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"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (MICROBIOLOGIA APLICADA)

DIVERSIDADE DE FUNGOS EM *Eucalyptus microcorys* F. MUELL DA FLORESTA ESTADUAL EDMUNDO NAVARRO DE ANDRADE, RIO CLARO-SP

LORENA TIGRE LACERDA

Tese apresentada ao Instituto de Biociências, do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Microbiologia Aplicada).

Rio Claro
Outubro – 2018

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TÍTULO DA TESE: DIVERSIDADE DE FUNGOS EM Eucalyptus microcorys F. MUELL E SUA APLICABILIDADE NA PRODUÇÃO DE LACASES

AUTORA: LORENA TIGRE LACERDA
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À minha mãe, pelo apoio e incentivo,

durante todo o tempo.

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BIOGRAFIA DA AUTORA

Lorena Tigre Lacerda

Iniciou o Curso de Ciências Biológicas em 2008, na Universidade Estadual de Santa Cruz (UESC), em Ilhéus-BA, quando foi bolsista de Iniciação Tecnológica, na Comissão Executiva do Plano da Lavoura Cacueira (CEPLAC), sob a orientação dos pesquisadores: Dr. João de Cássia e Dr. Givaldo Niella. Durantes os anos de 2009 a 2011, atuou como estágiaria no projeto "Desenvolvimento de metodologia analítica e amostral para a avaliação de conformidade e da inocuidade de produtos comerciais formulados a base de agentes microbianos de biocontrole".

Em março de 2012, iniciou o curso de Mestrado em Produção Vegetal, na mesma Universidade, sob a orientação do Prof. Dr. Jadergudson e co-orientação do Prof. Dr. José Luís Bezerra, elaborando, em fevereiro de 2014, a dissertação intitulada: "Fungos Xylariaceae associados a plantas em áreas de Mata Atlântica na Bahia, Ceará e Paraíba".

Em maio de 2014, foi bolsista de Desenvolvimento Tecnológico e Industrial (CNPq) no projeto "Informatização e disponibilização dos dados associados ao acervo do Centro de Recursos Microbianos da UNESP", sob a coordenação do Prof. Dr. André Rodrigues. Em outubro do mesmo ano, ingressou no doutorado no Programa de Pós-Graduação em Ciências Biológicas (Microbiologia Aplicada), na Universidade Estadual Paulista "Júlio de Mesquita Filho", sob a orientação do Prof. Dr. André Rodrigues e co-orientação do Prof. Dr. Luís Fernando Pascholati Gusmão (UEFS, Feira de Santana, BA).

The greater our knowledge increases the more our ignorance unfolds (KENNEDY, 1962).

Sabedoria é o ponto de encontro entre a dúvida e a certeza (SUKHORUKOV, 2005).

RESUMO

O eucalipto pode ser considerado uma das plantas economicamente mais importantes do mundo. Isso é devido às diversas aplicações na indústria, tais como: fabricação de móveis, papel e celulose, carvão vegetal, óleos essenciais, mel, entre outras. O Brasil é o maior produtor mundial de eucalipto, com mais de cinco milhões de ha plantados. A Floresta Estadual Edmundo Navarro de Andrade (FEENA) é considerada o "berço do eucalipto" no Brasil e apresenta a maior diversidade dessa planta fora do seu local de origem (Oceania). No entanto, pouco se sabe sobre a riqueza de espécies fúngicas associada à *Eucalyptus* spp. da FEENA. Assim, o objetivo desse trabalho foi descrever a comunidade fúngica presente em diferentes fases foliares de *Eucalyptus microcorys* F. Muell, uma espécie pouco explorada em relação a sua diversidade microbiana. Inicialmente foram avaliadas duas técnicas de isolamento (fragmentos foliares e filtração de partículas), a fim de delimitar o método que melhor representasse a comunidade fúngica endofítica associada às folhas frescas. Em seguida, selecionamos a técnica de filtração de partículas para investigar a comunidade sapróbia em diferentes fases foliares da serrapilheira do eucalipto. Os isolados fúngicos foram identificados quanto às características morfológicas e sequenciamento de fragmentos do DNA genômico. Ao todo, foram recuperadas 3.267 colônias fúngicas, distribuídas em 87 taxa. As principais conclusões desse estudo foram: (i) Sugerimos a utilização das técnicas de fragmentos foliares e filtração de partículas como métodos complementares de isolamento para avaliar a comunidade de fungos endofíticos; (ii) *E. microcorys* hospeda uma grande diversidade de espécies fúngicas associada às folhas. As comunidades de fungos da serapilheira dessa planta diferem significativamente da comunidade encontrada nas folhas frescas. Além disso, a diversidade de fungos encontrada no folhedo diminui conforme a fase de decomposição da folha. No entanto, o contrário pode ser observado em relação à abundância de algumas espécies; (iii) Relatamos novas ocorrências de fungos para o Brasil, bem como um novo gênero (*Mycrocylindroseptoria*), com base em marcadores morfológicos e moleculares. Portanto, nossos resultados demonstram a importância de estudos que abordem a diversidade fúngica associada às folhas de eucalipto, para descoberta de novas ocorrências e novos taxa fúngicos.

Palavras-chave: Endófitos. Ecologia. Folhas. Sapróbios. Taxonomia.

