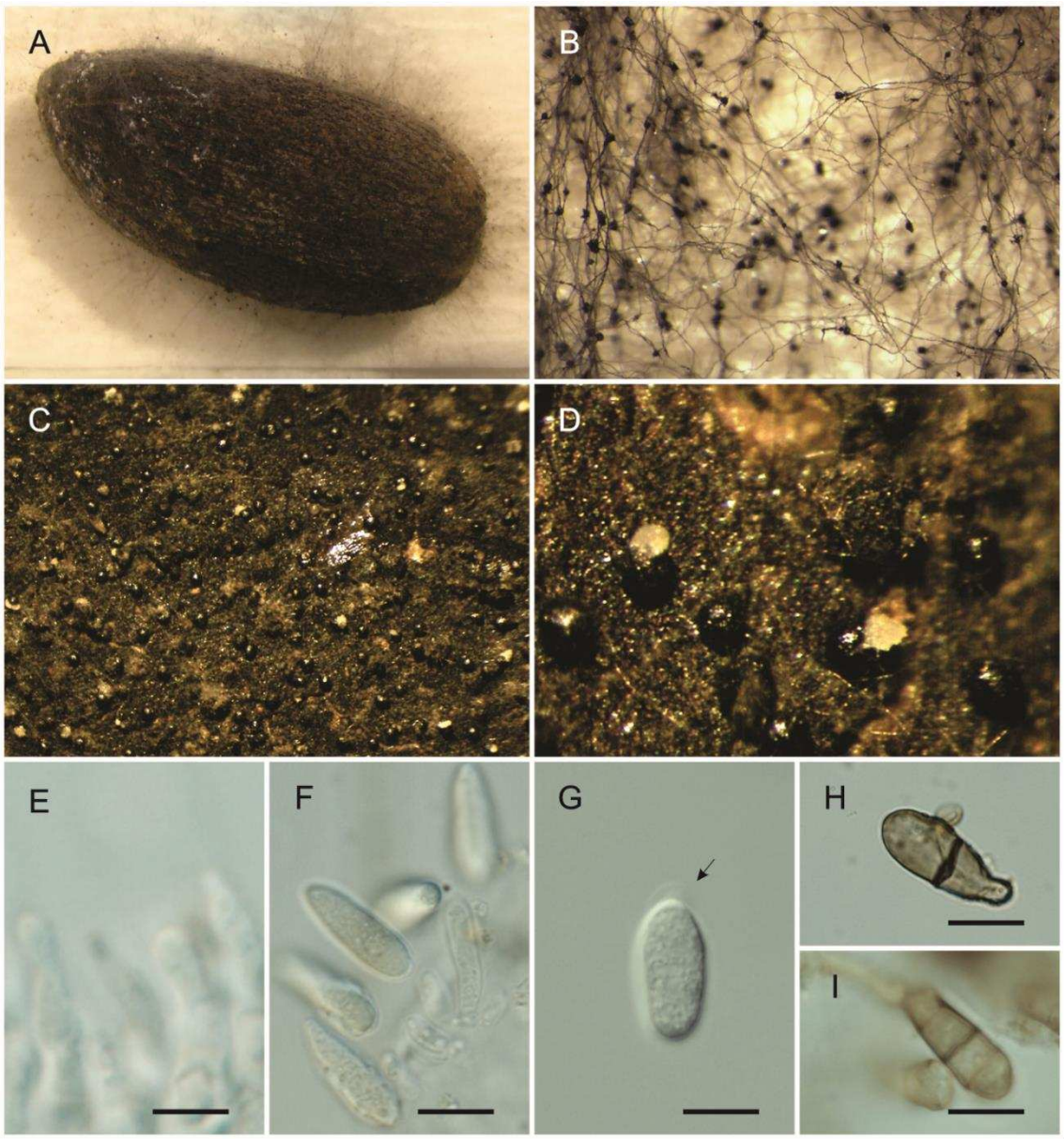

Fig. 2. *Macrophomina pseudophaseolina* (CMM4030). **A–B.** *Jatropha curcas* seed covered by mycelium and sclerotia in blotter test. **C–D.** Black sclerotia and conidiomata on seed in culture. **E.** Conidiogenous cells. **F.** Immature conidia. **G.** Apical mucoid appendages on conidia. **H–I.** Mature and up to three septate conidia. Bars = 10 µm.

ARTIGO 3

According to the guidelines of Fungal Diversity

Botryosphaeriaceae species causing dieback on Annonaceae in Brazil

Botryosphaeriaceae species causing dieback on Annonaceae in Brazil

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Abstract:

In Brazil, the Annonaceae species *Annona muricata*, *Annona squamosa*, *Annona cherimola*, and atemoya (a hybrid obtained with *A. cherimola* and *A. squamosa*) are cultivated in several regions and produce fruits highly appreciated by consumers and which have great economic importance. Many diseases affect these crops, however the dieback has been highlighted, since in more severe cases, it can causes death of plants. Due to the lack of suitable diagnostic studies until the moment, this work aimed to identify the Botryosphaeriaceae causing dieback on Annonaceae in Brazil. Based on combined phylogenetic analyses of ITS and TEF-1α, ten species of Botryosphaeriaceae were identified, *Lasiodiplodia brasiliense*, *L. crassispora*, *L. hormozganensis*, *L. iraniensis*, *L. jatrophicola*, *L. pseudotheobromae*, *L. subglobosa*, *L. theobromae*, *Pseudofusicoccum stromaticum* and *Lasiodiplodia* sp., which is proposed as new phylogenetic species. Except for *P. stromaticum* and *L. theobromae*, all species were pathogenic and reproduced the symptoms of dieback. The results showed that the dieback of Annonaceae in Brazil is caused by several species of *Lasiodiplodia* in Brazil.

Keywords: Aetiology, *Annona*, *Lasiodiplodia*, *Pseudofusicoccum*, plant pathogen, phylogeny.

INTRODUCTION

The Annonaceae family is a large group of Angiosperms that includes about 128 genera and 2300 species. In Brazil, there are 29 genera and 386 species distributed mainly in the Amazon biome, but also in the Atlantic Forest and Cerrado (Judd et al. 2009; Lopes and Mello-Silva 2014). Many species of Annonaceae are known by the production of fruits of great economic importance in many countries, such as *Annona muricata* L., *Annona squamosa* L., *Annona cherimola* Mill. and a hybrid obtained with *A. cherimola* and *A. squamosa* known as atemoya. In Brazil, these species are found in several regions, but it was in the semiarid region of the Minas Gerais and Northeast of Brazil that had a great expansion of cultivated areas. The largest producer is Bahia state, followed by Alagoas and São Paulo (Braga Sobrinho 2014).

The *Annona muricata* produces large fruits with up to eight kilograms per unit that are marketed mostly in the fruit pulp form. The *A. squamosa* is cultivated in small areas, but its fruits are highly appreciated in some regions of Brazil by its sweet taste. The atemoya (*A. cherimola* x *A. squamosa*) was introduced in Brazil in the 1980s and it has been successfully cultivated in several states of Brazil. Among the Annonaceae species, the atemoya fruits has been the highest preference of the consumers, once presents appreciated characteristics in the *A. cherimola* and *A. squamosa* (Braga Sobrinho 2014).

Several diseases and pathogens have been reported in Annonaceae in Brazil (Lopez 2005; Junqueira and Junqueira 2014; Mendes and Urben 2015; Farr and Rossman 2015), however despite of the great economic importance and of the expansion of cultivated areas in Brazil, there are a lack of more suitable studies of diseases diagnostics of these fruit crops.

It has been observed in several cultivated areas with Annonaceae in Brazil, the high occurrence of dieback that many times resulted in the death of plants. The symptoms start from tip of stems and can expand to the trunk causing rot. Often, the affected plant parts become invaded by termites. This disease has been frequently associated with the Botryosphaeriaceae fungus, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Ponte 1985; Lopez 2005; Junqueira and Junqueira 2014), however more suitable studies should be performed to find the causal agents of this disease, once the genus *Lasiodiplodia* is known to comprise species complexes (Alves et al. 2008; Abdollahzadeh et al. 2010; Marques et al. 2013a*;* Machado et al. 2014a; Netto et al. 2014).

In the past, the species discrimination of Botryosphaeriaceae was considered a difficult task and often confused, once only morphological characters, ecological and geographic data were used in taxonomic works (Slippers et al. 2013, 2014; Jami et al. 2014). However, in last years, the use of DNA sequences in phylogenetic studies of fungi has helped discriminate the cryptic species, solve taxonomic difficulties, diagnostics of diseases and the discovery of several new species to science (Taylor et al. 2000; Crous et al. 2006; Alves et al. 2008; Slippers et al. 2013, 2014). Thus, the use of molecular tools combined with the morphological studies is essential for studies with Botryosphaeriaceae.

The family Botryosphaeriaceae (Botryosphaeriales, Ascomycota) includes fungi with different life styles, such as saprophytic, endophytic and the more important, plant pathogens. It can incite a great variety of diseases in a broad range of hosts and it has been responsible for many losses in the world (Slippers and Wingfield 2007). Due to its phytopathological importance, in the last years Botryosphaeriaceae has been extensively studied by many research groups that helped clarify several complexes of species and solve many taxonomic difficulties (Crous et al. 2006; Phillips et al. 2008, 2013; Liu et al. 2012; Slippers et al. 2013, 2014). In Brazil, some few works using molecular tools allied to morphological studies have been made with Botryosphaeriaceae. These works revealed a great diversity of new species or new reports for this country, associated with several hosts, such as the cultures of cassava, coconut, grape, mango, papaya, physic nut, strawberries and ornamental plants (Marques et al. 2012, 2013a,b; Machado et al. 2012, 2014a,b; Silva et al. 2013; Lopes et al. 2014; Netto et al. 2014; Correia et al. 2015; Rosado et al. 2015). However, given the huge diversity of cultivated and native plants in Brazil that are potential hosts of Botryosphaeriaceae and the great amount of misdiagnosed diseases, these studies are still incipient.

Thus, based on the importance of Annonaceae species in Brazil and in other countries, in addition to severe damage caused by diseases in these crops and the lack of suitable etiologic studies of these diseases, the aims of this work were to identify the species of Botryosphaeriaceae causing dieback on Annonaceae in Brazil.

MATERIALS AND METHODS

Sample collection and isolation

Annonaceae plants with symptoms of dieback were collected during 2014. The visited areas were in Jaíba, Paraopeba and Piraúba in Minas Gerais state, Petrolina in

Pernambuco state and Juazeiro in Bahia state. Additionally, isolates from Trairi and Parambu in Ceará state, previously identified as *Lasiodiplodia theobromae* by morphological characters in the study of Lima et al. (2013) were ceded by authors. The samples were sent to the Laboratório de Micologia e Etiologia de Doenças Fúngicas de Plantas (Departamento de Fitopatologia, Universidade Federal de Viçosa). Longitudinal sections of the stems were made for observation of vascular necrosis, from which small fragments of areas of transition between the healthy tissue and the symptomatic tissue were obtained for fungal isolations. These fragments were disinfected in 70% ethanol for 1 min followed by 1% sodium hypochlorite for 3 min and washed in sterile distilled water. Later, these fragments were placed in Petri dishes with Potato Dextrose Agar (PDA - Acumedia®) and incubated at 25°C.

The isolates obtained were grown in Petri dishes with 2% Water Agar (WA - Agar Agar, type I Himedia®) and incubated at 25° for 1-2 days. After, the growing tips of hyphae of colonies were cut out and transferred to Petri dishes with PDA. The pure cultures were stored in tubes on PDA at 10°C. Upon the species identification, the isolates were deposited in the Coleção Octávio Almeida Drummond (COAD) culture collection (Viçosa, Brazil).

Morphological studies

The isolates were grown on Petri dishes containing 2% WA overlaid with double-sterilized needles and twigs of *Pinus* or corn straw and incubated at 25°C with a photoperiod of 12 hours to induce the formation of fruiting bodies and sporulation.

DNA extraction, Sequencing and Phylogenetic studies

To extract genomic DNA, the isolates were grown on PDA at 25 °C for one week. Approximately 40 mg of mycelia were collected and placed in a 2 mL microcentrifuge tube containing 600 µL of Nuclei Lysis Solution of the Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.), 100 mg of Polyvinylpyrrolidone (PVP; Sigma–Aldrich Co.) and four steal beads. Next, the samples were mixed and crushed in the L-Beader 3 (Loccus Biotecnologia). After the maceration, the extraction was continued as described by Pinho et al. (2012).

Target sequences of the Internal Transcribed Spacer regions 1 and 2 including the 5.8S rRNA gene (ITS) and Translation Elongation Factor 1-α (TEF1-α) were amplified using primers ITS1 and ITS4 for ITS (White et al. 1990); EF1-728F (Carbone

and Kohn 1999) and EF2R (Jacobs et al. 2004) or EF1-688F (Alves et al. 2008) and EF1-986R (Carbone and Kohn 1999) for partial TEF1-α. The PCR conditions and reagents were the same as those described by Machado et al. (2014a).

PCR products were purified and sequenced by Macrogen Inc., Korea (http://www.macrogen.com). The nucleotide sequences were edited with the BioEdit software (Hall 2012). All sequences were checked manually and nucleotides with ambiguous positions were clarified using both primer direction sequences. New sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (Table 1). Consensus sequences were compared against GenBank's database using their Mega BLAST program for a first identification. The ITS and TEF1-α sequences of additional species were retrieved from GenBank (Table 1) and aligned with sequences generated in this study using the multiple sequence alignment program MUSCLE® (Edgar 2004), built in MEGA v. 6 software (Tamura et al. 2013). Alignments were checked and manual adjustments were made when necessary. The resulting alignment was deposited into TreeBASE (http://www.treebase.org/) under accession number XXXX.

Bayesian inference concatenated (BI) analyses employing a Markov Chain Monte Carlo method were performed with all sequences, first with each gene/locus separately and then with the concatenated sequences (ITS and TEF1-α). Before launching the BI, the best nucleotide substitution model was determined for each gene with MrMODELTEST 2.3 (Posada and Buckley 2004). Once the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). The GTR+I+G and K80+I models of evolution were used for ITS in the analyses of *Lasiodiplodia* and *Pseudofusicoccum* respectively. While HKY+G was selected for TEF1-α in all analyses. The phylogenetic analysis of the concatenated alignment was performed with the CIPRES web portal (Miller et al. 2010) using MrBayes v.3.2.6 (Ronquist and Huelsenbeck 2003). In MrBayes, data were partitioned by locus, and the parameters of the nucleotide substitution models for each partition were set as described above. Four MCMC chains were run simultaneously, starting from random trees for 10 000 000 generations. Trees were sampled every 1000th generation for a total of 10 000 trees. The first 2 500 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a majority-rule consensus tree generated with the remaining 7 500 trees. Trees were visualized in FigTree v1.3.1 (Rambaut 2009) and exported to graphics programs. The *Lasiodiplodia* tree (Fig 2) was

rooted to *Neoscytalidium hyalinum* CBS499.66. The *Pseudofusicoccum* tree (Fig 3) was rooted to *Phyllosticta philoprina* CBS616.72.

Pathogenicity tests

Three one-year-old plants of *Annona muricata*, *Annona squamosa* and atemoya (*A. cherimola* x *A. squamosa)* were inoculated with representative isolates of Botryosphaeriaceae species in the respective hosts they were isolated. For inoculation, the isolates were grown in Petri dishes with PDA for 7 days at 25°C. The tips of stems of the healthy plants were wounded with a sterilized scalpel. Later, 6 mm-diameter disks containing mycelia from the margins of the growing culture were placed on the wounds. A portion of moistened cotton was placed just below each disk and subsequently covered with parafilm. On control plants, only PDA plugs were placed on wounded stems. After five days, the parafilm and cotton were removed. The inoculated plants were maintained in a greenhouse at 25°C for 20 days from which it was evaluated for the presence or absence of symptoms.

RESULTS

Symptomatology and fungal isolation

The symptoms frequently observed in the field were dieback that frequently resulted in the death of plants. The symptoms start from tip of stems, causing wilting, subsequent leaf fall and many times expanded to the trunk causing rot. Also, it could be observed a necrosis of vascular system like a black rot. Often, the affected plant parts become invaded by termites (Fig 1).

Fifty-four isolates of Botryosphaeriaceae were obtained from dieback of native and cultivated Annonaceae and additionally, two isolates obtained from post-harvest fruit rot of araticum (*Annona crassiflora* Mart.) were included in the analyses. Fourty-two isolates were obtained in a farm from Jaíba and two isolates from Paraopeba in north of Minas Gerais state, four isolates from Piraúba in Zona da Mata of Minas Gerais. Four isolates were obtained in Petrolina (Pernambuco state) and two isolates from Juazeiro (Bahia state). In addition, two isolates obtained in *Annona squamosa* and *Annona muricata* from Trairi and Parambu (Ceará state) used in the study of Lima et al. (2013) were ceded by authors. From the 56 isolates, 48 were classified in the genus *Lasiodiplodia* and 8 isolates as *Pseudofusicoccum*.