ABSTRACT

Eucalyptus is considered one of the most economically important plants in the world. This is due to its diverse industrial applications, including furniture manufacturing and production of pulp, paper, charcoal, essential oils, honey, among others. Brazil is the world's largest producer of eucalyptus, with more than five million hectares. The Floresta Estadual Edmundo Navarro de Andrade (FEENA) is considered the "cradle of eucalyptus" in Brazil and has the largest diversity of this plant outside its place of origin (Oceania). However, little is known about fungal diversity associated with *Eucalyptus* spp. at FEENA. The aim of this study was to assess the fungal community in different leaf stages of *Eucalyptus microcorys* F. Muell, an unexplored species in relation to its microbial diversity. Initially, two isolation techniques (fragment plating and particle filtration) were evaluated to determine the method that best represented the endophytic fungal community associated with fresh leaves. Then, we selected the particle filtration technique to investigate the saprobic community in different eucalyptus leaf stages. Fungal isolates were identified using morphological characteristics and DNA sequencing. A total of 3,267 fungal colonies were recovered, distributed in 87 taxa. We concluded that (i) the fragment plating and particle filtration techniques can be used as complementary isolation methods to evaluate the endophytic fungal community; (ii) *E. microcorys* harbors a large diversity of fungal species associated with its leaves. The fungal communities from eucalyptus litter leaves differ significantly from that found in fresh leaves. In addition, the diversity of fungi found in the litter decreases depending on the stages of leaf decomposition. However, the opposite was observed regarding the abundance of some species; (iii) We report new occurrences of fungi for Brazil, as well as a new genus (*Mycrocylindroseptoria*), based on morphological and molecular markers. Therefore, our results demonstrate the importance of addressing the fungal diversity associated with eucalyptus leaves for discovering new occurrences and new fungal taxa.

Keywords: Endophytes. Ecology. Leaves. Saprobes. Taxonomy.

SUMÁRIO

1 INTRODUÇÃO

1.1 *Eucalyptus* L'Hér.

Eucalyptus lato sensu são plantas lenhosas da família Myrtaceae Juss. (Myrtales), nativas da Oceania e compreendem três grandes gêneros: *Angophora* Smith, *Corymbia* K.D. Hill & L.A.S. Johnson e *Eucalyptus* L'Hér. (BROOKER, 2000). O último abriga a maior diversidade, com mais de 600 espécies e subespécies, sendo descrito por Charles L´Héritier em 1788, baseado em *Eucalyptus obliqua* L'Hér. (BROOKER et al., 2005; SILVA-PANDO; PINO-PÉREZ, 2016). O gênero *Eucalyptus* é caracterizado, principalmente, por apresentar frutos secos e deiscentes, flores andrógenas com simetria radial, folhas perenes e heteroblásticas (BROOKER, 2000). As folhas, flores, frutos, gemas, raiz e casca do eucalipto apresentam como componentes dominantes: óleos essenciais, compostos fenólicos, alcalóides, esteróides, terpenos, lactonas insaturadas e derivados de cumarinas. Esses compostos são propelidos no ar ou expelidos pelas raízes (FERREIRA; ÁQUILA, 2000) e podem atuar na dormência, na germinação das sementes, no crescimento de plântulas, bem como no vigor vegetativo de plantas adultas (YAMAGUSHI; GUSMAN; VESTENA, 2011). Além disso, a presença de terpenos arómaticos pode proporcionar para a planta: defesa contra herbivoria, resistência a baixas temperaturas, redução na perda de água e alelopatia (DORAN, 1991). Devido a essas características físico-químicas, o eucalipto tornou-se uma das espécies florestais mais importantes do mundo (TURNBULL, 2000). Ele é cultivado para os mais diversos fins, como: fabricação de papel e celulose, carvão, móveis, postes, serraria, produtos de limpeza, alimentos, perfumaria, remédios, mel, ornamentação, produção de energia elétrica, entre outros (KHAEKHUM et al., 2017).

Originário da Austrália e ilhas adjacentes da Oceania, o eucalipto adaptou-se a praticamente todas as condições climáticas, ocupando uma área mundial de aproximadamente 22 milhões de ha (MENDES; TREICHEL; BELING, 2016). Atualmente, está distribuído em mais de 90 países, em todos os continentes, com exceção da Antártida, sendo considerada a madeira mais amplamente plantada das regiões tropicais e subtropicais (TIBBITS; BOOMSMA; JARVIS, 1997; BRANCO et al., 2015). O sucesso do cultivo comercial de eucalipto pode ser atribuído a vários fatores bióticos e abióticos, incluindo: a alta tolerância à diferentes condições climatológicas, solos pobres em nutrientes, doenças e a rápida taxa de crescimento (BRANCO et al., 2015).

A distribuição mundial do eucalipto iniciou-se na Europa, no século XVIII, em seguida se expandiu para a Ásia, África e para a América (BRANCO et al., 2015). Apesar do gênero *Eucalyptus* ser originário da Oceania, atualmente, esse é o continente que apresenta a menor área plantada de eucalipto do mundo (1 milhão/ha), ficando atrás da Ásia (8,4 milhões/ha), América (7,5 milhões/ha), África (2,4 milhões/ha) e Europa (1,3 milhões/ha) (SBS, 2015). Brasil, Índia e China são os maiores produtores de eucalipto. Juntos, esses países representam mais de 50% da produção mundial (SBS, 2015).

O Brasil possui a maior área plantada de eucalipto (7,4 milhões de ha), sendo o maior produtor mundial (IBGE, 2018). A silvicultura gerou para o país R$ 12.681.426 no ano de 2017 e desses, R$ 10.504.592 foram provenientes somente do cultivo de eucalipto (IBGE, 2018). A celulose é um dos produtos historicamente mais exportado pelo Brasil (SNIF, 2017). Em 2017, o país exportou 12,9 milhões de toneladas de celulose, se mantendo no topo dos maiores produtores desse polímero vegetal (IBÁ, 2018). Isso tornou a silvicultura responsável por 6,2% do Produto Interno Bruto (PIB) do país, gerando aproximadamente 5 milhões de empregos (IBÁ, 2018).

O sucesso do plantio de eucalipto no Brasil iniciou há mais de 200 anos. Em 1824, o Jardim Botânico do Rio de Janeiro recebeu as duas primeiras mudas de *E. gigantea* Hook. (FOELKEL, 2005). A partir daí, essa planta colonizou os demais estados brasileiros: Rio Grande do Sul (1868), São Paulo (1880), entre outros (HASSE, 2006). Inicialmente, o cultivo do eucalipto se deu apenas para fins ornamentais (ANDRADE, 1928). No entanto, em 1904, o engenheiro agrônomo, Edmundo Navarro de Andrade, iniciou a produção de eucalipto com a finalidade de atender as demandas da Companhia Paulista de Estradas de Ferro (ANDRADE, 1941). Com isso, a produção dessa planta começou a crescer no Estado de São Paulo e, consequentemente, no Brasil. Entre os anos de 1909 e 1966, o país apresentava 470 mil ha de área plantada, sendo que 80% concentrava-se no Estado de São Paulo (SAMPAIO, 1975). Contudo, o grande salto da eucaliptocultura ocorreu em 1965, com a Lei nº 5106/66 de incentivos fiscais ao reflorestamento. Essa lei proporcionou aos produtores a possibilidade de contratação de terceiros para o serviço de reflorestamento, com direito ao abatimento de impostos (TRES; REIS, 2009). Estima-se que naquela década a área de plantio de eucalipto passou de cerca 500 mil para 3,2 milhões de ha (FERREIRA, 1993). Desde então, o Brasil tem crescido e atingido vários avanços na eucaliptocultura, tornando-se o maior produtor mundial de eucalipto.

1.2 Breve histórico sobre a Floresta Estadual Edmundo Navarro de Andrade

No final do século XIX, o Brasil era o maior produtor e exportador de café do mundo (MILLIET, 1941). O interior da província de São Paulo foi destaque nesse avanço da economia cafeeira, principalmente, a região oeste, que compreende desde Campinas até Ribeirão Preto (GARCIA, 1992). O município de Rio Claro chegou a ser o terceiro maior produtor de café do Brasil (GARCIA, 1992). No entanto, com o aumento da cafeicultura, os produtores enfrentavam dificuldades para transportar o café do interior para o litoral Paulista, uma viagem que poderia durar até 15 dias (GARCIA, 2009). Tal retardo no escoamento do café acarretava em grandes perdas da safra e, consequentemente, no aumento dos custos da produção (GRANDI, 2010). Com isso, o governo da província de São Paulo, junto com os grandes produtores de café da época, a fim de otimizarem o transporte do grão, investiram na implementação e melhoramento das estradas de ferro (GARCIA, 1992).

Assim, em 1868, foi fundada a Companhia Paulista de Estradas de Ferro (CPEF). A construção de ferrovias gerou a diminuição de cerca de 20% do custo da produção do café, à vista disso, ocorreu uma grande expansão da cafeicultura no interior Paulista (GARCIA, 1992). Em 1876, a ferrovia chegou ao município de Rio Claro, sendo conhecida como a cidade “fim da linha” que ligava Jundiaí a Santos (GRANDI, 2010). Por conseguinte, o progresso ferroviário trouxe a Rio Claro um avanço na industrialização e expansão da área urbana (GRANDI, 2010). Com a expansão da cidade, houve um aumento no número de imigrantes, o crescimento de trabalhadores livres e assalariados e a redução da mão de obra escrava (GARCIA, 1992). Contudo, conforme o desenvolvimento das ferrovias era necessário uma maior quantidade de matéria prima para as caldeiras, construção de vagões e oficinas, fabricação de utensílios industriais e residenciais (devido à urbanização das cidades), entre outros (MARTINI, 2004). No entanto, o desmatamento das florestas nativas para a fabricação de matéria prima, em longo prazo seria inviável, assim os empresários da CPEF buscaram novas fontes de madeira a fim de suprir as necessidades da ferrovia, sem proporcionar o desmatamento das florestas nativas (GRANDI, 2010).

Em 1904, a CPEF contratou o engenheiro agrônomo Edmundo Navarro de Andrade para resolver o problema da escassez de madeira. Navarro comparou as características físico-químicas de 95 espécies de plantas nativas e exóticas, como: peroba, o jacarandá, o jequitibá, o cedro, a cabreúva, a canela, o pinheiro do Paraná, o cedro do Bussaco, o carvalho português, a casuarina, a tristânia, a grevílea, o eucalipto, entre outras (SAMPAIO, 1959). Após seis anos

de estudo, Navarro concluiu que o eucalipto era a planta mais indicada para as caldeiras, pois é de crescimento rápido e de boa qualidade para a utilização como carvão (MARTINI, 2004).

Em 1909, a CPEF adquiriu a fazenda Santa Gertrudes, na época, com cerca 1.260 ha, e as fazendas Cachoeirinha e Santo Antônio, nas redondezas do munícipio de Rio Claro e deu início ao cultivo de espécies de eucalipto em larga escala (GARCIA, 1992). Para iniciar seus estudos, Navarro reuniu sementes de 150 espécies de eucalipto, oriundas da Austrália, Argélia, Argentina, África do Sul e outras localidades (MARTINI, 2004). Assim, se originou o Horto Florestal de Rio Claro, conhecido internacionalmente como o "berço do eucalipto no Brasil" (Figura 1). Em 1959, a CPFP possuía 18 hortos florestais distribuídos pelo interior paulista, ocupando uma área de 24.387 ha, sendo os principais os hortos de Rio Claro, Tatuí, Sumaré e Cordeirópolis (GARCIA, 2009). O Horto Florestal situado no município de Rio Claro foi por muitas décadas o maior da América Latina, chegando a ocupar 3.012,90 ha (GRANDI, 2010). Navarro fundou o museu do eucalipto, o primeiro museu temático do Brasil (GARCIA, 1992). No entanto, com o tempo, a área sofreu várias desocupações com o avanço da cidade para a construção de novos bairros (TRES; REIS, 2009). Assim, com intuito de preservar esse patrimônio histórico, em 11 de junho de 2002, o Horto Florestal foi transformado na Floresta Estadual "Edmundo Navarro de Andrade" (FEENA), pelo Decreto nº. 46.819, artigo 1° (TRES; REIS, 2009).

Figura 1. Vista da área de visitação da Floresta Estadual Edmundo Navarro de Andrade (FEENA).

Fonte: Elaborada pela autora.

Atualmente, a FEENA apresenta 2.230,53 ha, abrangendo os munícipios de Rio Claro e Santa Gertrudes, localizada na latitude 22º25'S e 47º33'W de longitude (GARCIA, 2009). Após 100 anos de implantação, ainda existem mais de 40 espécies de eucalipto nesse local (CASTELLANO, 2013). Navarro estudou o genótipo e o melhoramento genético de diversas espécies e vários clones foram criados por ele (GARCIA, 1992). Toda essa coleção de espécimes de eucalipto criada por Navarro abriga uma grande diversidade de micro-organismos, no entanto, nada se sabe sobre a diversidade microbiana presente em *Eucalyptus* spp. da FEENA.