PCR amplification, sequencing and phylogenetic analyses

Initially, sequences of TEF1-α were generated for all isolates, in which allowed to identify preliminarily the species of each isolate. Later, representative isolates of each species were selected for ITS sequencing and the sequences obtained were used in phylogenetic analysis to confirm the identity of isolates. PCR reactions were conducted successfully for all gene regions used, however the pair of primers EF1-728F and EF2R for TEF1-α did not produce sequences with a good quality for several isolates. Many times, the more variable region 5' of these sequences was smaller than sequences of databank. Thus, several isolates were sequenced with the pair of primers EF1-688F and EF1-986R as recommended by Alves et al. (2008). The amplicons for ITS were approximately 500bp in size, those for TEF1-α were 700bp for EF1-728F/EF2R and 300bp for EF1-688F/EF1-986R. The sequences used in the phylogenetic analysis were deposited in Genbank. The accession numbers are available in Table 1.

The concatenated analysis of ITS and TEF1-α was performed separately for each genus found in this work (*Lasiodiplodia* and *Pseudofusicoccum*), once using genera genetically distant in the same analysis can produce an inaccurate alignment or can reduce the support of nodes. The analyses resulted in two trees with well-supported clades (Fig 2 and 3). The analysis of *Lasiodiplodia* included 74 taxa and contained 802 characters, of which 107 were parsimony-informative, 187 were variable and 569 were conserved. The analysis of *Pseudofusicoccum* included 18 taxa and contained 890 characters, of which 37 were parsimony-informative, 164 were variable and 646 were conserved.

Based on phylogenetic analyses (Fig 2 and 3), the isolates were separated in ten different species of Botryosphaeriaceae, in which eight are known species: *Lasiodiplodia brasiliense* (on *Annona muricata*), *L. crassispora* (on *Annona muricata*, *Annona squamosa* and a native Annonaceae), *L. hormozganensis* (on *Annona squamosa*), *L. iraniensis* (on *Annona muricata*, *Annona squamosa* and atemoya), *L. jatrophicola* (on *Annona muricata*), *L. pseudotheobromae* (on *Annona crassiflora*, *Annona muricata*, *Annona squamosa* and atemoya), *L. subglobosa* (*Annona muricata* and a native Annonaceae), *L. theobromae* (*Annona muricata* and *Annona squamosa*), *Pseudofusicoccum stromaticum* (*Annona crassiflora*, *Annona muricata* and atemoya); and a new phylogenetic species (on *Annona squamosa*) to be proposed. The analyses of TEF1-α alone (data not shown), provided a preliminary identification of all isolates of this study and enabled to verify the frequency of each species (Fig 4).

Taxonomy

***Lasiodiplodia* sp.** (To be proposed as new) (Fig 2)

MycoBanK: XXXX

Etymology: XXXX.

Cultures sterile. *Lasiodiplodia* sp. differs from its closest phylogenetically species *Lasiodiplodia subglobosa* by nucleotide polymorphisms on TEF based on separate alignments of species types: TEF positions 7(T), 8(T) 9(C), 10(G), 11(A), 12(G), 13(A), 14(A), 15(G), 16(T), 17(G), 18(A), 19(G), 20(T), 21(GAP), 22(GAP), 23(GAP), 43(T) and 46(G). ITS sequences did not show variation.

Habitat: On *Annona squamosa*

Known distribution: Jaíba, Minas Gerais state

Holotype: BRAZIL, Jaíba, Minas Gerais, on dieback of *Annona squamosa*, 2014, A. R. Machado & O. L. Pereira, VIC XXXX (holotype designated here), COADXXXX (Ex-type culture)

Pathogenicity tests

From all Botryosphaeriaceae species inoculated in *Annona muricata*, the species *Lasiodiplodia brasiliense*, *L. crassispora*, *L. iraniensis*, *L. jatrophicola*, *L. pseudotheobromae* and *L. subglobosa* reproduced the symptoms of dieback observed in the field (Fig 5 A–G). Under the test conditions, only the *Lasiodiplodia theobromae* and *Pseudofusicoccum stromaticum* did not produce symptoms. From the species inoculated in *Annona squamosa*, the species *Lasiodiplodia* sp., *L. crassispora*, *L. hormozganenesis* and *L. iraniensis* showed to be pathogenic and reproduced the symptoms of dieback (Fig 5 H–L). In the same way of the results for *Annona muricata*, under the test conditions, *L. theobromae* was not pathogenic in *Annona squamosa*. The isolates of the species *L. iraniensis* and *L. pseudotheobromae* found associated with atemoya were pathogenic (Fig 5 M–P). However, the species *Pseudofusicoccum stromaticum* was not pathogenic until the last evaluation, since it did not differentiate the control plants.

DISCUSSION

In the past, the dieback of Annonaceae was associated only with *Lasiodiplodia theobromae* (Ponte 1985; Lopez 2005; Junqueira and Junqueira 2014). But, in this study,

through combined phylogenetic analyses of ITS and TEF-1α, it were identified ten species of Botryosphaeriaceae causing disease or associated with *Annona muricata*, *Annona squamosa*, atemoya (*A. cherimola* x *A. squamosa*), *Annona crassiflora* and a native unidentified Annonaceae plant from north of Minas Gerais state, frequently found in riparian forests. Until now, the morphological characterization of species was not possible, because of the sporulation inability of the isolates in several substrates described in the methodology. Nevertheless, new methodologies are being tested.

Among the species found in this work, only *Pseudofusicoccum stromaticum* and *Lasiodiplodia theobromae* did not produce symptoms on the inoculated plants. Possibly, the isolates of these species used in the pathogenicity tests were associated with the hosts endophytically and grown in culture during the isolations. New tests with other isolates should be performed to discard this species as pathogens of these crops. *Pseudofusicoccum stromaticum* was firstly described associated with asymptomatic branches of *Eucalyptus* and *Acacia mangium* trees in Venezuela, suggesting its endophytic nature (Phillips et al. 2013). However, this species was already reported causing dieback and stem-end rot in fruits of mango in Brazil (Marques et al. 2012, 2013b; Phillips et al. 2013).

In several works carried out in Brazil, *Lasiodiplodia theobromae* is one of the most dominant species in the populations studied associated with different hosts (Marques et al. 2013a; Netto et al. 2014; Correia et al. 2015; Rosado et al. 2015). However, in the present study, this species showed a lower frequency among the isolates (5%), followed by *L. brasiliense* (4%), *L. subglobosa* (3%), *L. jatrophicola* (2%) and *L. hormozganensis* (2%). The most prevalent *Lasiodiplodia* species was *L. iraniensis* (36%) followed by *L. pseudotheobromae* (18%), *L. crassispora* (9%) and *Lasiodiplodia* sp. (7%). Although this study has examined a reasonable amount of isolates obtained from different locations in Brazil, it would be appropriate to obtain more isolates from other regions, mainly of the Northeast region of Brazil, in which it is considered the largest producer of Annonaceae in this country, to access more information about the species frequency in these hosts.

Lasiodiplodia iraniensis was firstly described associated with *Mangifera indica*, *Eucalyptus* sp., *Citrus* sp., *Salvadora persica*, *Juglans* sp. and *Terminalia catappa* (Abdollahzadeh et al. 2010). In Brazil, this species was associated with mango causing stem-end rot and it was one of the most prevalent species in Northeastern of Brazil

(Marques et al. 2013a). The results of this work have shown the wide distribution of *L. iraniensis* (Jaíba, Minas Gerais state; Parambú, Ceará state), infecting different hosts (*Annona squamosa*, atemoya and *Annona muricata*). Probably, this fungus is widely distributed in other regions of Brazil associated with several other hosts. However, more studies of Botryosphaeriaceae diversity are needed to confirm this hypothesis.

Lasiodiplodia pseudotheobromae is another prevalent species found in this work. This species was firstly described on *Acacia mangium*, *Citrus aurantium*, *Coffea* sp., *Gmelina arborea* and *Rosa* sp. from Costa Rica, The Netherlands, Suriname and Zaire (Alves et al. 2008). Recently, this fungus was reported on cassava (Machado et al. 2014b), coconut (Rosado et al. 2015), grapevine (Correia et al. 2013, 2015), mango (Marques et al. 2013a), physic nut (Machado et al. 2014a) and papaya (Netto et al. 2014) in Brazil. In this study, *L. pseudotheobromae* was associated with *Annona squamosa*, *Annona muricata*, atemoya, on fruits of *Annona crassiflora* and causing dieback in a native unidentified Annonaceae from Minas Gerais state, which show its wide distribution and wide host range in Brazil.

The other known species less frequently isolated, *Lasiodiplodia brasiliense* (on *Annona muricata* from Jaíba, Minas Gerais state), *L. crassispora* (on *Annona muricata*, *Annona squamosa* and native Annonaceae from Jaíba, Minas Gerais state), *L. hormozganensis* (on *Annona squamosa* from Petrolina, Pernambuco state), *L. jatrophicola* (on *Annona muricata* from Trairi, Ceará state), *L. subglobosa* (*Annona muricata* and native Annonaceae from Jaíba, Minas Gerais state) and *L. theobromae* (in *Annona muricata* from Jaíba and *Annona squamosa* from Piraúba, Minas Gerais state) were already related in Brazil associated with several hosts (Marques et al. 2013a; Correia et al. 2013, 2015; Machado et al. 2014a; Netto et al. 2014; Rosado et al. 2015). The isolates from the municipalities of Trairi and Parambú (Ceará state), obtained by Lima et al. (2013), were identified erroneously only by morphological characters, once in this study, these isolates were identified as *L. jatrophicola* and *L. iraniensis* respectively.

Thus, according to the results of this work, the dieback of Annonaceae is caused by several species of *Lasiodiplodia* and this is first report of *Lasiodiplodia* sp., *L. brasiliense*, *L. crassispora*, *L. hormozganensis*, *L. iraniensis*, *L. jatrophicola*, *L. pseudotheobromae*, *L. subglobosa* and *Pseudofusicoccum stromaticum* associated with Annonaceae in Brazil. In addition, as already showed in several works, the use of molecular tools is essential for identification of *Lasiodiplodia* species, since based only

on morphological characters, it would be not possible (Alves et al. 2008; Slippers et al. 2014).

An important fact to be observed in this study is the presence of species *Lasiodiplodia crassispora* and *L. pseudotheobromae*, originally described in other countries, on native plants of Brazil (*Annona crassiflora* and with a native unidentified Annonaceae). This show a wide distribution of these species, and similar as observed for *L. theobromae*, these species were related in several native or cultivated hosts from other countries and continents (Burgess et al. 2006; Begoude et al. 2010; Mehl et al. 2011; Marques et al. 2013a; Slippers et al. 2013; Machado et al. 2014a; Trakunyingcharoen et al. 2015). In future, this information can helps studies about the origin and distribution of plant pathogenic Botryosphaeriaceae. In addition, it can helps future studies with control of diseases caused by Botryosphaeriaceae, once the native plant species can serve as alternative hosts for these plant pathogens.

To our knowledge, this is the first etiologic study using molecular tools for identification of pathogens of Annonaceae, focusing in dieback, in which we can consider as the main disease of these crops, since it can cause death of the plants. The information showed here will certainly be useful for future studies of the managements measures of dieback control in Annonaceae. In addition, will provide data for quarantine programs and possibly for the development of resistant Annonaceae varieties for dieback.

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Table 1 Genbank accession numbers of DNA sequences of Botryosphaeriaceae used in phylogenetic analyses.

Species	Isolates	Host/Substrate	Genbank acession n°	
			ITS	EF1-α
Lasiodiplodia venezuelensis	CMW13513	*Acacia mangium*	DQ103549	DQ103570
L. venezuelensis	WAC12539	*Acacia mangium*	DQ103547	DQ103568
L. brasiliense	CMM4015	*Mangifera indica*	JX464063	JX464049
L. brasiliense	CMM2313	*Carica papaya*	KC484793	KC481524
L. brasiliense	**CDA1170**	***Annona muricata***	-	-
L. crassispora	CBS110492	Unknown	EF622086	EF622066
L. crassispora	CMW22653	*Pterocarpus angolensis*	FJ888465	FJ888452
L. crassispora	**CDA1347**	**native Annonaceae**	-	-
L. crassispora	**CDA1203**	***Annona squamosa***	-	-
L. crassispora	**CDA1204**	***Annona muricata***	-	-
L. citricola	IRAN1521C	*Citrus* sp.	GU945353	GU945339
L. citricola	IRAN1522C	*Citrus* sp.	GU945354	GU945340
L. euphorbiicola	CMM3651	*Jatropha curcas*	KF234553	KF226711
L. euphorbiicola	CMM3652	*Jatropha curcas*	KF234554	KF226715
L. euphorbicola	CMM3609	*Jatropha curcas*	KF234543	KF226689
L. egyptiacae	CBS130992	*Mangifera indica*	JN814397	JN814424
L. egyptiacae	BOT-29	*Mangifera indica*	JN814401	JN814428
L. exigua	CBS 137785	*Retama raetam*	KJ638317	KJ638336
L. exigua	BL 184	*Retama raetam*	KJ638318	KJ638337
L. gilanensis	IRAN1523C	Unknown	GU945351	GU945342
L. gilanensis	IRAN1501C	Unknown	GU945352	GU945341
L. gonubiensis	CBS115812	*Syzygium cordatum*	DQ458892	DQ458877
L. hormozganensis	IRAN1500C	*Olea* sp.	GU945355	GU945343
L. hormozganensis	IRAN1498C	*Mangifera indica*	GU945356	GU945344
L. hormozganensis	**CDA1363**	***Annona squamosa***	-	-
L. iraniensis	IRAN1517C	*Citrus* sp.	GU945349	GU945337
L. iraniensis	IRAN1519C	*Mangifera indica*	GU945350	GU945338
L. iraniensis	**CDA1200**	***Annona squamosa***	-	-
L. iraniensis	**CDA1134**	***Annona muricata***	-	-
L. iraniensis	**CDA1147**	***Annona squamosa***	-	-
L. iraniensis	**CDA1185**	**atemoya**	-	-
L. jatrophicola	CMM3610	*Jatropha curcas*	KF234544	KF226690
L. jatrophicola	**CDA1146**	***Annona muricata***	-	-
L. macrospora	CMM3833	*Jatropha curcas*	KF234557	KF226718
L. margaritacea	CBS122519	*Adansonia gibbosa*	EU144050	EU144065
L. mahajangana	CMW27801	*Terminalia catappa*	FJ900595	FJ900641
L. mahajangana	CMW27820	*Terminalia catappa*	FJ900597	FJ900643
L. mediterranea	CBS 137783	*Quercus ilex*	KJ638312	KJ638331
L. mediterranea	CBS 137784	*Vitis vinifera*	KJ638311	KJ638330
L. missouriana	UCD2193MO	*Vitis vinifera*	HQ288225	HQ288267
L. missouriana	UCD2199MO	*Vitis vinifera*	HQ288226	HQ288268
L. parva	CBS456.78	Cassava-field soil	EF622083	EF622063
L. parva	CBS495.78	Cassava-field soil	EF622085	EF622065
L. plurivora	STE-U5803	*Vitis vinifera*	EF445362	EF445395
L. pseudotheobromae	CBS116459	*Gmelina arborea*	EF622077	EF622057
L. pseudotheobromae	CMM3887	*Jatropha curcas*	KF234559	KF226722
L. pseudotheobromae	**CDA1138**	***Annona muricata***	-	-
L. pseudotheobromae	**CDA1182**	**atemoya**	-	-
L. pseudotheobromae	**CDA1400**	***Annona crassiflora***	-	-
L. pseudotheobromae	**CDA1352**	***Annona muricata***	-	-
L. pseudotheobromae	**CDA1399**	***Annona crassiflora***	-	-
L. pseudotheobromae	**CDA1196**	***Annona squamosa***	-	-
L. pyriformis	CBS 121770	*Acacia mellifera*	EU101307	EU101352
L. pyriformis	CBS 121771	*Acacia mellifera*	EU101308	EU101353
L. rubropurpurea	WAC12536	*Eucalyptus grandis*	DQ103554	DQ103572
L. subglobosa	CMM3872	*Jatropha curcas*	KF234558	KF226721
L. subglobosa	CMM4046	*Jatropha curcas*	KF234560	KF226723
L. subglobosa	**CDA1137**	***Annona muricata***	-	-
L. subglobosa	**CDA1344**	**native Annonaceae**	-	-

***Lasiodiplodia* sp.**	**CDA1190**	***Annona squamosa***	**-**	**-**
***Lasiodiplodia* sp.**	**CDA1191**	***Annona squamosa***	**-**	**-**
***Lasiodiplodia* sp.**	**CDA1192**	***Annona squamosa***	**-**	**-**
***Lasiodiplodia* sp.**	**CDA1193***	***Annona squamosa***	**-**	**-**
L. thailandica	**CPC 22755**	*Phyllanthus acidus*	KM006433	KM006464
L. thailandica	CPC22795	*Mangifera indica*	KJ193637	KJ193681
L. theobromae	CBS164.96	Unknown	AY640255	AY640258
L. theobromae	CBS124.13	Unknown	DQ458890	DQ458875
L. theobromae	CBS111530	Unknown	EF622074	EF622054
L. theobromae	**CDA1211**	***Annona squamosa***	**-**	**-**
L. theobromae	**CDA1212**	***Annona squamosa***	**-**	**-**
L. theobromae	**CDA1169**	***Annona muricata***	**-**	**-**
L. viticola	UCD2553AR	*Vitis vinifera*	HQ288227	HQ288269
L. viticola	UCD2604MO	*Vitis vinifera*	HQ288228	HQ288270
Neoscytalidium hyalinum	CBS499.66	*Mangifera indica*	AY819727	EU144063
Pseudofusicoccum adansoniae	CBS122055	*Adansonia gibbosa*	EF585523	EF585571
P. adansoniae	WAC12689	*Mangifera indica*	EF585534	EF585567
P. ardesiacum	CBS122062	*Adansonia gibbosa*	EU144060	EU144075
P. ardesiacum	WAC13294	*Mangifera indica*	GU172405	GU172437
P. kimberleyense	CBS122058	*Acacia synchronicia*	EU144057	EU144072
P. kimberleyense	CBS 122059	*Eucalyptus* sp.	EU144056	EU144071
P. olivaceum	CBS 124939	*Pterocarpus angolensis*	FJ888459	FJ888437
P. olivaceum	CBS 124940	*Pterocarpus angolensis*	FJ888462	FJ888438
P. stromaticum	CBS 117448	*Eucalyptus* sp.	AY693974	AY693975
P. stromaticum	CBS 117449	*Eucalyptus* sp.	DQ436935	DQ436936
P. stromaticum	**CDA1208**	**atemoya**	**-**	**-**
P. stromaticum	**CDA1209**	***Annona muricata***	**-**	**-**
P. stromaticum	**CDA1207**	***Annona muricata***	**-**	**-**
P. stromaticum	**CDA1167**	***Annona muricata***	**-**	**-**
P. stromaticum	**CDA1365**	***Annona crassiflora***	**-**	**-**
P. violaceum	CBS 124936	*Pterocarpus angolensis*	FJ888474	FJ888442
P. violaceum	CBS 124937	*Pterocarpus angolensis*	FJ888458	FJ888440
Phyllosticta philoprina	*CBS 616.72*	*Ilex aquifolium*	KF154279	KF289205

The specimens obtained in this study are highlighted in bold. *Ex-type cultures obtained in this study.

Fig. 1 Symptoms of dieback in *Annona muricata* **(A)** and *Annona squamosa* **(B)** in the field. Necrotic symptoms on the vascular system of *Annona muricata* **(C)**. Trunk rot and cracks in the bark of *Annona muricata* **(D)**. Postharvest fruit rot of *Annona crassiflora*

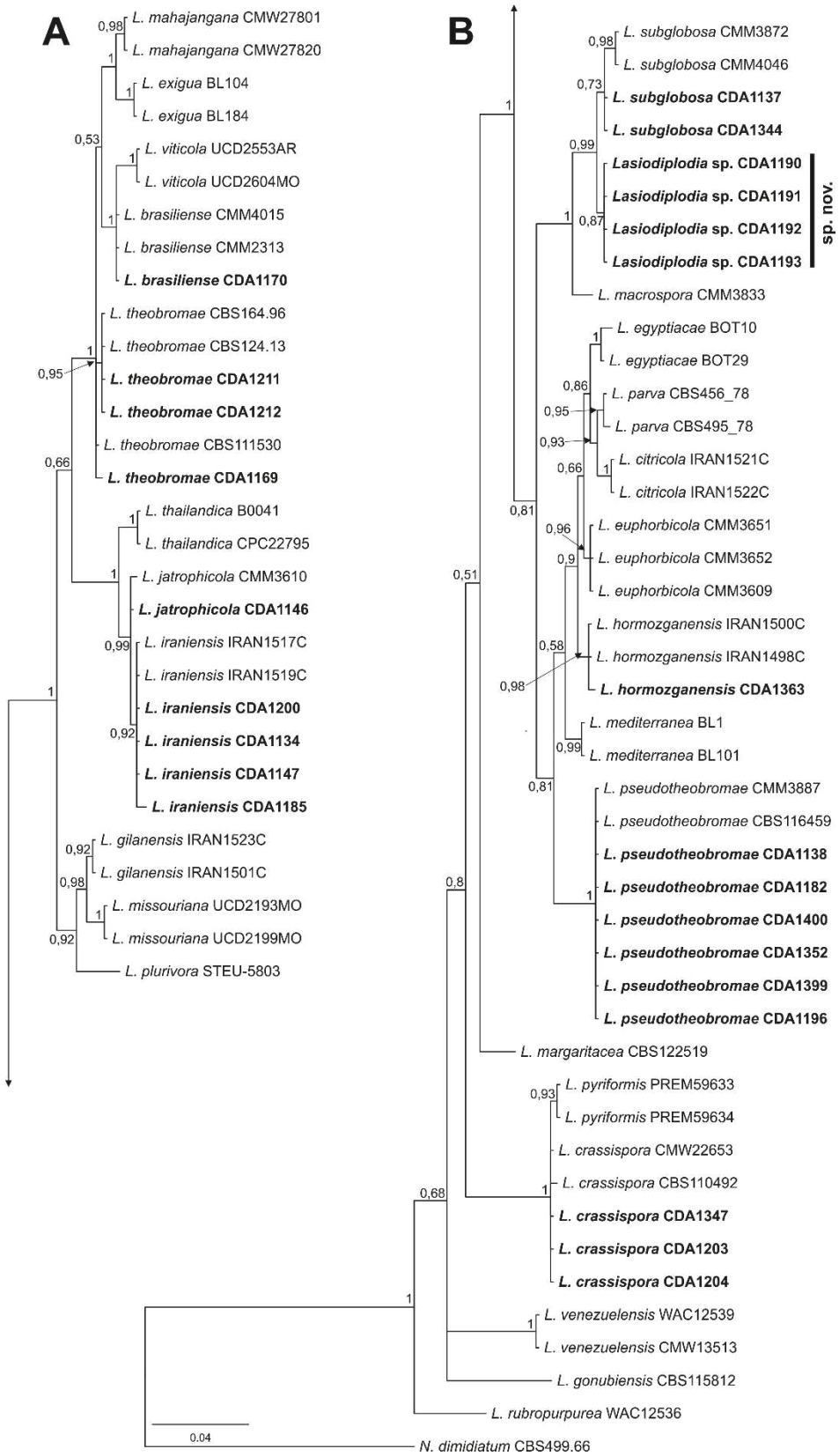

Fig. 2 Multilocus phylogenetic tree of *Lasiodiplodia* species inferred from Bayesian analysis based on the combined sequences of the ITS and TEF-1α **(A–B)**. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Neoscytalidium hyalinum* CBS499.66. The bar represents the number of changes in the nucleotide sequence of each 100 bp. The species in this study are highlighted in bold

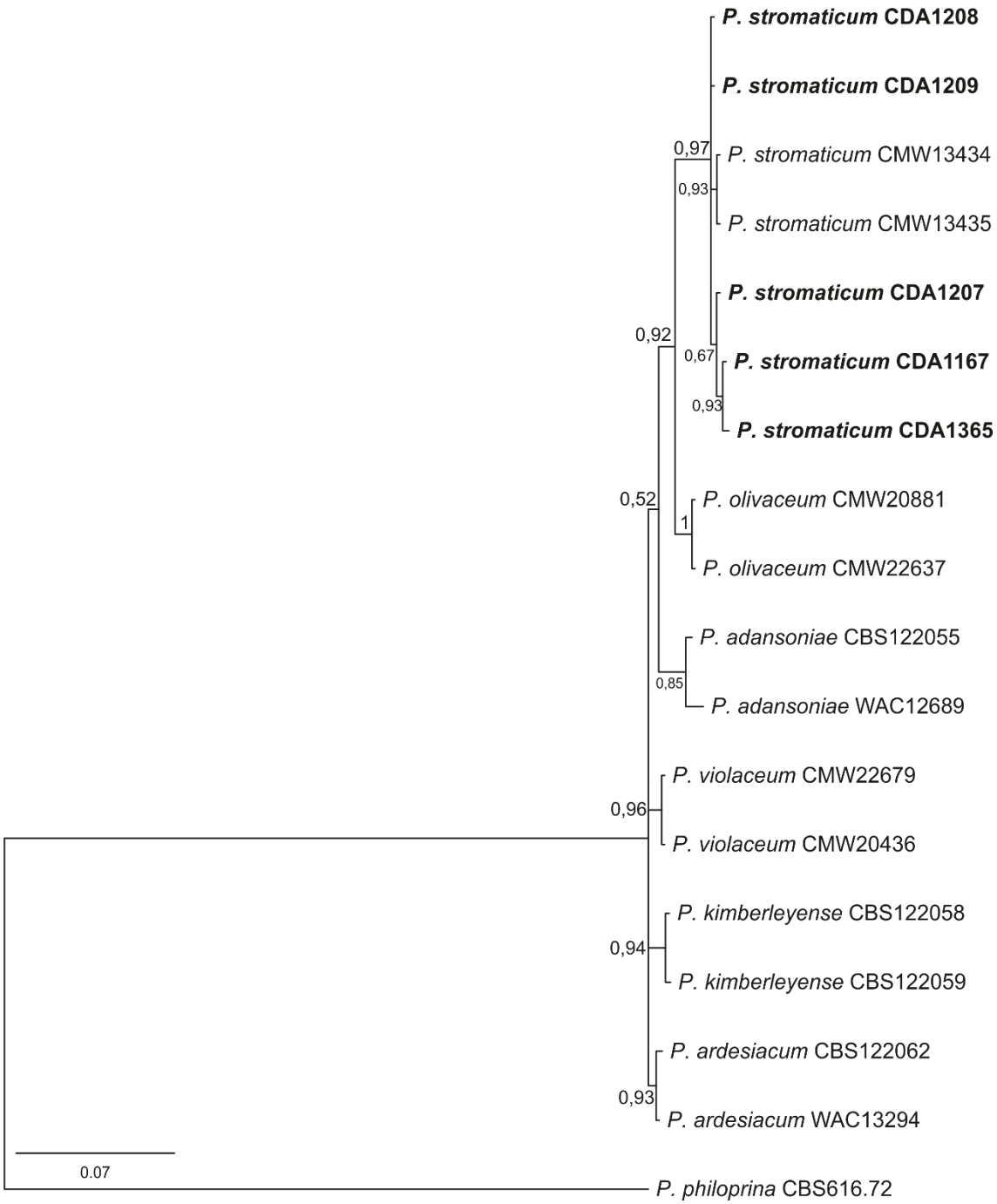

Fig. 3 Multilocus phylogenetic tree of *Pseudofusicoccum* species inferred from Bayesian analysis based on the combined sequences of the ITS and TEF-1α. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta philoprina* CBS616.72. The bar represents the number of changes in the nucleotide sequence of each 100 bp. The species in this study are highlighted in bold

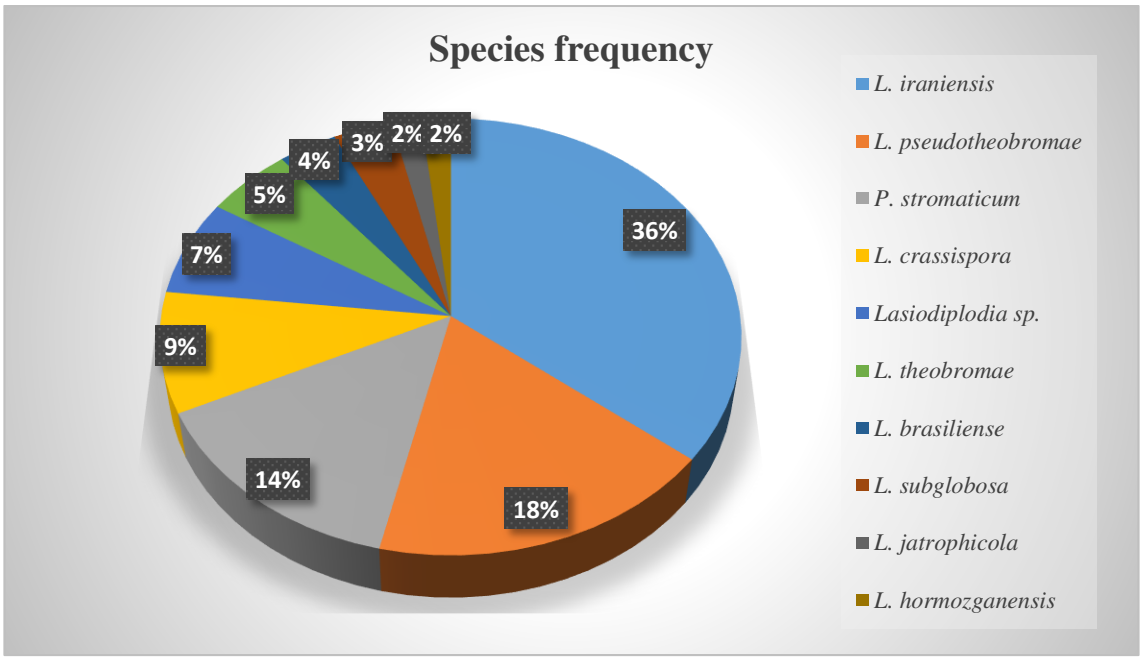

Fig. 4 Frequencies (%) of Botryosphaeriaceae species isolated associated with dieback on Annonaceae in Brazil

Fig. 5 Pathogenicity test results on *Annona muricata* **(A–G)**, *Annona squamosa* **(H–L)** and atemoya **(M–P)**. Symptoms caused by *Lasiodiplodia brasiliense* **(A)**, *L. crassispora* **(B)**, *L. iraniensis* **(C)**, *L. jatrophicola* **(D)**, *L. pseudotheobromae* **(E)**, *L. subglobosa* **(F)** and control plants of *Annona muricata* **(G)**; Symptoms caused by *Lasiodiplodia* sp. **(H),** *L. crassispora* **(I)**, *L. hormozganensis* **(J)**, *L. iraniensis* **(K)**, control plants of *Annona squamosa* **(L)**; Symptoms caused by *L. iraniensis* **(M)**, *L. pseudotheobromae* **(N)**, control plants of atemoya **(O–P)**

ARTIGO 4

According to the guidelines of Molecular Phylogenetics and Evolution

A six-gene phylogeny reveals potential markers for DNA barcode of Botryosphaeriales

A six-gene phylogeny reveals potential markers for DNA barcode of Botryosphaeriales

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ABSTRACT

The gene regions commonly used in phylogenetic studies of Botryosphaeriales are LSU, SSU, RPBII, β-tubulin, ITS and TEF-1α, however it is known that most of these genes are not appropriate for phylogenetic studies. Thus, the aim of this work was to compare the performance of single-copy gene regions RPBII, MS204 and MCM7 with the gene regions ITS, TEF-1α and β-tubulin for phylogenetic studies of Botryosphaeriales, its potential in taxonomic studies and as a possible marker for the species recognition. In this study, we evaluated the phylogenetic performance of six gene regions for the discrimination of 16 species of Botryosphaeriales, focusing in *Lasiodiplodia* species. The gene regions ITS and BT widely used in phylogenetic studies of Botryosphaeriales obtained the worst performance in the species differentiation. Thus, despite the ITS to be historically used in phylogenetic studies of fungi, with the development of more appropriate gene regions for studies with Botryosphaeriales, we highly recommend the abandonment of this gene region. The gene region TEF1-α was better than other gene regions, confirming its potential in Botryosphaeriales species discrimination. However its use should be done carefully, because highly variable introns makes the alignments a difficult task. Among the single copy genes evaluated, RPBII was better, followed by MS204 and MCM7. New pair of primers were developed for MCM7 and provided good results, once amplified all isolates used in the trials and generated sequences with a good quality. Thus, the new pair of primers developed will be useful in future studies for a wide range of Botryosphaeriales species, providing a good amplification and sequencing. The combination of TEF1-α, RPBII and MS204 resolved most of species with high posterior probabilities values, once performed better than reference tree with the six gene regions combined. Possibly due to small species number used in this study, all gene regions and

combinations were unable to discriminate *L. theobromae* and *L. brasiliense*. Thus, a condition for that we can recommend these gene regions for future studies, would be the development of reference datasets with RPBII and MS204 sequences for all species of Botryosphaeriales, that will be a great challenge.

Keywords: Botryosphaeriaceae, *Lasiodiplodia*, orthologous genes, species complex.

1. Introduction

Botryosphaeriales (Dothideomycetes, Ascomycota) are known by its phytopathological importance and can cause several diseases in a wide host range, such as fruit rots, leaf spots, damping-off, canker, gummosis, dieback, colar and root rot, frequently resulting in great losses in several crops of economic importance (Slippers and Wingfield, 2007; Slippers et al., 2009; 2013; Alves et al., 2013; Wikee et al., 2013; Jami et al., 2014; Machado et al., 2014). This group of fungi can also include saprophytic and endophytic species, in which the last represents a challenge for quarantine programs, since Botryosphaeriales eventually survive in a latent phase as endophytic and can rapidly incite diseases when their hosts are subjected to some kind of abiotic stress. This fact allows its spread to other regions of the world in apparently healthy plants (Slippers and Wingfield, 2007).

Historically, the taxonomy of Botryosphaeriales has been a source of controversy between different research groups by many factors, especially due to the morphological characters typically used for species identification (size and shape of conidia or ascospores, septation and pigmentation, etc.) which are considered uninformative and ecological and geographic data which are difficult to interpret due the wide host range and geographic distribution (Slippers et al., 2013; 2014; Jami et al., 2014). Thus, the use of these characters alone in the species description (widely used in the past) can lead to several errors and to underestimate the true species diversity. However, in recent years, advances in molecular techniques and the use of DNA sequences in phylogenetic studies of fungi have contributed substantially in solving taxonomic difficulties of the Botryosphaeriales (Crous et al., 2006; Phillips et al., 2008, 2013; Slippers et al., 2009, 2013; Liu et al., 2012; Jami et al., 2014; Slippers et al., 2014; Trakunyingcharoen et al., 2015).