1.3 Fungos associados ao eucalipto

As associações fungo-planta podem variar desde relações harmônicas a inarmônicas (ALY; DEBBAB; PROKSCH, 2011). Estas interações promovem a biodiversidade vegetal e podem ser consideradas como uma das mais importantes do planeta (HEIJDEN et al., 2016). Os fungos podem interagir com a planta e influenciar o funcionamento do ecossistema de diversas maneiras, por exemplo, fornecendo nutrientes (HEIJDEN et al., 2016). Estima-se que para cada espécie de planta, existe pelo menos uma espécie de fungo. No entanto, esse número pode variar de dezenas a centenas, dependendo da espécie hospedeira e de sua localização (ARNOLD, 2005). O gênero *Eucalyptus*, por exemplo, abriga mais de 1.400 espécies fúngicas (HYDE et al., 2007; LOMBARD et al., 2010; MARIN-FELIX et al., 2017).

Devido à variedade de espécies de fungos encontrada em eucalipto, esse pode ser considerado um *hotspot* de diversidade (CHEEWANGKOON et al., 2009). Entre o final do século XX e o início do século XXI, 180 novos taxa foram descritos como associados ao eucalipto, sendo 34 deles apenas em *E. globulus* Labill. (HYDE et al., 2007). Entre 2010 e 2018, 37 novas espécies de *Calonectria* foram descobertas em eucaliptos no Brasil e em outros países (ALFENAS et al., 2015; CHEN et al., 2011a; CROUS et al., 2012, 2013; LI et al., 2017; LOMBARD et al., 2010, 2015, 2016; MARIN-FELIX et al., 2017). Novas espécies pertencentes aos gêneros: *Castanediella*, *Harknessia*, *Neofusicoccum*, *Neophaeomoniella*, *Pseudoplagiostoma*, *Teratosphaeria*, *Xenogliocladiopsis* e *Xyladictyochaeta* também foram encontradas em eucaliptos (ANDJIC et al., 2010; CHEEWANGKOON et al., 2010; CROUS et al., 2012, 2015, 2016; CROUS; KENDRICK, 1993; HERNÁNDEZ-RESTREPO et al., 2017; LOMBARD et al., 2015; SUMMERELL et al., 2006). Essa grande diversidade de espécies demonstra a importância do eucalipto como *hotspot* para a descoberta de novos taxa fúngicos.

Plantas do gênero *Eucalyptus* abrigam uma comunidade exclusiva de fungos (HYDE et al., 2007). Algumas famílias são recorrentes e frequentemente encontradas em eucalipto, como: Botryosphaeriaceae Theiss. & Syd., Mycosphaerellaceae Lindau, Nectriaceae Tul. & C. Tul., Teratosphaeriaceae Crous & U. Braun, entre outras (ALFENAS et al., 2015; CROUS et al., 2009; JAMI et al., 2017; PÉREZ et al., 2009a). Por exemplo, mais de 25 espécies de Botryosphaeriaceae, distribuídas em vários gêneros, foram relatadas em *Eucalyptus* spp. (BARRADAS et al., 2016). Algumas dessas são consideradas específicas de seu hospedeiro, como: *Botryosphaeria fabicerciana* (S.F. Chen bis, Pavlic, M.J. Wingf. & X.D. Zhou) A.J.L. Phillips & A. Alves e *Neofusicoccum andinum* (Mohali, Slippers & M.J. Wingf.) Mohali, Slippers & M.J. Wingf (PAVLIC-ZUPANCA et al., 2017). Isso também pode ser observado em outras famílias como: Nectriaceae (*Calonectria aciculata* Jie Q. Li, Q.L. Liu & S.F. Chen; *C. amazonica* L. Lombard & Crous; *C. amazoniensis* L. Lombard & Crous; *C. brassiana* R.F. Alfenas, L. Lombard, Crous & Alfenas; *C. eucalypticola* R.F. Alfenas, L. Lombard, Crous & Alfenas; *C. glaebicola* R.F. Alfenas, L. Lombard, Crous & Alfenas; *C. honghensis* Jie Q. Li, Q.L. Liu & S.F. Chen; *C. lageniformis* L. Lombard & Crous; *C. longiramosa* L. Lombard & Crous; *C. maranhensis* R.F. Alfenas, L. Lombard, Crous & Alfenas; *C. multinaviculata* R.F. Alfenas, L. Lombard, Crous & Alfenas; *C. nemoralis* L. Lombard & Crous; *C. paraensis* R.F. Alfenas, L. Lombard, Crous & Alfenas; *C. piauiensis* R.F. Alfenas, L. Lombard, Crous & Alfenas; *C. propaginicola* R.F. Alfenas, L. Lombard, Crous & Alfenas; *C. pseudocerciana* R.F. Alfenas, L. Lombard, Crous & Alfenas; *C. pseudoyunnanensis* Jie Q. Li, Q.L. Liu & S.F. Chen, *C. tucuruiensis* L. Lombard & Crous; *C. yunnanensis* Jie Q. Li, Q.L. Liu & S.F. Chen) e Teratosphaeriaceae (*Teratosphaeria novaehollandiae* Andjic, T.I. Burgess & A. Maxwell e *T. tiwiana* Andjic, T.I. Burgess & A. Maxwell) (ALFENAS et al., 2015; ANDJIC et al., 2016; LOMBARD et al., 2016; LI et al., 2017; MARIN-FELIX et al., 2017).