Recently several works have combined morphological and phylogenetic analyses for delimitation of cryptic species in Botryosphaeriales, such as *Botryosphaeria* spp., *Diplodia* spp., *Lasiodiplodia* spp., *Macrophomina* spp., *Neofusicoccum* spp., *Pseudofusicoccum* spp. and *Phyllosticta* spp. (Alves et al., 2008; Liu et al., 2012; Phillips et al., 2008; 2013; Wikee et al., 2013; Alves et al., 2014; Machado et al., 2014; Sarr et al., 2014; Trakunyingcharoen et al., 2015). Moreover, using this approach, Botryosphaeriales was recognized as distinct order in Dothideomycetes and currently include six families, Aplosporellaceae, Botryosphaeriaceae, Melanopsaceae, Planistromellaceae, Phyllostictaceae and Saccharataceae (Slippers et al., 2013).

The first and more important step for phylogenetic studies of fungi is the definition of which gene regions will be utilized in these analyses. The choose of the gene regions for phylogenetic studies has been based simply in the sequences availability in the Genbank or in gene regions traditionally used by other research groups, without concern whether these sequences represent the evolutionary history of species. Thus, the use of inappropriate gene regions can lead to an underestimation or overestimation of biodiversity or even establish an inappropriate phylogenetic position of species (Aguileta et al., 2008; Feau et al., 2011).

The gene regions commonly used in phylogenetic studies of Botryosphaeriales are Large Ribosomal Subunit (LSU), Small Ribosomal Subunit RNA (SSU), RNA Polymerase Subunit II (RPBII), β-tubulin (βT), Internal Transcribed Spacer of ribossomal DNA (ITS) and Translation Elongation Factor 1α (TEF-1α), since the last three are the most used and recommended (Hyde et al., 2014). However several works showed that most of these genes are not appropriate for phylogenetic studies (Aguileta et al., 2008; Schmitt et al., 2009; Schoch et al., 2012; Walker et al., 2012).

The ITS region has been the most used in phylogenetic studies of fungi and recently it was selected as the most appropriate marker for the proposal of "DNA barcode" (Schoch et al., 2012). However some studies have shown the occurrence different copies of this region in fungal genome that may constitute a serious problem for phylogenetic analyses and for the species recognition as proposed by the "DNA barcode" of fungi (O'Donnell and Cigelnik, 1997; Ko and Jung, 2002; Lindner and Banik, 2011; Schoch et al., 2012; Harrington et al., 2014). In addition to ITS, multiple copies and paralogous copies has been shown in protein-coding genes such as β-tubulin (Ayliffe et al., 2001; Landvik et al., 2001; Corradi et al., 2004), TEF-1α (James et al., 2006) and evidence of

the pseudogenes existence of TEF-1α in a single isolate of *Cladosporium sphaerospermum* (Dugan et al., 2008). The existence of multiple copies, paralogous copies or pseudogenes are factors that can affect the amplification of the target DNA, the quality of the sequencing, the phylogenetic analyses, the discrimination of species, etc.

From this premise, several studies have been tested new gene regions that could better represent the evolutionary history of fungal species. Aguileta et al. (2008) evaluated the phylogenetic performance of single-copy protein-coding genes that resulted in two promising genes, MCM7 (a DNA replication licensing factor required for DNA replication initiation and cell proliferation) and Tsr1 (required for rRNA accumulation during biogenesis of the ribosome) , once it were obtained topologies, resolution power and similar support to the reference tree constructed with 246 concatenated orthologous genes and proved the potential of these genes for phylogenetic studies of fungi. Later, Schmitt et al. (2009) developed degenerate primers to amplify these regions for a wide range of Pezizomycotina. Other studies confirmed the phylogenetic utility of MCM7 gene for various taxonomic levels (class and below) in Ascomycota and it was more phylogenetically informative than LSU (Raja et al., 2011).

Walker et al. (2012) identified two single-copy genes, FG1093 (60S ribosomal protein L37) and MS204 (guanine nucleotide-binding protein subunit beta-like protein), developed primers useful for a wide range of Ascomycota species and compared the phylogenetic performance of these genes with genes commonly used in multilocus analyses (β-tubulin, ITS and TEF-1α) for species delimitation. The single-copy genes identified in this study were equal to or better than β-tubulin, ITS, and TEF-1α in resolving species and showed be potential for studies involving other fungi groups. Similarly Fourie et al. (2015) compared the phylogenetic performance of single-copy genes CAL (calmodulin), RPBII, MS204, FG1093 and MCM7 for species delimitation of *Ceratocystis* with genes commonly used for this group (EF 1-α and βT1). Of the seven gene regions evaluated in this study, analysis of βT1, RPBII and MS204 alone as well as concatenated provided best resolution and distinguished the greatest number of species.

Although many studies cited above showed the effectiveness of single-copy genes in phylogenetic studies in several fungi groups, until now, no work was done with Botryosphaeriales. In addition, although many advances in taxonomy and systematic of this group have been reached in recent years, studies that provided the positioning of the order, families, genera or even proposed phylogenetic species have been based on a few

genetic regions which are not suitable. In addition, the ITS region selected as the "DNA barcode" of fungi does not allow discrimination of most species in Botryosphaeriales (Schoch et al., 2012; Machado et al., 2014; Hyde et al., 2014).

Thus, based on the worldwide importance of Botryosphaeriales, on the increase of the taxonomic and phylogenetic studies of this group in the last years and of the need to adapt the methodologies used in these studies, the the aim of this work was to compare the performance of single-copy gene regions RPBII, MS204 and MCM7 with the gene regions ITS, TEF-1α and β-tubulin for phylogenetic studies of Botryosphaeriales, its potential in taxonomic studies and as a possible marker for the species recognition.

2. Materials and Methods

2.1 Isolates

The isolates used in this study were obtained and characterized in previous works by Silva et al. (2013), Machado et al. (2014), Lopes et al. (2014), Rosado et al. (2015), Adamčík et al. (2015) and Machado et al. (in prep.). A total of 30 isolates comprising 16 Botryosphaeriales species were used in the analyses. Whenever possible, sequences of ex-type were used.

2.2 DNA extraction, sequencing and phylogenetic studies

DNA extraction and sequencing was performed as described by Pinho et al. (2012) and Machado et al. (2014). PCR reactions included the following ingredients for each 25 µL reaction: 12.5 µL of Dream Taq TM PCR Master Mix 2X (MBI Fermentas, Vilnius, Lithuania), 1 µL of 10 µM of each forward and reverse primer synthesized by Invitrogen (Carlsbad, U.S.A), 1 µL of dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO, U.S.A.), 5 µL of 100× (10 mg/mL) bovine serum albumin (BSA, Sigma–Aldrich, St. Louis, MO, U.S.A.), 2 µL of genomic DNA (25 ng/µl) and 2.5 µL of nuclease-free water.

The genes regions and primers utilized in the analyses were described in Table 1. The thermal cycle consisted of 95 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min (denaturation), annealing temperatures are described in Table 1, 72 ºC for 2 min (elongation), and 72 ºC for 10 min (final extension). PCR products were analyzed with 2% agarose electrophoresis gels stained with GelRed™ (Biotium Inc., Hayward, CA, U.S.A.) in a 1× TAE buffer and visualized under UV light to check for amplification size and purity. PCR products were purified and sequenced by Macrogen Inc., South Korea (http://www.macrogen.com). The nucleotide sequences were edited with BioEdit

software (Hall, 2015). All sequences were checked manually and nucleotides with ambiguous positions were clarified using both primer direction sequences. New sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (Table 2).

Consensus sequences were aligned using the multiple sequence alignment program MUSCLE® (Edgar, 2004), built in MEGA v. 6 software (Tamura et al., 2013). The resulting alignments were deposited into TreeBASE (http://www.treebase.org/).

Bayesian inference concatenated (BI) analyses employing a Markov Chain Monte Carlo method were performed with all sequences, first with each gene/locus separately and then with the concatenated sequences (Table 3). Before launching the BI, the best nucleotide substitution model was determined for each gene with MrMODELTEST 2.3 (Posada and Buckley, 2004). After the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). The evolution models selected for each gene region are shown in Table 3. The phylogenetic analyses were performed with the CIPRES web portal (Miller et al., 2010) using MrBayes v.3.2.3 (Ronquist and Huelsenbeck, 2003). For concatenated analyses in the MrBayes, the data were partitioned by locus, and the parameters of the nucleotide substitution models for each partition were set as described above. Four MCMC chains were run simultaneously, starting from random trees, for 10,000,000 generations. Trees were sampled every 1000th generation for a total of 10,000 trees. The first 2500 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala and Yang, 1996) were determined from a majority-rule consensus tree generated with the remaining 7500 trees. Trees were visualized in FigTree v1.3.1 (Rambaut, 2009) and exported to graphics programs.

2.3 Primer design

In order to optimize the amplification of MCM7 gene region for all species used in this study, it was necessary to design suitable primers for Botryosphaeriales. First, some few sequences previously generated using the original MCM7 primers (Schmitt et al., 2009) and MCM7 sequences retrieved from the genome of *Macrophomina phaseolina* MS6 (GenBank: AHHD00000000) and *Neofusicoccum parvum* UCRNP2 (GenBank: AORE00000000) were aligned using program MUSCLE® (Edgar, 2004), built in MEGA v. 6 software (Tamura et al., 2013) from which the new primers “MCM7 BotryPF” / “MCM7BotryPR” (not degenerated) and “MCM7 BotryPFDeg” / “MCM7 BotryPRDeg” (degenerated) were manually designed. Additionally, the primers MCM7R / MCM7F (not

degenerated) and MCM7DR / MCM7DF (degenerated) were designed using the online tool Primer3web version 4.0.0 (Untergrasser et al., 2012). Thus, isolates of Botryosphaeriales unsuccessfully amplified using original primers were tested using the four new primers designed. The primer sequences and annealing temperatures are available in the Table 1.

2.4 Evaluation of phylogenetic informativeness of six gene regions

First, phylogenetic analyses were carried out for all gene regions ITS (Fig. S1), TEF-1α (Fig. S2), BT (Fig. S3), RPBII (Fig. S4), MCM7 (Fig. S5) and MS204 (Fig. S6) separately. After, phylogenetic analyses for genes combination ITS/TEF-1α (Fig. S7), TEF-1α/ RPBII (Fig. S8), TEF-1α/MCM7 (Fig. S9), TEF-1α/MS204 (Fig. S10), ITS/TEF-1α/BT (Fig. S11) and TEF-1α/ RPBII /MS204 (Fig. S12) were carried out and compared with reference tree of all gene regions combined ITS/TEF-1α/βt/RPBII /MCM7/MS204 (Fig. 1). Posterior probabilities of nodes in trees were evaluated for each species and showed in the heat map (Table 3) for a visual analysis, similarly as shown by Woudenberg et al. (2013).

Additionally, the phylogenetic performance of each individual gene regions were assessed through of the online program PhyDesign (http://phydesign.townsend.yale.edu/index.html) in which implements an empirical metric of phylogenetic informativeness (PI) through an automated analysis of the evolution rates in a given period in a reference phylogenetic tree. The PI analysis provide a quantitative prediction of the utility of loci to solve specific phylogenetic questions. This software requires an ultrametric tree file in Newick (recommended) or nexus format and an alignment in Nexus or Phylip format (Townsend, 2007; López-Giráldez and Townsend, 2011). The alignment including all genera analyzed in this work affected negatively the PI results of gene regions, increasing the phylogenetic signal for some locus (for example ITS) contradicting the results of phylogenetic trees. Thus, only the sequences of *Lasiodiplodia* species were included in a partitioned alignment necessary for this analysis. The required tree was obtained by Bayesian inference (Fig. 1) in the same conditions described above and converted to an ultrametric tree using FigTree v1.3.1(Rambaut, 2009) and exported in Newick format. The substitution rates were obtained with program HyPhy (Pond et al., 2005), as recommended by the author.

3. Results and Discussion

In this study, we evaluated the phylogenetic performance of six gene regions for the discrimination of 16 species of Botryosphaeriales, focusing on *Lasiodiplodia* species complex. A total of 30 isolates previously characterized by several works (Silva et al., 2013; Machado et al., 2014; Lopes et al., 2014; Adamčík et al., 2015; Rosado et al., 2015; Machado et al., in prep.) were utilized in the analyses. The phylogenetic signal of each gene regions and combinations are shown in a heat map (Table 3) and the phylogenetic informativeness (PI) of these were assessed by a graphical interface (Fig. 2). The shape of curves in PI profiles can be used to estimate the utility of genes for phylogenetic studies, once flat curves indicate less usefull genes, curves with sharp peaks indicate genes most useful for a narrow timescale and curves steadily increasing or broad curves representing genes useful over longer times scales (Fong and Fugita, 2011).

As shown in heat map (Table 3), despite of the ITS to be the gene region most widely used for phylogeny of fungi, it has the largest number of data in Genbank and it has been chosen as the most appropriate marker for the proposal of "DNA barcode", several evidences have shown that this gene region is not appropriate for phylogenetic studies of Botryosphaeriales. In this study, ITS alone did not provide sufficient support for species differentiation of *Lasiodiplodia* and *Macrophomina*. The net PI of ITS shown in a flat curve (Fig. 2), indicated a low phylogenetic resolving power for *Lasiodiplodia* species discrimination, corroborating with results of phylogenetic trees generated (Fig. S1) and summarized in the heat map (Table 3). Several studies have already demonstrated the inefficiency of ITS in separation of the Botryosphaeriales species, thus, need to be used in combination with protein-coding genes such as TEF1-α, β-tubulin and RPBII (Phillips et al., 2008; Pavlic et al., 2009b; Wikee et al., 2011; Marques et al., 2013; Machado et al., 2014).

In addition to inefficiency of ITS for species discrimination in several groups of fungi, some works have shown the existence of intragenomic variation, in which can obscure the phylogenetic relationships (O'Donnell and Cigelnik, 1997; Ko and Jung, 2002; Lindner and Banik, 2011; Schoch et al., 2012; Harrington et al., 2014). Thus, despite the ITS to be historically used in phylogenetic studies, with the development of more appropriate gene regions, we highly recommend the abandonment of this gene region in studies of Botryosphaeriales, in the same way that occurred with others fungi groups, such as *Fusarium* (Hyde et al., 2014).

The gene region β-tubulin (BT) is a protein-coding gene often recommended for increase of support in phylogenetic analysis of Botryosphaeriales combined with others gene regions (Hyde et al., 2014). However, in the same manner that ITS, phylogenetic analysis of this gene region alone, showed a low phylogenetic resolving power for *Lasiodiplodia* species discrimination (Table 3 and Fig. S3), since it did not obtain support for any *Lasiodiplodia* species. Also showed a low net PI in a flat curve (Fig. 2). In addition, paralogous copies of β-tubulin have been shown in some works (Ayliffe et al., 2001; Landvik et al., 2001; Corradi et al., 2004). Thus, despite the widely use in phylogenetic studies of fungi, this region has shown not be appropriate for works with Botryosphaeriales.

Another widely used gene region for phylogenetic studies of Botryosphaeriales is the TEF1-α. This protein-coding gene has been considered as marker very useful for species discrimination of Botryosphaeriales (Marques et al., 2013; Machado et al., 2014; Hyde et al., 2014). Phylogenetic analysis of this gene region alone, showed a high phylogenetic resolution power for *Lasiodiplodia* species discrimination, since distinguish the greatest number of species (Table 3 and Fig. S2) and curves with sharp peaks in net PI profile (Fig. 2) indicating its utility for recent divergences times. The presence of highly variable introns in the TEF1-α becomes this gene region recommended to resolve relationships between closely related species (Irimia and Roy, 2008). Thus, this gene region can be used for routine identifications as DNA barcode and for resolution of taxonomic questions in Botryosphaeriales, since this gene region presents the largest database for Botryosphaeriales. However, this should be done carefully, because highly variable introns makes the alignments a difficult task, due to large number of gaps generated, in which can result in subjective alignments and in an incorrect phylogenetic inferences. In addition, some works have shown the existence of paralogous copies in several fungi (James et al., 2006; Matheny et al., 2007) and evidences of occurrence of pseudogenes (Dugan et al., 2008) that can causes problems in phylogenetic analyses. The desirable would be to use single copy protein-coding genes for phylogenetic studies, but since it is not yet possible for Botryosphaeriales, we can use TEF1-α in combination with single copy genes, which will be discussed below.

In this work, the major goal was to evaluate the performance of single copy genes MCM7, MS204 and RPBII for phylogenetic studies of Botryosphaeriales in comparison with gene regions traditionally used. Some works have shown the potential of MCM7 and MS204 for phylogenetic studies (Aguileta et al., 2008; Schmitt et al., 2009; Raja et al.,

2011; Walker et al., 2012; Fourie et al., 2015). In this study, we had difficulty in obtain MCM7 sequences for several species using original degenerate primers developed by Schmitt et al. (2009). Thus, in this study, degenerate and non-degenerate primers were designed. From the four primers pairs developed (Table 1), two primers, one degenerated and one non-degenerated (MCM7F/MCM7R; MCM7DR/MCM7DF) provided good results (not shown) and amplified all isolates used in the trials. Thus, the new pair of primers developed can be used in future studies for a wide range of Botryosphaeriales species, providing a good amplification and sequencing.