Esses taxa apresentam diversos papéis ecológicos em eucalipto, como saprotróficos e parasitas (CHEN et al., 2011b; PÉREZ et al., 2008, 2009b; SLIPPERS et al., 2009). Muitos deles estão associados à doenças foliares do eucalipto (ALFENAS et al., 2015; LOMBARD et al., 2010; PARTE; MIRANDA; DÍAZ, 2016). Esses agentes podem afetar a produtividade e a qualidade das plantações (FERREIRA, 1989). O fungo *Ceratocystis fimbriata* Ellis & Halst., por exemplo, causou mais de 40% de mortalidade de plantas em monoculturas de eucalipto no sudeste da Bahia (FERREIRA et al., 2000, 2006). A produtividade também pode ser afetada por níveis de desfolha superiores a 25% na presença de *Pseudoteratosphaeria* spp. Quaedvl. & Crous (LUNDQUIST, 1987; SMITH et al., 2017), podendo chegar a 75% em alguns casos (HUNTER et al., 2011). *Calonectria candelabrum* (Viégas) Rossman, L. Lombard & Crous é

um fungo que também pode acarretar em desfolha intensa (SANTOS; AUER; GRIGOLETTI, 2001). Esse micro-organismo sobrevive em material em decomposição pela formação de escleródios no solo e consegue se disseminar para folhas e ramos das árvores (ALFENAS et al., 2009). Os prejuízos causados por esses micro-organismos nas plantações tendem a crescer nos próximos anos, devido ao aumento nas áreas de monocultura e às mudanças climáticas (ZHOU; WINGFIELD, 2011). Por isso, a identificação da comunidade microbiana e estudos sobre a diversidade fúngica presente nas folhas de eucalipto, além de atribuir um maior entendimento das interações planta-micro-organismo, é essencial na otimização do manejo de culturas e avanço da silvicultura brasileira (MIGUEL et al., 2017).

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2 OBJETIVOS

2.1 Objetivo geral

Descrever a diversidade de fungos associados às folhas de *Eucalyptus microcorys.*

2.2 Objetivos específicos

2.2.1 ***Capítulo 1:*** Descrever a diversidade de fungos endofíticos em *E. microcorys* e comparar duas técnicas de isolamento.

2.2.2 ***Capítulo 2:*** Descrever a comunidade fúngica sapróbia presente em três diferentes estágios foliares de *E. microcorys* na serapilheira.

2.2.3 ***Capítulo 3:*** Descrever um novo gênero, *Microcylindroseptoria*.

3 CONTEXTUALIZAÇÃO DA TESE

Capítulo 1: Devido o árduo trabalho de Navarro, a FEENA pode ser considerada o local de maior diversidade de eucalipto fora do centro de origem (Oceania). Vários estudos pesquisaram a diversidade de organismos presentes nesse local, como: mamíferos, anfíbios e insetos. Contudo, pouco se sabe sobre a comunidade fúngica ali presente. Considerando que a FEENA abriga muitas espécies de eucalipto e que essa planta hospeda uma comunidade fúngica singular, selecionamos *Eucalyptus microcorys* para iniciar os estudos micológicos dessa área. Em seguida, o próximo passo foi definir o método de isolamento, tendo em vista reunir uma coleção de fungos provenientes de eucalipto. Para tanto, utilizamos dois métodos de isolamento dependentes de cultivo: filtração de partículas e fragmentos foliares. Considerando que as comunidades recuperadas se difereriam quanto à técnica de isolamento, em termos de composição de fungos, e que não houve diferença significativa na diversidade, sugerimos os dois métodos como complementares no isolamento de fungos endofíticos. Acreditamos que este capítulo servirá como base para futuros trabalhos sobre a diversidade de endófitos em eucalipto e de outras plantas.

Capítulo 2: O eucalipto abriga uma grande diversidade de fungos. Esses engajam em várias simbioses com seu hospedeiro, podendo ser endofíticos, sapróbios ou parasitas. A fim de acessar as comunidades fúngicas presentes em diferentes fases de decomposição foliar de *E. microcorys*, selecionamos a técnica de filtração de partículas, pois no trabalho anterior observamos que esse método recuperou um maior número de espécies endofíticas. Aqui relatamos pela primeira vez, para *E. microcorys*, que a comunidade fúngica presente nesse hospedeiro, além de ser bastante diversa, varia conforme o avanço da decomposição foliar. Além disso, foi abordada a diversidade de fungos associados ao eucalipto, compilando os dados existentes na literatura e acrescentando novos relatos e novas ocorrências. Assim, esperamos que este capítulo ofereça uma melhor compreensão das relações ecológicas entre fungos e plantas.

Capítulo 3: Apesar da grande diversidade de espécies recuperadas, um táxon chamou nossa atenção, por ser encontrado em todas as fases foliares e plantas estudadas. Esse também foi encontrado pelo nosso grupo de pesquisa em outro substrato (jardim de fungo de formigas cortadeiras). Assim, intensificamos os estudos com esse micro-organismo e o descrevemos como um novo gênero, provavelmente associado ao eucalipto. Com base em marcadores

morfológicos e moleculares, descrevemos *Microcylindroseptoria eucalypti*, como um novo táxon pertencente à família Dothideaceae (Dothideales). Esse se difere morfogicamente dos outros gêneros da família, por apresentar conidioma picnidial estéril e dois tipos de conídios. As análises filogenéticas demonstraram que *M. eucalypti* se separa das outras linhagens de Dothideaceae, formando um clado a parte.

4 DIVERSITY OF ENDOPHYTIC FUNGI IN *Eucalyptus microcorys* assessed BY COMPLEMENTARY ISOLATION METHODS

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Abstract

Brazil is the world's largest producer country of eucalyptus. Although widely applied in the charcoal industry, no studies have focused on the microorganisms associated with *Eucalyptus microcorys*. Here, we evaluated the composition and structure of endophytic fungal communities in leaves of *E. microcorys* through two isolation techniques. A total of 120 fresh leaves were collected in a year-long survey at an eucalyptus plantation in the State of São Paulo (Brazil). Endophytic fungi were isolated by particle filtration (PF) and direct leaf fragment plating (LP) in two media: modified dicloran and synthetic nutrient agar, both supplemented with rose bengal and chloramphenicol. The isolates were grouped into morphospecies and identified by morphology and DNA sequencing.We recovered a total of 709 isolates, representing 59 taxa. All taxa found are reported as endophytic for the first time for *E. microcorys*. *Castanediella eucalypticola* and *Neophaeomoniella eucalypti* are new occurrences reported for Brazil. The LP technique recovered a higher number of taxa and isolates than the PF. However, the PF technique retrieved a higher species/isolate ratio than the LP method, 0.12 and 0.09, respectively. Fungal diversity assessed by diversity metrics did not significantly differ between isolation methods. Both techniques recovered a high number of unique taxa, demonstrating that neither method would individually represent the species richness from *E. microcorys*. The use of LP and PF provided a greater number of observed taxa and consequently new occurrence of species for Brazil.