MCM7 and MS204 alone were better than ITS and β-tubulin, discriminating more *Lasiodiplodia* species. In addition, MS204 was better than MCM7 (Table 3, Fig. S5 and S6), maybe due to largest sequences and presence of introns in the first, indicating its potential use in combination with other gene regions. These genes were easily aligned, which represent an advantage over ITS and TEF1-α, moreover these can be very useful in phylogenetic studies of higher taxonomic levels (genus, family, order and class).

RPBII is a single copy gene very useful in phylogenetic studies of Ascomycota (Liu et al., 1999), Basidiomycota (Matheny et al., 2007) and it has been successfully used for discrimination of *Neofusicoccum* species (Pavlic et al., 2009a; Pavlic et al., 2009b; Begoude et al., 2010; Sakalidis et al., 2011). In this study, phylogenetic analysis of this gene region alone, showed high resolving power for *Lasiodiplodia* and *Macrophomina* species discrimination, high support for genera clades (Table 3 and Fig. S4) and a better net PI than ITS, β-tubulin, MS204 and MCM7 (Fig. 2). In addition, the sequences were easily obtained and aligned. However, it was lower than TEF1-α, since it did not separate the species *L. egyptiacae* from *L. theobromae* and *L. brasiliense*, and showed a broad curve in a PI profile in contrast with sharp peak of TEF1-α (Fig. 2). Thus, RPBII can be used for phylogenetic studies of several taxonomic ranks of Botryosphaeriales, but for closest species differentiation, it is necessary to combine with other gene regions for increase the phylogenetic support.

From six gene regions evaluated in this study, TEF1-α, RPBII and MS204 were identified as the three most informative for species discrimination and potential for use in combination. For two gene regions combinations, TEF/ RPBII (Fig. S8) provided a good resolution (Table 3), once it was better than ITS/TEF1-α (Fig. S7) that is the combination more used in Botryosphaeriales works. The combination of TEF1-α/RPBII/MS204 (Fig. S12) provided the best resolution (Table 3), since it was better than

reference tree (Fig. 1) with six gene regions concatenated, that was not expected. Similarly, Walker et al. (2012) observed that the increasing the markers number in the combined analysis, it was accompanied with the decrease of branches support. This occurred likely due to incongruences between evolutionary histories in the genes or perhaps due to insufficient time for fixation of gene lineages (Walker et al., 2012). According with Aguileta et al. (2008), the ideal would be the choice of few genes for with high phylogenetic informativeness and that converge in a single answer for allow the construction of robust and reliable phylogenies. Furthermore, choosing few gene regions would reduce the amount of data to be sequenced.

The TEF1-α/RPBII/MS204 combination showed posterior probabilities values above 95% for all species, except for *Lasiodiplodia theobromae* and *L. brasiliense* (Table 3), although these species presented small differences in trees, they did not obtain sufficient support for the separation. We believe that the use of a larger number of sequences would be more representative and could increase the support for these species. Thus, a condition for that we can recommend these gene regions for future studies, would be the development of reference datasets with RPBII and MS204 sequences for all species of Botryosphaeriales, that will be a great challenge. This work only represents a start and it will be an incentive for other research groups that work with Botryosphaeriales to adopt this promising way.

Currently, a new trend of search for promising genes for phylogenetic studies and for "DNA barcode" has been observed (Aguileta et al., 2008; Schmitt et al., 2009; Walker et al., 2012; Fourie et al., 2015; Stielow et al., 2015), in which has shown a concern of research groups in become the phylogenetic analysis and fungi identification through the "DNA barcode" more reliable and reproducible, once the fungal taxonomic community still lacks the resources for work with genomes for species identification (Stielow et al., 2015).

In this way, this work will provide bases for futures studies of fungi taxonomy and systematic, it will contribute for a more appropriate discrimination of Botryosphaeriales species.

4. Conclusion

The gene regions ITS and BT obtained the worst performance, once they were unable to separate the *Lasiodiplodia* and *Macrophomina* species. Thus, with the development of more appropriate gene regions for studies with Botryosphaeriales, we

highly recommend the abandonment of these gene regions. The gene region TEF1-α was better than all the gene regions, confirming its potential in Botryosphaeriales species discrimination. However its use should be done carefully, because highly variable introns makes the alignments a difficult task. Among the single copy genes evaluated, RPBII was better, followed by MS204 discriminating many species. The MCM7 showed be a potential region for phylogenetic studies of higher taxonomic levels. The new pair of primers developed for MCM7 provided good results, once amplified all isolates used in the trials and generated sequences with a good quality. Thus, the primers developed will be useful in future studies for a wide range of Botryosphaeriales species, providing a good amplification and sequencing. The combination of TEF1-α, RPBII and MS204 resolved most of species with high posterior probabilities values, once performed better than reference tree with the six gene regions combined. Possibly due to small species number used in this study, all gene regions and combinations were unable to discriminate *L. theobromae* and *L. brasiliense*. Thus, a condition for that we can recommend these gene regions for future studies, would be the development of reference datasets with RPBII and MS204 sequences for all species of Botryosphaeriales.

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Table 1. Gene regions and primers utilized in the analyses.

GENE REGION	PRIMER	ANNEALING T°C	SEQUENCES (5' TO3')	REFERENCES
MCM7	**BotryPF**	**55°C**	**ACCGCTGTGGTTGTGAGATT**	This study
MCM7	**BotryPR**		**ACCCATCTCCTTCGTCACAC**	
MCM7	**BotryPFDeg**		**ACCGCTGYGGYTGYGARATT**	
MCM7	**BotryPRDeg**		**CCCATYTCCTTNGTSACACC**	
MCM7	**MCM7R***		**CCATCAAGCAGACGTTGATGTCGCC**	
MCM7	**MCM7F***		**AATGCCTACTCTTGCGACCGCTGCGG**	
MCM7	**MCM7DR***		**CCATCARRCADAYGTTGATRTCDCC**	
MCM7	**MCM7DF***		**AAYGCVTAYWCKTGYGAYCGYTGYGG**	
MCM7	709f		ACI MGI GTI TCV GAY GTH AAR CC	Schmitt et al. (2009)
MCM7	1348r		GAY TTD GCI ACI CCI GGR TCW CCC AT	
MS204	E1F1	56°C	AAG GGC ACC CTG GAG GGC CAC	Walker et al., (2012)
MS204	E5R1		GAT GGT GAC GGY GTT GAT GTA	
RPBII	5F2	54°C	GGG GWG AYC AGA AGA AGG C	Sung et al. 2007
RPBII	7cR		CCC ATR GCT TGY TTR CCC AT	Liu et al. (1999)
β-tubulin	Bt2a	56°C	GGT AAC CAA ATC GGT GCT GCT TTC	Glass & Donaldson (1995)
β-tubulin	Bt2b		ACC CTC AGT GTA GTG ACC CTT GGC	
ITS	ITS1	52°C	TCC GTA GGT GAA CCT GCG G	White et al. (1990)
ITS	ITS4		TCC TCC GCT TAT TGA TAT GC	
TEF-1α	EF1F	56°C	TGC GGT GGT ATC GAC AAG CGT	Jacobs et al. (2004)
TEF-1α	EF2R		AGC ATG TTG TCG CCG TTG AAG	

The primers generated in this study are highlighted in bold. * indicate the pair of primers that performed better.

Table 2. Genbank accession numbers of DNA sequences of Botryosphaeriales used in phylogenetic analyses. Newly deposited sequences are shown in bold.

Species	[1]Culture accession n°	Genbank acession n°					
		ITS	TEF1-α	BT	RPBII	MCM7	MS204
Phyllosticta capitalensis	CDA117	-	-	-	-	-	-
Phyllosticta yuccae	CMM1843	JX227945	JX227947	-	-	-	-
Phyllosticta yuccae	CMM1844	JX227946	JX227948	-	-	-	-
Neoscytalidium hyalinum	CMM3649	KF234550	KF226707	KF254934	-	-	-
Neoscytalidium hyalinum	CMM3607	KF234542	KF226688	KF254925	-	-	-
Macrophomina phaseolina	CMM3615	KF234547	KF226693	KF254930	-	-	-
Macrophomina phaseolina	CMM3875	KF369263	KF553897	-	-	-	-
Macrophomina phaseolina	CMM3650	KF234552	KF226710	KF254936	-	-	-
Macrophomina pseudophaseolina	CMM4032	KF369272	KF553905	-	-	-	-
Macrophomina pseudophaseolina	CMM3653	KF369262	KF553906	-	-	-	-
Neofusicoccum kwambonambiense	CMM1842	KC507813	KC507810	KC507807	KC507804	-	-
Neofusicoccum parvum	CMM1846	KC507812	KC507809	KC507806	KC507803	-	-
Neofusicoccum parvum	CMM1845	KC507814	KC507811	KC507808	KC507805	-	-
Neodeightonia licuriensis	COAD1780	KP165429	KP165430	KP165431	-	-	-
Lasiodiplodia pseudotheobromae	COAD1785	KP244690	KP308464	KP308523	-	-	-
Lasiodiplodia pseudotheobromae	CMM3887	KF234559	KF226722	KF254943	-	-	-
Lasiodiplodia euphorbicola	CMM3651	KF234553	KF226711	KF254937	-	-	-
Lasiodiplodia euphorbicola	CMM3652	KF234554	KF226715	KF254938	-	-	-
Lasiodiplodia euphorbicola	CMM3609	KF234543	KF226689	KF254926	-	-	-
Lasiodiplodia egyptiacae	CMM3648	KF234549	KF226705	KF254933	-	-	-
Lasiodiplodia egyptiacae	CMM3611	KF234545	KF226691	KF254928	-	-	-
Lasiodiplodia subglobosa	CMM3872	KF234558	KF226721	KF254942	-	-	-
Lasiodiplodia subglobosa	CMM4046	KF234560	KF226723	KF254944	-	-	-
Lasiodiplodia macrospora	CMM3833	KF234557	KF226718	KF254941	-	-	-
Lasiodiplodia jatrophicola	CMM3610	KF234544	KF226690	KF254927	-	-	-
Lasiodiplodia theobromae	CMM3831	KF234556	KF226717	KF254940	-	-	-
Lasiodiplodia theobromae	CMM3612	KF234546	KF226692	KF254929	-	-	-
Lasiodiplodia theobromae	CMM3647	KF234548	KF226704	KF254932	-	-	-
Lasiodiplodia brasiliense	COAD1786	KP244696	KP308471	KP308526	-	-	-
Lasiodiplodia brasiliense	COAD1784	KP244693	KP308469	KP308524	-	-	-

[1]CMM = Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes", Universidade Federal Rural de Pernambuco, Recife, Brazil; COAD = Coleção Octávio Almeida Drummond, Universidade Federal de Viçosa, Viçosa, Brazil.

Table 3.Summary of the phylogenetic results in a heat map showing the posterior probabilities support values for all gene regions and combinations tested.

	SINGLE REGIONS						COMBINED REGIONS						
	ITS	**TEF**	**BT**	**RPBII**	**MCM7**	**MS204**	**TEF MCM7**	**TEF MS204**	**TEF RPBII**	**ITS TEF**	**TEF RPBII MS204**	**ITS TEF BT**	**ITS TEF BT RPBII MCM7 MS204**
Aligned length	504	311	427	921	435	766							
Nucleotide substitution model	GTR+I	HKY+I+G	HKY+G	GTR+I+G	GTR+I+G	GTR+I+G							
Lasiodiplodia theobromae (3)													
Lasiodiplodia brasiliense (2)													
Lasiodiplodia egyptiacae (2)													
Lasiodiplodia jatrophicola (1)													
Lasiodiplodia macrospora (1)													
Lasiodiplodia subglobosa (2)													
Lasiodiplodia pseudotheobromae (2)													
Lasiodiplodia euphorbicola (3)													
Neodeightonia licuriensis (1)													
Macrophomina phaseolina (3)													
Macrophomina pseudophaseolina (2)													
Neoscytalidium hyalinum (2)													
Neofusicoccum parvum (2)													
Neofusicoccum kwambonambiense (1)													
Phyllosticta capitalensis (1)													
Phyllosticta yuccae (2)													

*Numbers in parentheses represent the number of isolates for each species.

100	95 - 99	90-94	80-89	70-79	50-69	25-49	1-24	NO SUPPORT	MISSING DATA

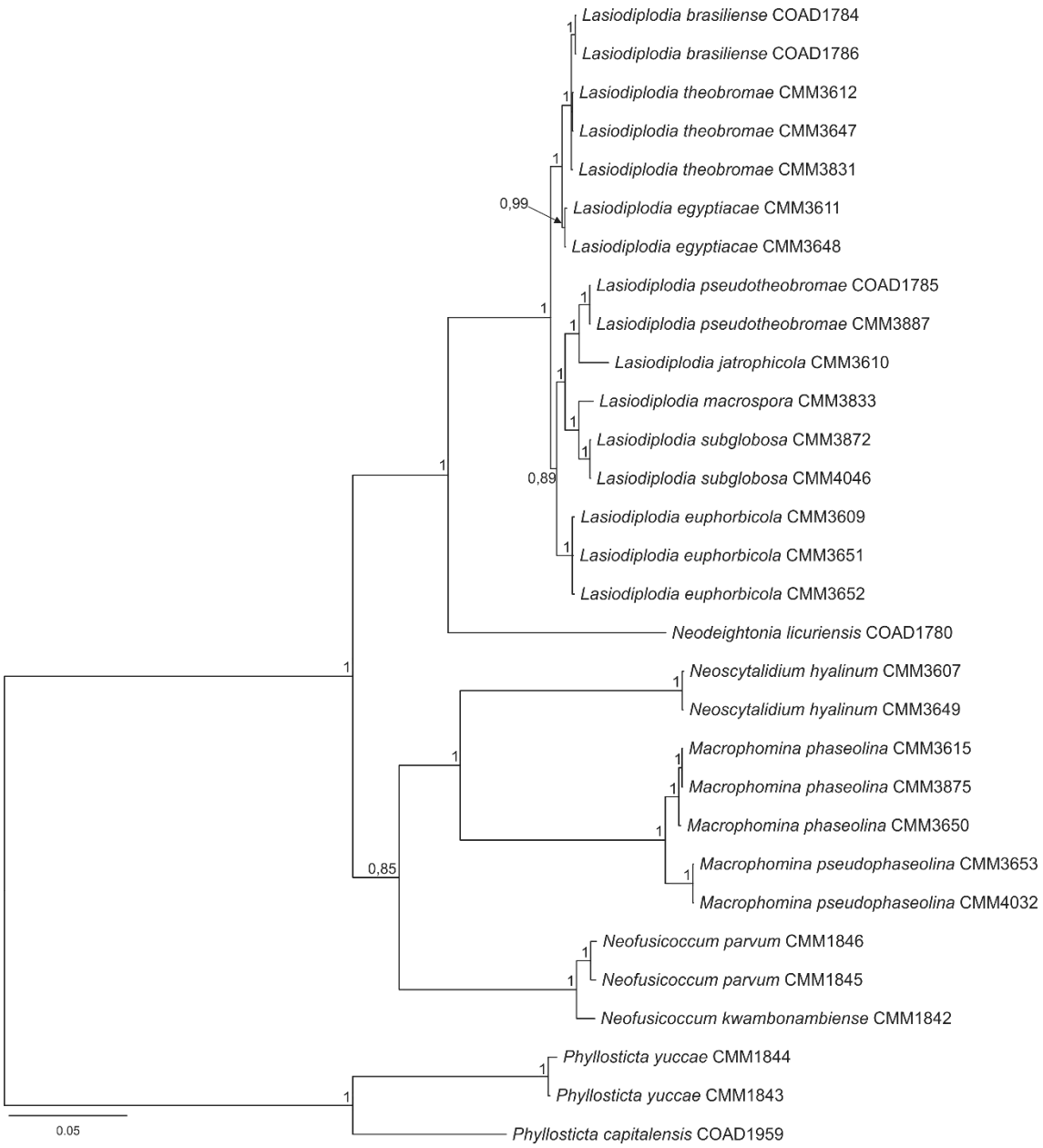

Fig. 1. Multilocus phylogenetic reference tree inferred from Bayesian analysis based on the combined sequences of the ITS, TEF-1α, BT, RPBII, MCM7 and MS204. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

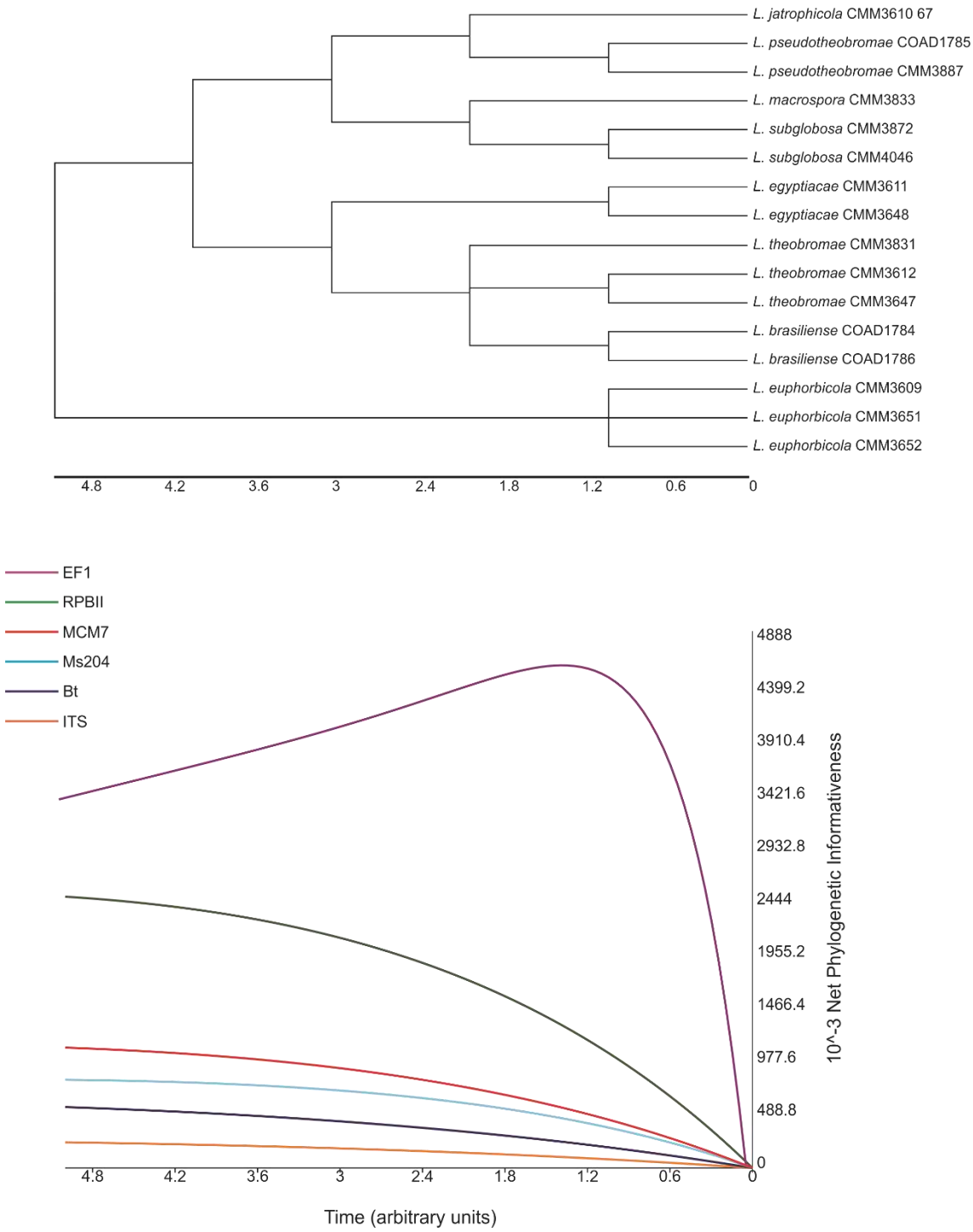

Fig.2 Net phylogenetic informativeness (PI) of the six gene regions in the same time scale of the phylogenetic tree above.

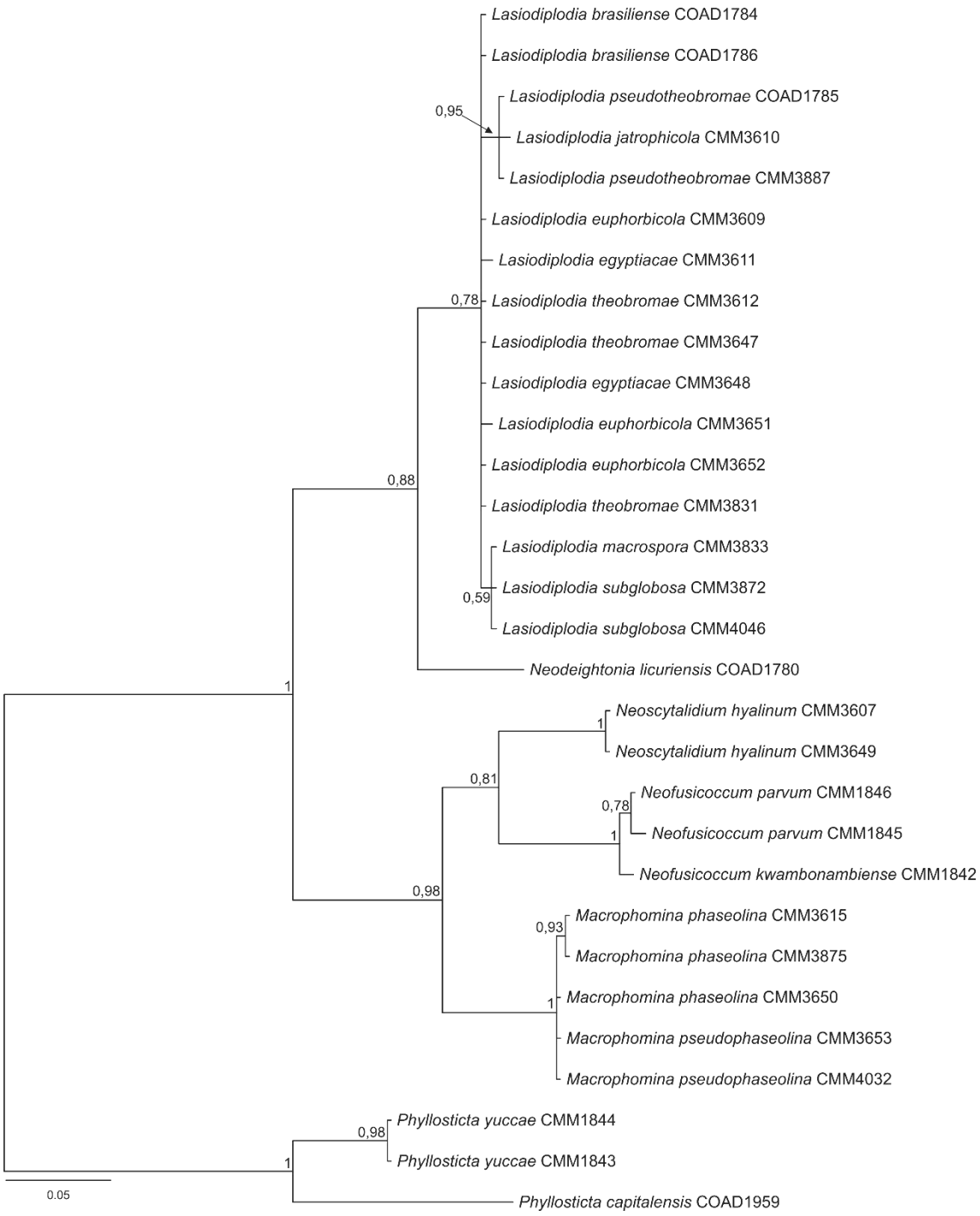

Fig. S1. Phylogenetic tree inferred from Bayesian analysis based on the ITS sequences. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

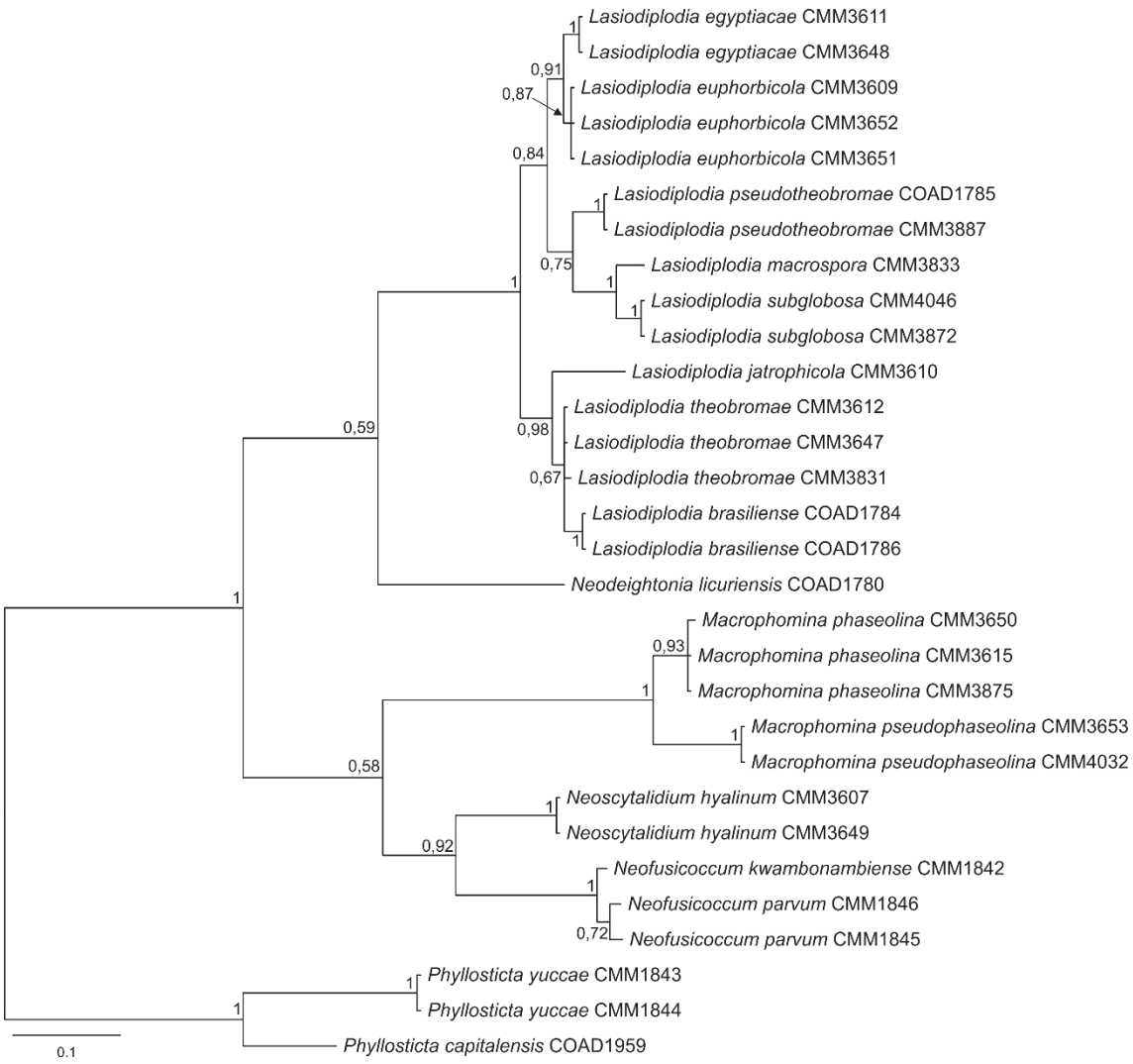

Fig. S2. Phylogenetic tree inferred from Bayesian analysis based on the TEF1-α sequences. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

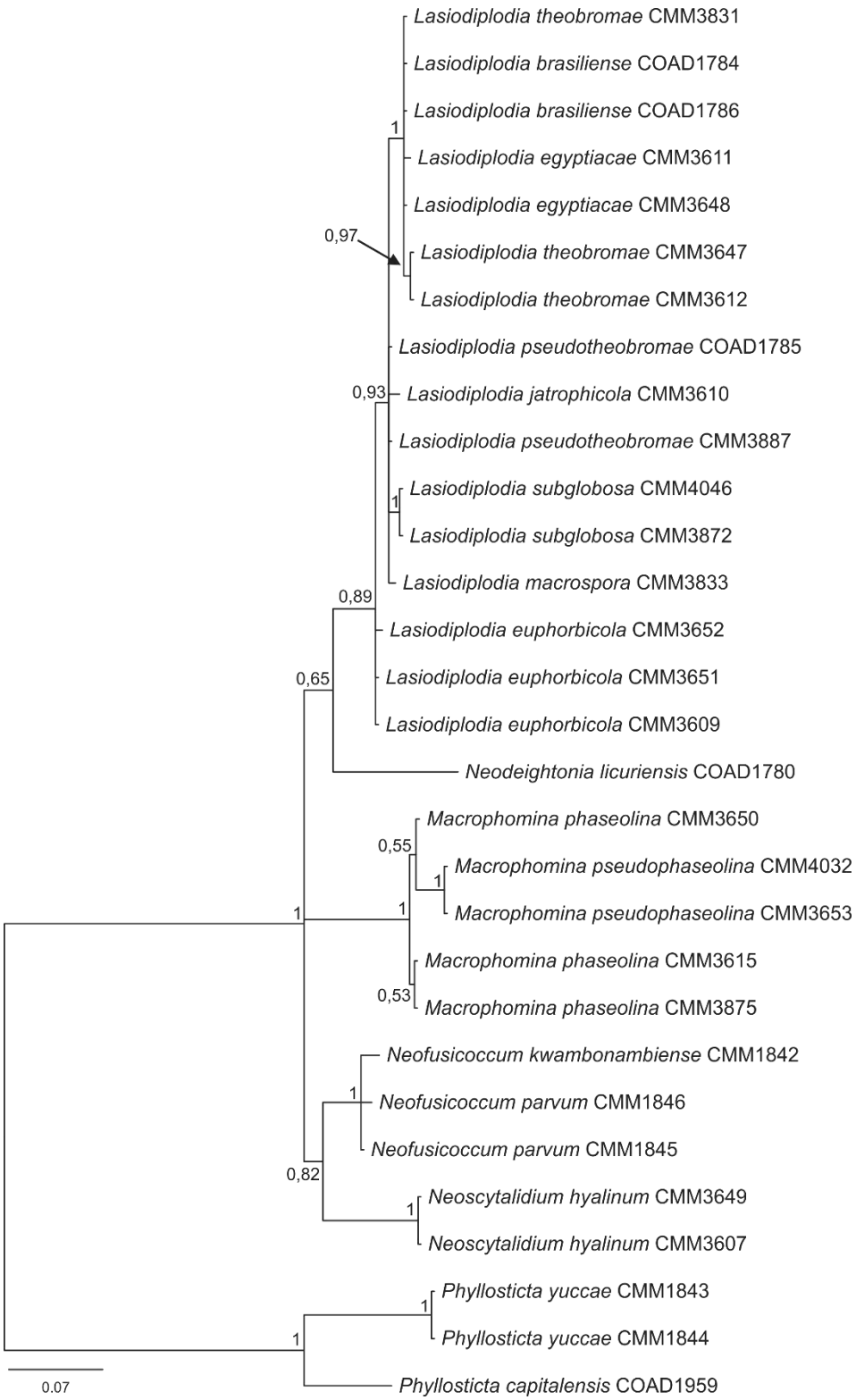

Fig. S3. Phylogenetic tree inferred from Bayesian analysis based on the BT sequences. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

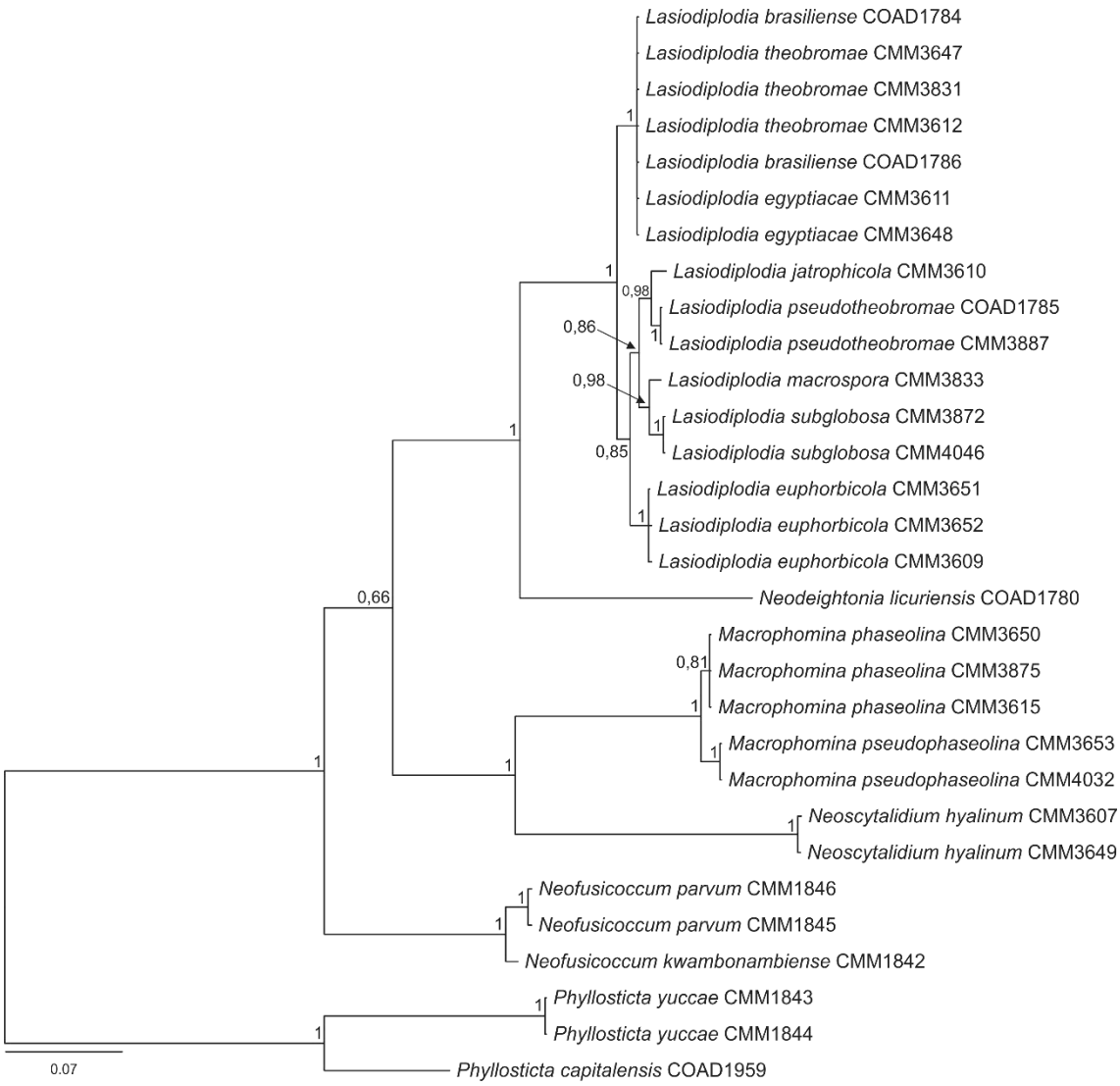

Fig. S4. Phylogenetic tree inferred from Bayesian analysis based on the RPBII sequences. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