Keywords Culture-dependent methods. Leaf plating. Particle filtration. Endophytes

Introduction

The genus *Eucalyptus* is native to Australia and adjacent islands of Oceania, but Brazil has become the world's largest producer country (Sociedade Brasileira de Silvicultura 2015). Eucalyptus is used as raw material for the production of paper, cellulose, and other derivatives that represent 4% of Brazil's GDP and 8% of all Brazilian exports (Jonker et al. 2015).). Although *E. grandis* Hill ex Maiden is the most planted species in Brazil, *E. microcorys* F.Muell is also widely cultivated in the country, due to its feasible chemical and physical properties for the production of charcoal (Oliveira et al. 2014).

There are about 700 species and sub-species of eucalyptus and at least 150 of them are associated with more than 1,400 fungal species (Hyde et al. 2007). Between the years 1995 and 2003 alone, 34 new fungal species were described from eucalyptus trees (Hyde et al. 2007). In addition, Cheewangkoon et al. (2009) described 15 new fungal species associated with *Eucalyptus* spp. Although several studies continue to focus on the microbial diversity in eucalyptus (Lima et al. 2013; Massenssini et al. 2016; Souza et al. 2017; Pavlic-Zupanc et al. 2017), there are no reports in the fungal community associated with *E. microcorys* in Brazil.

Many fungi have been considered recurrent and exclusive in eucalyptus. For example, the order Capnodiales has more than 150 species associated with eucalyptus leaves (Crous and Wingfield 1997; Crous 1998; Hunter et al. 2006; Crous et al. 2006; Cheewangkoon et al. 2008; Crous et al. 2009; Aguín et al. 2013). Many of these fungi are endophytic or latent pathogens such as *Cladosporium*, *Mycosphaerella*, *Pseudocercospora*, *Readeriella*, and *Teratosphaeria* (Kharwar et al. 2010; Marsberg et al. 2014; Bernreiter et al. 2016). *Eucalyptus* spp. are considered a cache of fungal diversity and studies that explore the microbiota associated with these plants are essential for the discovery of new species (Cheewangkoon et al. 2009).

Diversity studies of fungi in eucalyptus have been based on culture-dependent techniques, usually involving fungal isolation from disinfected plant surfaces, fragmented and inoculated in artificial media (Schulz et al. 1993). However, the direct plating of plant tissues may have limitations on fungal diversity analysis due to the potential bias favoring fast-growing species that sporulate profusely (Paulus et al. 2003). To circumvent these drawbacks, alternative methods that better represent the endophytic community of a plant host have been applied. The particle filtration technique is used to isolate fungi from leaf litter (Bills and

Polishook 1994). This method is based on substrate fragmentation and removal of conidia from the plant surface. As a result, the reduced particle size favors isolation from somatic mycelia embedded in the plant matrix, which increases the chance of recovering isolates that actually colonize the plant matrix, including slow-growing fungi otherwise missed by other methods (Bills and Polishook 1994). This technique better represents the community in the substrate, since the presence of species with a high sporulation rate is less. Some studies have recommended a particle filtration protocol to isolate fungi of litter due to its efficiency in evaluating the diversity of these microorganisms (Bills and Polishook 1994; Paulus et al. 2003; Collado et al. 2007). This technique also has been used for isolation of fungi associated with marine environments such as coral reefs (Barathikannan et al. 2017) and deep-sea sediments (Damare et al. 2006; Singh et al. 2010; Zhang et al. 2014). De Abreu and collaborators (2010) recovered over a thousand endophytic fungal isolates using this technique from the plant species *Phoradendron perrottettii* (DC.) Eichler and *Tapirira guianensis* Aubl. Aside from the latter study, little is known about the efficiency of this technique for endophytic fungi.

Considering the lack of knowledge about endophytic fungi of *E. microcorys*, the aim of this study was to evaluate the diversity of these fungi in a plant species not yet investigated for its endophytes, using two isolation techniques: direct leaf plating and particle filtration.

Material and methods

Study site and sampling

We sampled *E. microcorys* at "Floresta Estadual Edmundo Navarro de Andrade" (FEENA), in the municipality of Rio Claro, State of São Paulo, Brazil. FEENA has more than 2000 ha of eucalyptus planted, and it is known as the "cradle of eucalyptus" in Brazil. Currently, the site is considered to have the highest concentration of eucalyptus species (more than 100) outside their native range in Australia. The climate of this area is characterized by hot and humid summers and cold and dry winters.

Three plants of *E. microcorys* were selected: specimen #1(S22° 24.876' W47° 32.616′), specimen #2 (S22° 24.891'W47° 32.622′), and specimen #3 (S22° 24.891' W47° 32.648′), approximately 7 m apart. During a year-long survey (April 2015 to April 2016), we sampled each plant specimen twice (in wet and dry seasons). At each sampling, 20 fresh and

apparently healthy leaves were collected, thus a total of 120 leaves were sampled at the end of the experiment (20 leaves × 2 sampling periods × 3 plant specimens).

Endophytic fungi isolation

After collection, leaves were processed according to the method described in Crous et al. (1997) with modifications. First, the leaves were washed in running tap water and gently rubbed by hand for 5 min to remove debris. Then, the leaves were surface disinfected, which consisted of immersion for 1 min in 2% NaOCl, 30 s in 70% ethanol, and 1 min in sterile distilled water (SDW). To check for the absence of epiphytic fungi (control), 100 μL of the washing water was surfacespread on the culture media. To compare media with different levels of carbon sources, we selected dicloran rose bengal (DRB, without dicloran, nutrient-rich medium, Paulus et al. 2003) and synthetic nutrient agar (SNA, nutrient-poor medium, Nirenberg 1976), both supplemented with 150 μg mL^{-1} of chloramphenicol and bengal rose.