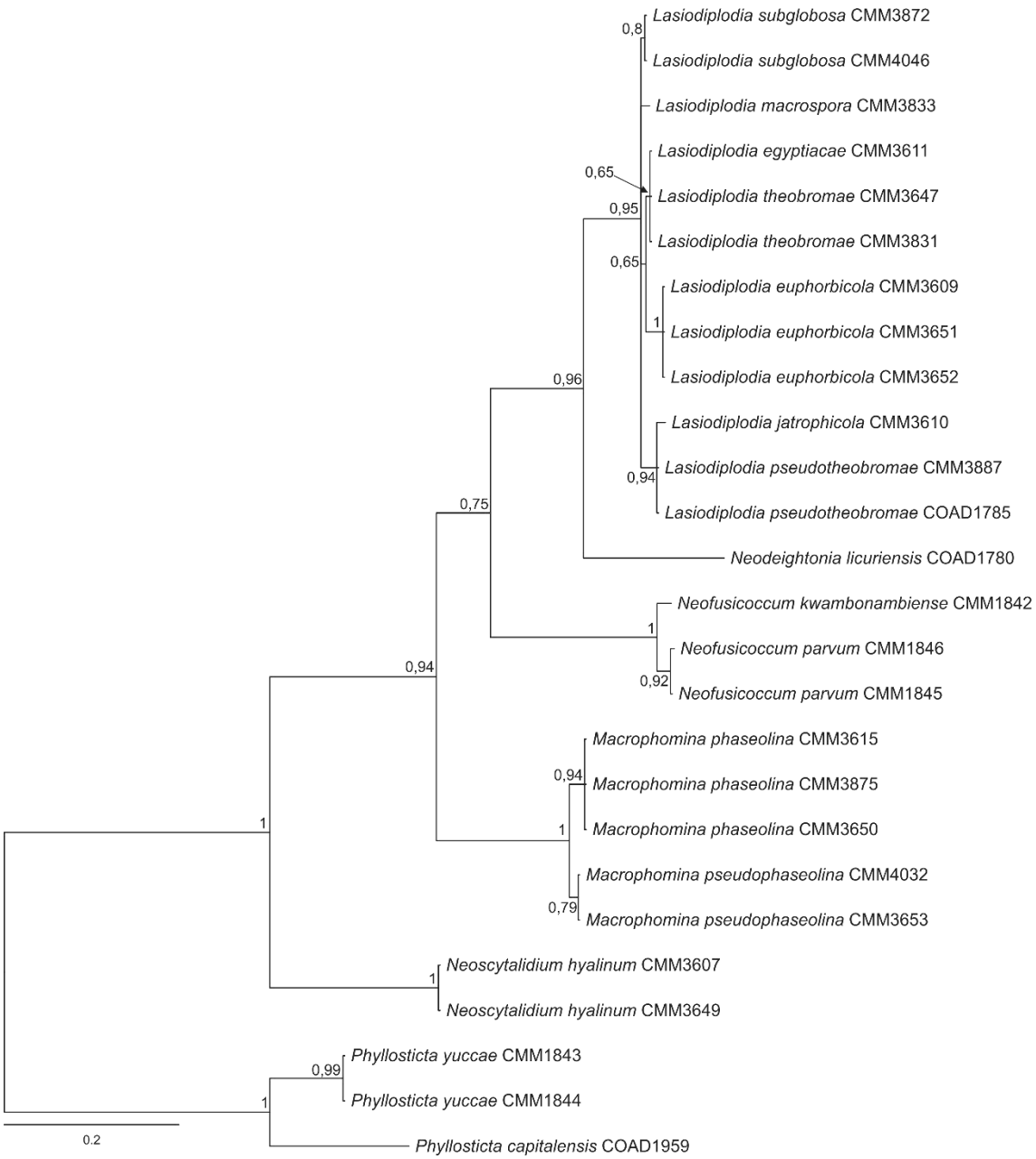

Fig. S5. Phylogenetic tree inferred from Bayesian analysis based on the MCM7 sequences. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

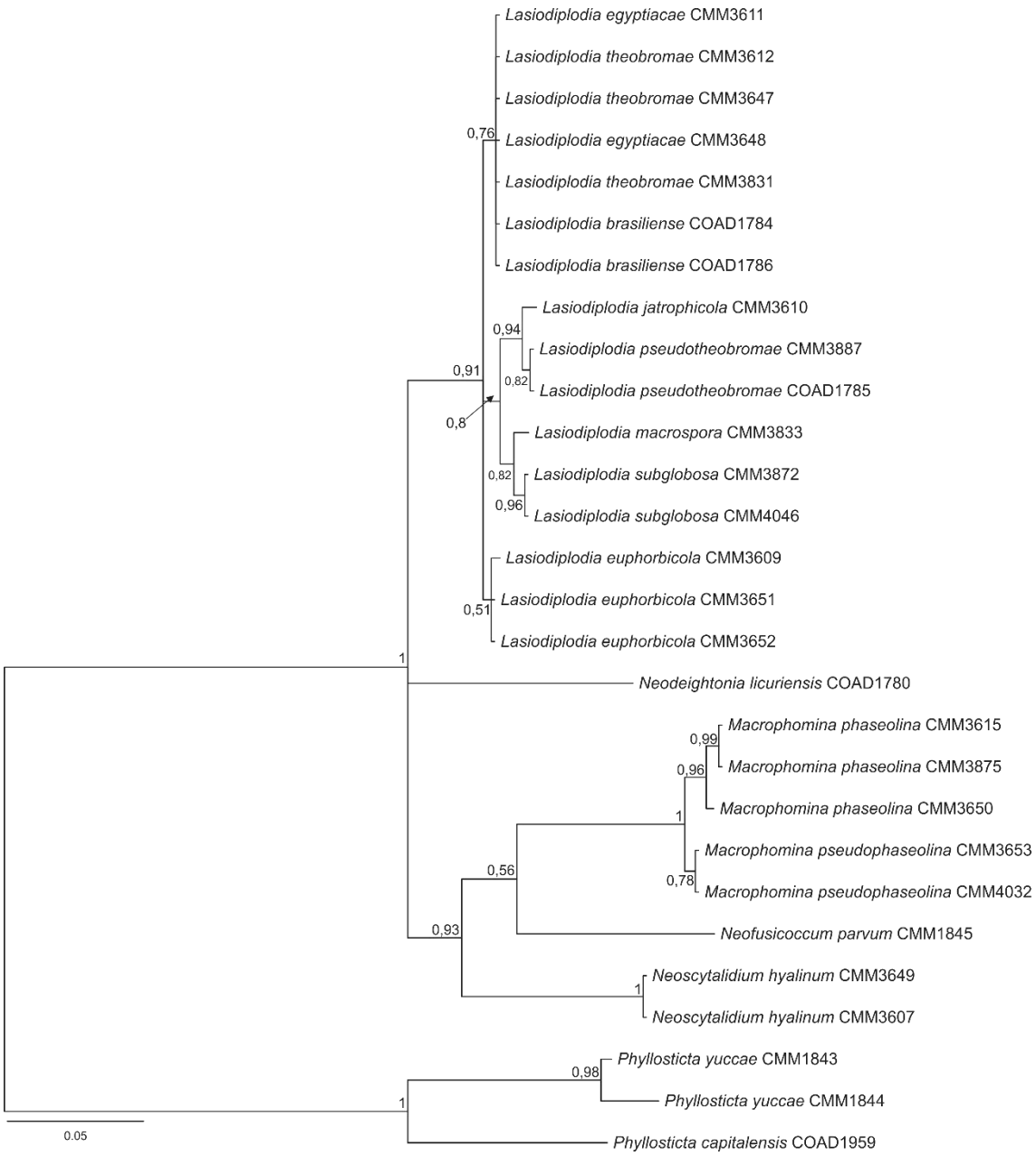

Fig. S6. Phylogenetic tree inferred from Bayesian analysis based on the MS204 sequences. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

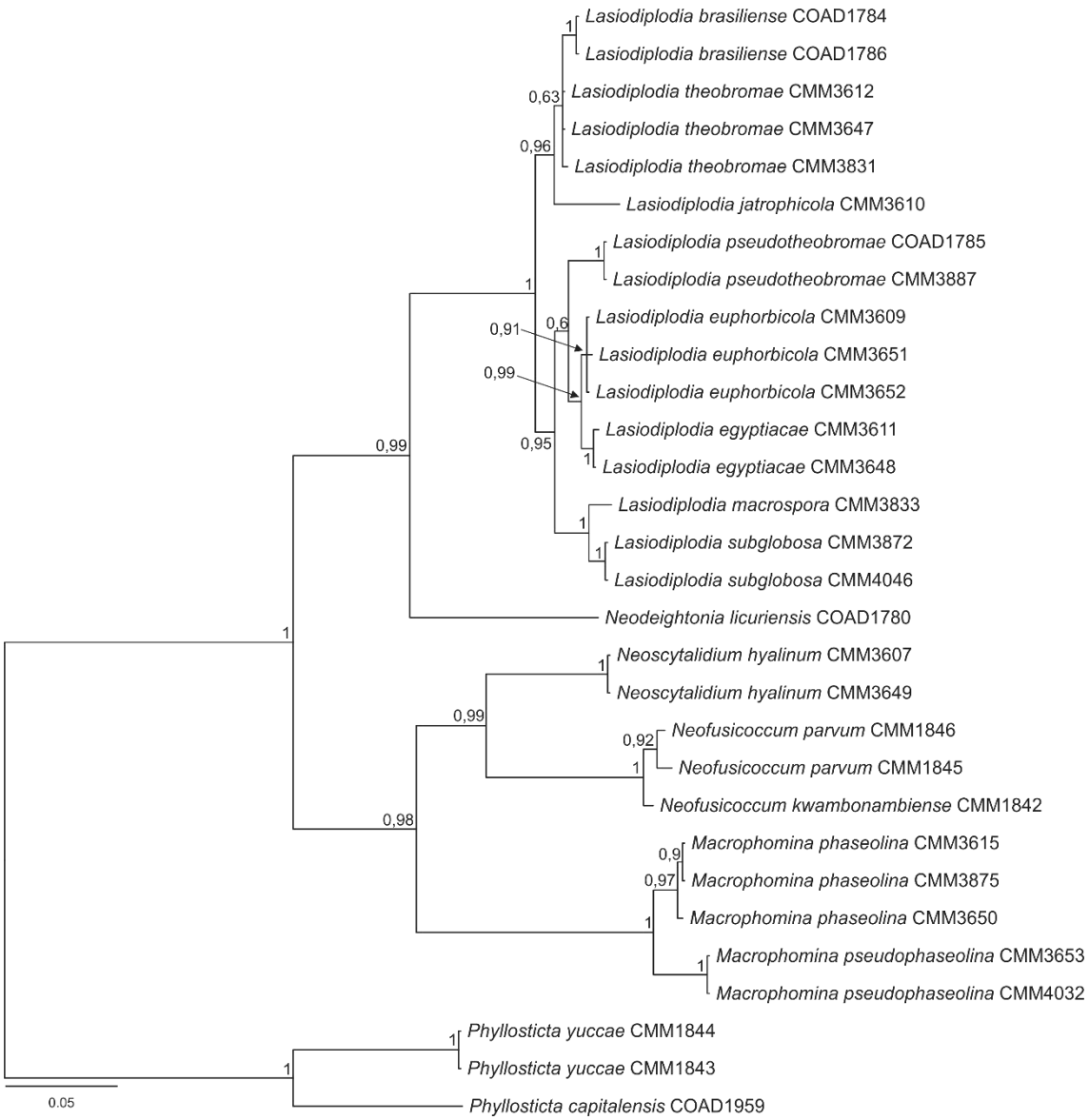

Fig. S7. Phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the ITS and TEF-1α. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

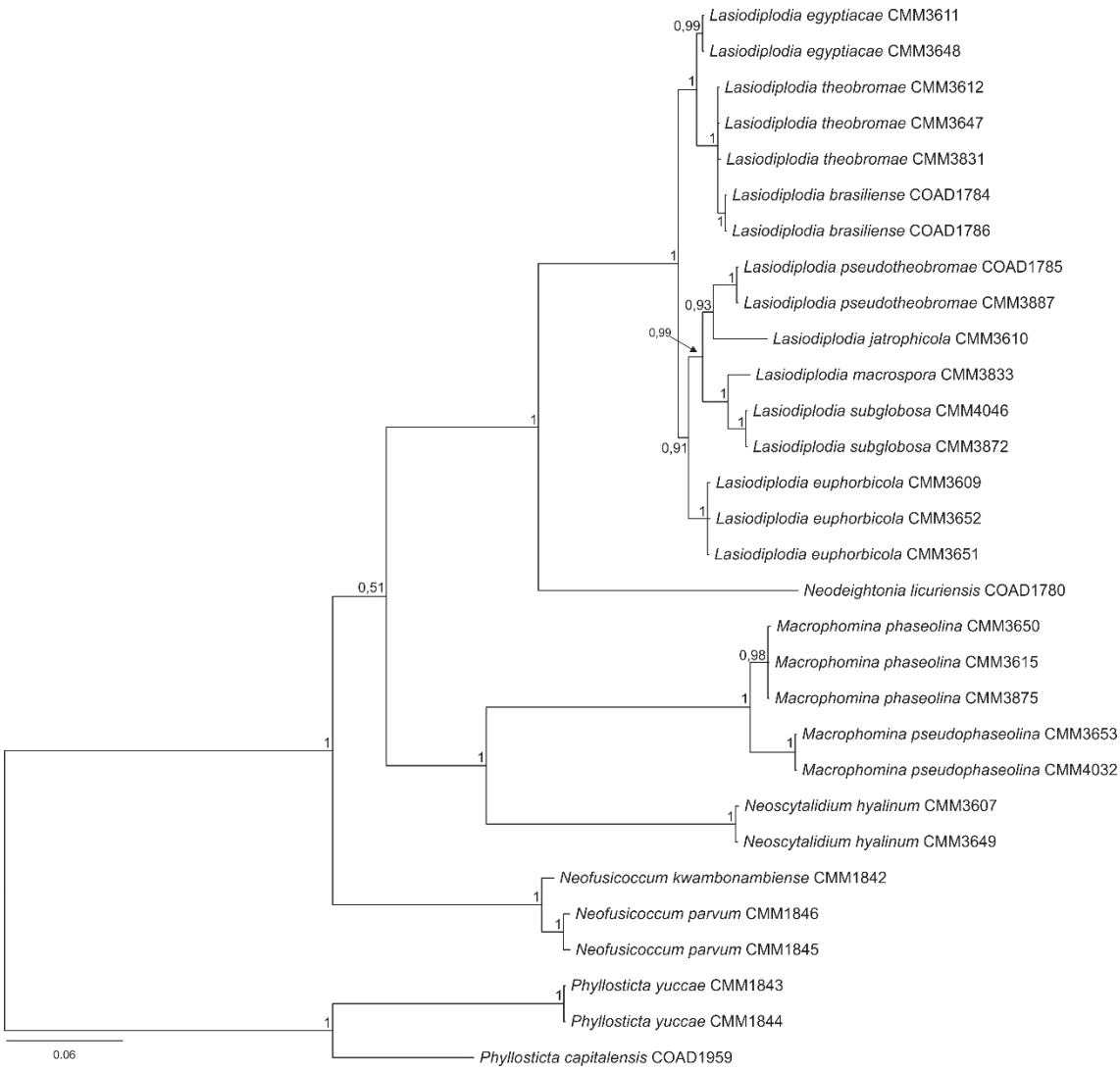

Fig. S8. Phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the TEF-1α and RPBII. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

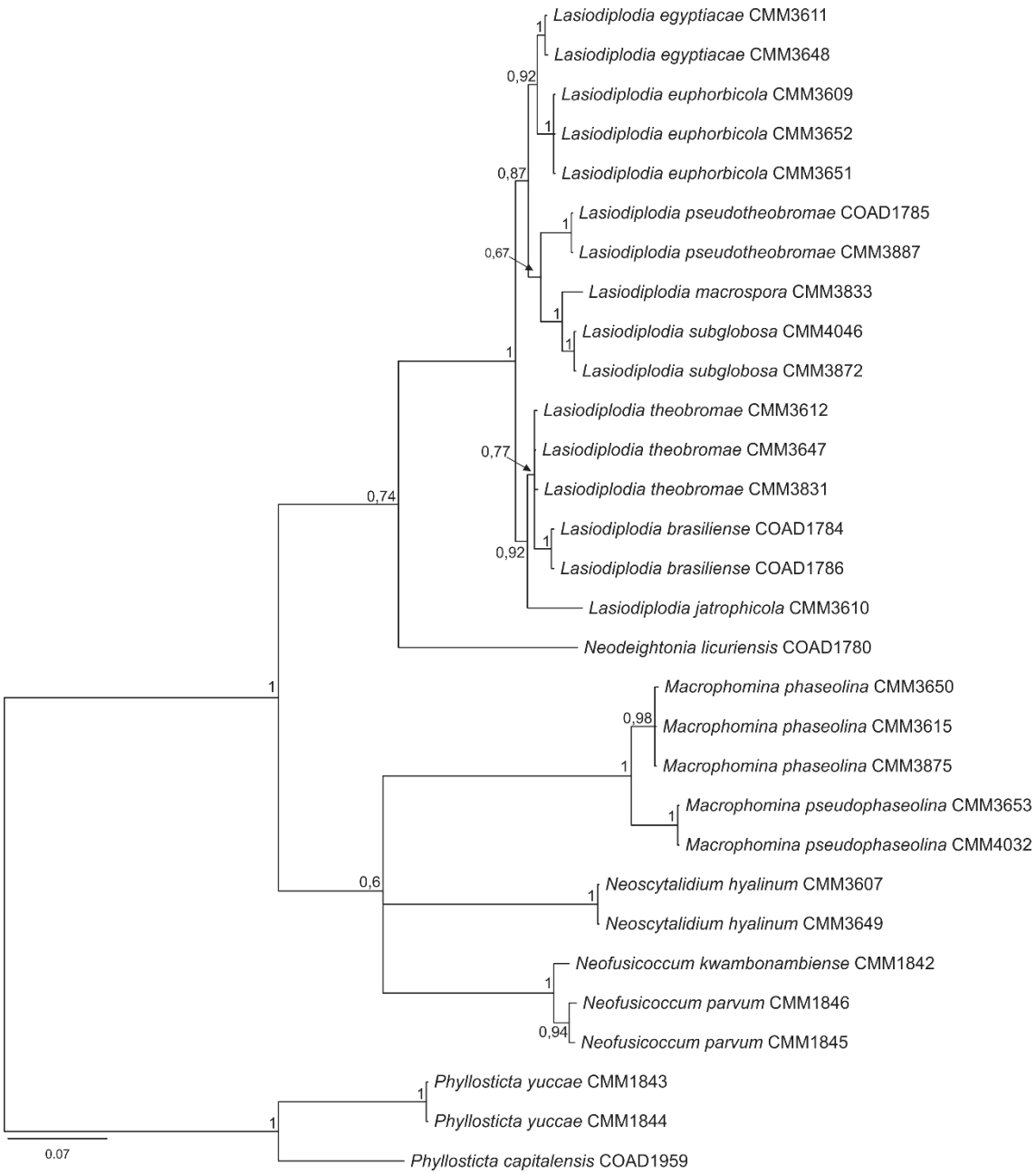

Fig. S9. Phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the TEF-1α and MCM7. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

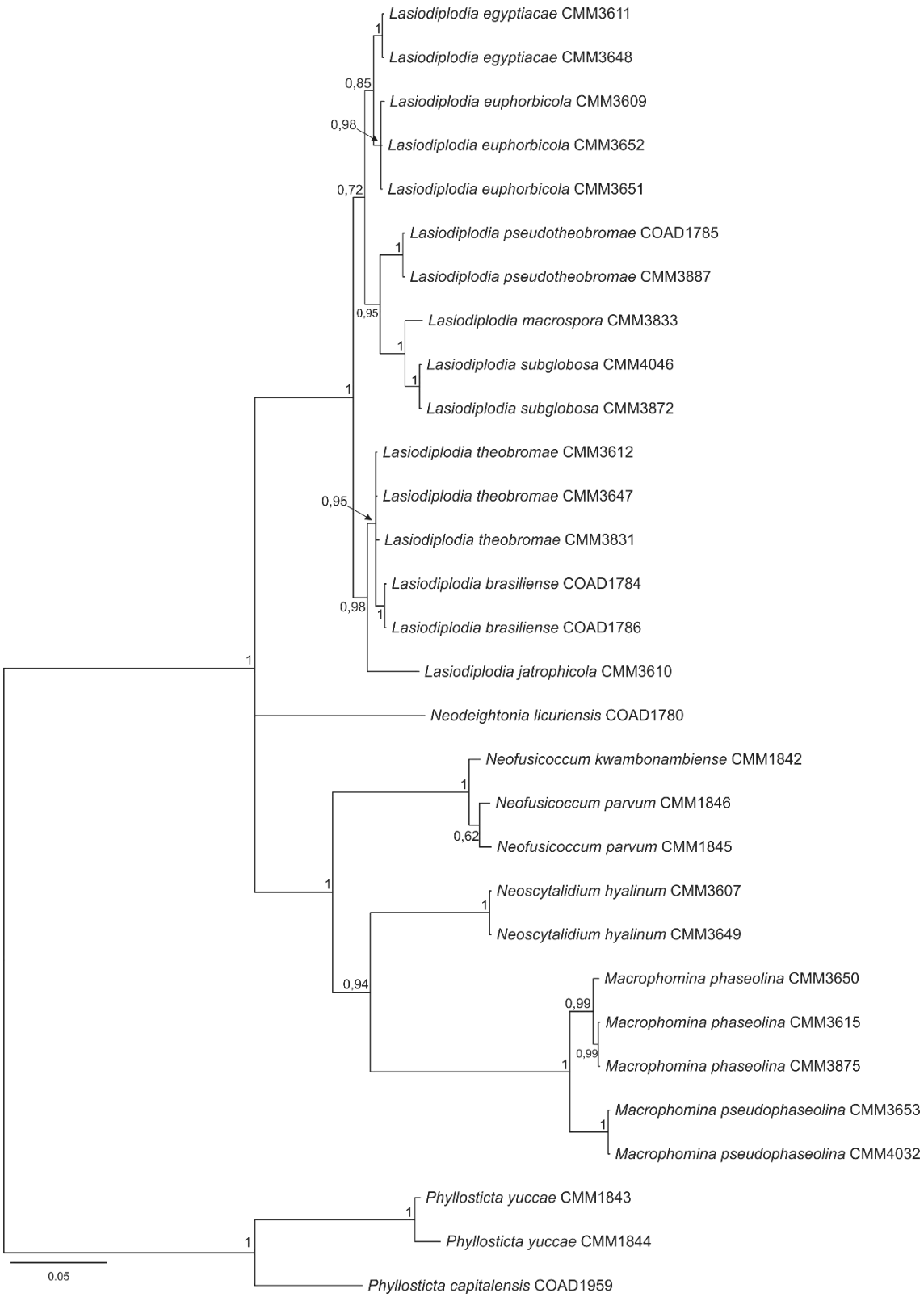

Fig. S10. Phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the TEF-1α and MS204. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

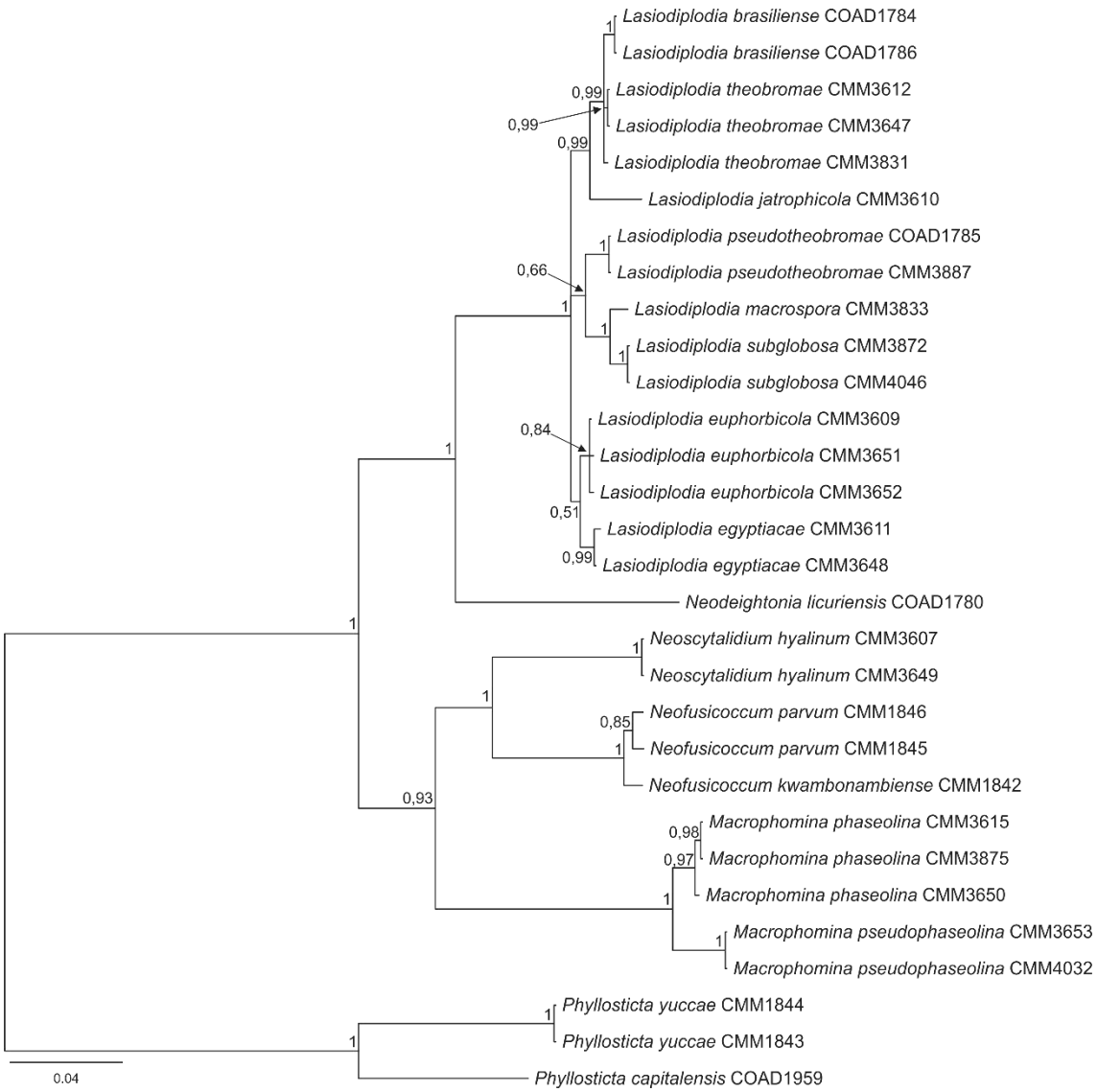

Fig. S11. Phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the ITS, TEF-1α and BT. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

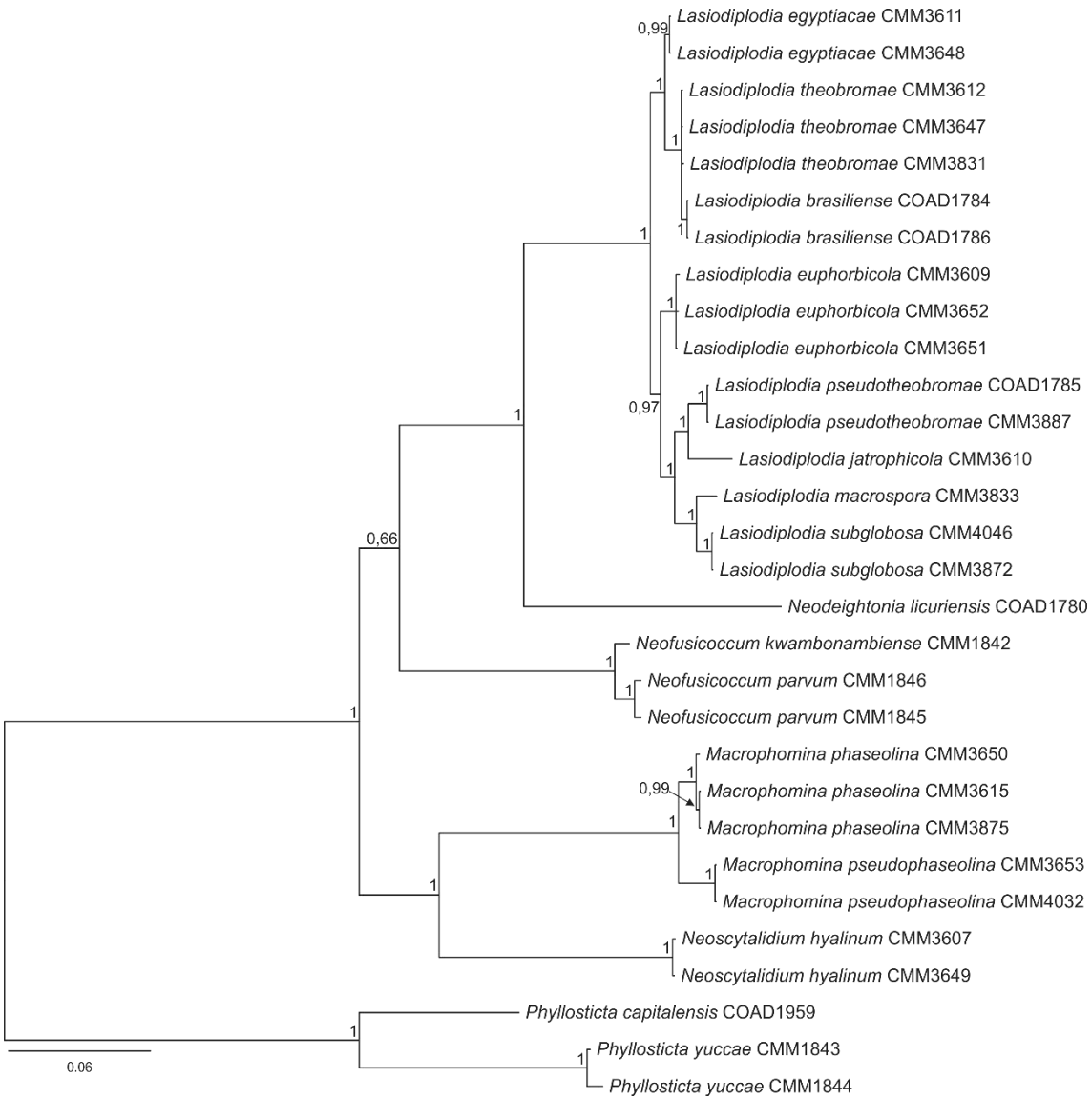

Fig. S12. Phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the TEF-1α, RPBII and MS204. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Phyllosticta yuccae* (CMM1843 and CMM1844) and *Phyllosticta capitalensis* (COAD1959).

CONCLUSÕES GERAIS

- A podridão negra das raízes da mandioca no Brasil é causada por duas espécies de *Lasiodiplodia* (*L. euphorbicola* e *L. pseudotheobromae*) e uma espécie de *Neoscytalidium* (*N. hyalinum*);
- A identificação molecular de espécies de *Macrophomina* associados a várias culturas oleaginosas (mamoneira, amendoim, pinhão-manso, soja, gergelim, girassol, algodão e *Jatropha gossypifolia*) no Brasil revelaram três espécies distintas, sendo uma nova espécie a ser proposta associada a mamoneira e *Jatropha gossypifolia*, e novas ocorrências *M. pseudophaseolina* associadas a mamoneira, algodão e amendoim;
- A seca descendente em Annonaceae no Brasil é causada por oito de espécies de *Lasiodiplodia*, sendo *Lasiodiplodia brasiliense* (em *Annona muricata*), *L. crassispora* (em *Annona muricata*, *Annona squamosa* e Annonaceae nativa), *L. hormozganensis* (em *Annona squamosa*), *L. iraniensis* (em *Annona muricata*, *Annona squamosa* e atemóia), *L. jatrophicola* (em *Annona muricata*), *L. pseudotheobromae* (em *Annona crassiflora*, *Annona muricata*, *Annona squamosa* e atemóia), *L. subglobosa* (em *Annona muricata* e Annonaceae nativa), e uma nova espécie filogenética a ser proposta (em *Annona squamosa*);
- As regiões gênicas TEF1-α, RPB2 e MS204 são as mais informativas para a diferenciação de espécies de Botryosphaeriales, enquanto ITS (região amplamente utilizada) e β-tubulina possuem baixo sinal filogenético. Adicionalmente, as regiões gênicas de cópia única RPBII e MS204 foram recomendadas para serem utilizadas em análises filogenéticas de espécies de *Lasiodiplodia* e *Macrophomina*. No entanto, uma condição para que se possa utilizar essas regiões gênicas em futuros estudos, será o desenvolvimento de banco de dados de referência com sequências de RPB2 e MS204 para todas as espécies possíveis de Botryosphaeriales;
- Novos oligonucleotídeos foram propostos para a amplificação da região gênica de cópia única MCM7 para diferentes espécies de Botryosphaeriales. E esta região gênica mostrou-se útil para estudos filogenéticos de categorias taxonômicas superiores (gêneros, famílias, ordens, etc.).