We carried out a modified version of the LP technique (Petrini 1991). Briefly, leaf fragments of approximately 1 × 1 cm^2 were cut and three fragments were inoculated on DRB and SNA media. An adaptation of the PF technique by Bills and Polishook (1994) was used. Two grams of leaves were triturated in a blender for 1 min with 50 mL of SDW. Then, fragments were washed with SDW jets and filtered through a set of four sieves with descending mesh openings (1.0, 0.7, 0.25, and 0.18 mm). The particles retained in the smallest mesh were transferred to disposable tubes and re-suspended in SDW (up to 50 mL). This suspension was shaken for 1 min and allowed to rest for 10 min. The supernatant was discarded and the tube was again filled with SDW (up to 50 mL). We repeated this procedure four times and, finally, the fragments were re-suspended in 20 mL of SDW. Aliquots of 100 μL of this suspension were surface-spread on DRB and SNA media.

All plates were incubated at 25 °C in the dark for 14 days and observed daily. Once a fungus colony appeared this was transferred onto PCA (potato carrot agar) medium to check for axenic cultures and for further identification.

Morphological and molecular identification

We preliminary grouped fungal isolates in morphospecies based on the colony morphology and microscopic characteristics of reproductive structures following taxonomic keys (Carmichael et al. 1980; Barber et al. 2005; Cheewangkoon et al. 2008; Lombard et al.

2010). Representative isolates of each morphospecies were submitted to molecular identification using the internal transcribed spacer region (ITS), as the universal barcode marker. Genomic DNA was extracted from isolates grown on PCA medium for 7 days at 25 °C. Mycelia were harvested and mechanically broken by agitation in lysis buffer supplemented with glass beads (425–600 μm in diameter, SIGMA), following a modified protocol by Möller et al. (1992). Briefly, we added 5 μL of proteinase K and incubated it at 65 °C for 30 min. Then, 140 μL of 5M NaCl and 64 μL of 10% CTAB were added and incubated at 65 °C for 60 min. The tubes were centrifuged at 10,000 rpm (Eppendorf microcentrifuge, 5424) for 30 s and 600 μL of chloroform with isoamyl alcohol (24:1) were added. Then, the tubes were centrifuged at 12,000 rpm for 10 min. The supernatant was collected and transferred to 1.5 mL tubes. After this procedure, 300 μL of 100% ice-cold isopropanol and 50 μL of 3M sodium acetate pH 5.2 were added. The suspension was centrifuged at 10,000 rpm for 10 min and removed by single inversion of the tubes, followed by washing with 600 μL of 70% ethanol and centrifuged at 10,000 rpm for 10 min. The ethanol was removed by single inversion. After ethanol drying, 30 μL of Tris EDTA buffer (10 mM Tris; 1 mM EDTA) were added.

The ITS region was amplified with primers ITS4 and ITS5 (White et al. 1990). Amplification reactions consisted of 0.2 mM of each dNTP, ×1 KCl buffer, 2 mM MgCl2, 0.4 μM of each primer, and 0.04 U of Taq polymerase to a final volume of 25 μL. The reactions followed these conditions: 94 °C/3 min, 35 cycles of 94 °C/1 min, 55 °C/1 min, 72 °C/2 min. For the isolates that were not identified with ITS, we also amplified the partial ribosomal large subunit (LSU) and small subunit (SSU) regions, using primers LR0/LR5 and NS1/NS4, respectively, according to Thambugala et al. (2014). Amplicon purification was performed with Illustra GFX PCR DNA and gel band purification kit (GE Healthcare). The sequencing reaction was performed using BigDye® Terminator v. 3.1 Cycle Sequencing Kit ThermoFisher), according to the manufacturer's protocol, and then applied in a ABI 3500 sequencer. (Life Technologies).

Forward and reverse sequences were assembled in BioEdit v. 7.0.5.3 (Hall 1999). Then, contigs were compared with homologous sequences deposited in the databases of the National Center for Biotechnology Information (NCBI) — GenBank (http://www.ncbi.nlm.nih.gov/genbank) by BLASTn (Altschul et al. 1997) and MycoBank (http://www. mycobank.org/). In addition, for the majority of fungal isolates, we carried out phylogenetic analysis to help in the barcode identification. The phylogenetic trees (not shown) were generated in MEGA v.5.2 (Tamura et al. 2011), using the neighbor-joining

algorithm, Kimura 2-parameter as nucleotide substitution model and 1000 bootstrap pseudoreplicates. Representative sequences generated in this study were deposited at the NCBI—GenBank under accessions: MF663538–MF663596 (for ITS) and MF664751 (for LSU).

Comparison between isolation techniques

We used a combination of diverse metrics with multivariate analysis to compare both isolation techniques in recovering the fungal community. Simpson (1-*D*) and Shannon (*H*′) diversity indices were calculated and compared by the nonparametric Mann-Whitney test to verify significant differences. Rarefaction curves with 95% confidence intervals were generated using the analytical solution of "Mao tau" to compare the fungi richness obtained by the different isolation techniques. The similarity index (SIMPER) was calculated to verify the contribution of each species to the general differences in fungal composition. In addition, to explore hidden patterns in the structure of fungal communities, we carried out an ordination nonmetric multidimensional scaling analysis (NMDS). For this analysis, we built a matrix with taxa and samples displayed into columns and rows, respectively. The Bray-Curtis index was applied as the distance measure, whose principle is the use of the abundance of the taxa to quantify the dissimilarity between samples. To measure differences observed in NMDS, we carried out a similarity analysis (ANOSIM). The diversity analyses were performed in Past v. 2.17c. (Hammer et al. 2001) and statistical analyses in R v. 3.0.1 (R Core Team2016). For a better comparison between the techniques, we normalized the data (using the species/isolate ratio).

Results

We recovered a total of 709 isolates from leaves of *E. microcorys*. After identification, the isolates were grouped in 59 taxa (Table S1). All of them are reported for the first time as endophytic for *E. microcorys*. The phylum Ascomycota was the most dominant (99% of the isolates), regardless of the technique used, and was represented by the orders: Xylariales (334 isolates), Botryosphaeriales (113), Diaporthales (110), Capnodiales (28), Pleosporales (8), Sordariales (7), Hypocreales (4), Mytilinidiales (1), and Phaeomoniellales (1). Cantharellales (1) and Polyporales (3) were the only orders representing the phylum Basidiomycota (1% of the isolates). A total of 99 out of 709 isolates could not be identified or assigned to an order.

The ascomycetous species *Castanediella eucalypticola* and *Neophaeomoniella eucalypti* were reported for the first time in Brazil.

Regarding the isolation techniques, the LP and PF methods recovered 489 and 220 isolates, respectively. The LP method obtained higher species richness than the PF technique (48 and 27, respectively, Table 1). However, the species/isolates ratio was higher in PF (0.12) when compared to LP (0.09, Table 1). The LP and PF methods recovered similar diversity of fungi, as noted by the absence of significant differences in the Simpson and Shannon indices (Mann-Whitney, $P > 0.05$). Although the techniques presented similar diversity indices, only 16 out of 59 taxa (27.1%) were shared.

Rarefaction curves did not reach an asymptote, even considering both techniques (Fig. 1). This suggests that the richness would continue to increase with a greater sampling effort, regardless of the isolation technique. The LP technique recovered a larger number of rare species (singletons and doubletons), including *Annulohypoxylon moriforme*, *Cytospora austromontana*, *Marssonina californica*, *Nemania abortiva*, *Neophaeomoniella eucalypti*, *Pantospora guazumae*, and *Preussia minimoides* (Table 2).

The ANOSIM showed significant differences between community compositions obtained by the two techniques($R = 0.56$, $P = 0.004$). The SIMPER test showed 87.3% dissimilarity among the communities recovered in both techniques, with *Xylaria globosa* (19%), *Xylaria apiculata* (8.3%), *Pseudoplagiostoma eucalypti* (5.7%), *Phyllosticta capitalensis* (5.5%), *Neofusicoccum* sp.2 (4.7%), and *Diaporthe* sp. (4.3%) as the fungi that accounted for the majority of the dissimilarity. These were also the most abundant species (Table 2).

The NMDS analysis showed a clear pattern in the distribution of fungal communities with stress coefficient equal 0. Coordinate 1 (representing 98% of the distribution) suggests structural differences in fungal communities as samples separates by isolation technique: LP (left) and PF (right, Fig. 2). On the other hand, coordinate 2 suggests no structuring of fungal communities by sampling periods (Fig. 2).

Another interesting result was in relation to culture media. The DRB medium was more efficient in the recovery of isolates compared to the SNA, regardless of the technique, 56 and 29 taxa, respectively, because 30 taxa were only observed on the DRB and 3 only by SNA (Table 2). Regarding the isolation time, there was no growth after 10 days of cultivation, regardless of the technique used. Both techniques had the highest number of taxa recovered between the second and fifth day of inoculation.

Discussion

This is the first study on the diversity of endophytic fungi in *E. microcorys*. Our evaluation of endophytes from this previously unexplored eucalyptus species suggests a high fungal diversity, in addition to new species records for Brazil. We also showed the impact of the isolation method on the recovery of endophytic fungi. Either LP or PF alone would not recover the breadth of fungal taxa found when considering both methods together. The low number of shared taxa between isolation techniques indicates that culture-dependent studies focused on the endophytic fungal diversity would benefit when different isolation methods are considered.

The use of two or more methods to assess the microbial diversity is expected to increase the number of species recovered. Although LP is widely used for endophytic fungi isolation, PF was useful in the isolation of these microorganisms. Despite the fact that LP recovered a greater number of isolates, PF recovered a more diverse community, according to the species/isolates ratio. This may have occurred due to the diversity of cultivable species increases as the density of the inoculum (leaf fragments) decreases (Collado et al. 2007). This technique also favored sporulating fungi found in leaves of *E. microcorys*, as *Pseudoplagiostoma eucalypti*, *R. eucalypti*, and *Satchmopsis brasiliensis*. Previous studies in various substrates showed the efficiency of PF in recovering an increased diversity compared to traditional isolation methods (Bills and Polishook 1994; Paulus et al. 2003; Collado et al. 2007). Because there is little knowledge about the applicability of this technique for endophytic fungi, our results suggest that PF may be considered when assessing the endophytic fungal diversity, supporting previous claims on this matter (Unterseher and Schnittler 2009).

The order Xylariales was the most abundant, and *Xylaria* was the most recurrent taxa. This genus is considered cosmopolitan (except for the poles) and apparently has no host specificity. *Xylaria* spp. are frequently reported as endophytes in several plant species such as: *Arundina graminifolia* (D. Don) Hochr. (Orchidaceae), *Embothrium coccineum* Forst. & Forst. (Proteaceae), *Khaya anthotheca* (Welw.) C. DC. (Meliaceae), *Piper aduncum* L. (Piperaceae), *Vanilla planifolia* Jacks. Ex Andrews (Orchidaceae), including *Eucalyptus* spp. (Lupo et al. 2001; Oliveira et al. 2011; Linnakoski et al. 2012; González-Teuber 2016; Rajulu et al. 2016). The LP technique allowed the isolation of a large number of xylariaceous fungi, recovering 296 out of 334 isolates. Perhaps, these fastgrowing fungi may have outnumbered

others in the isolation plates due to the size of the leaf fragment. In contrast, the PF method may have reduced the load of these microorganisms from the samples used for isolation. Nevertheless, *C. eucalypticola* (Xylariaceae) was only isolated by the PF method. This species was described by Crous et al. (2016) on leaf spots of *E. robusta* Sm. in France, and it is reported here as a new occurrence for Brazil (and America).

Botryosphaeriales were also largely represented in this study. More than 25 species in several genera of Botryosphaeriaceae have been reported from *Eucalyptus* leaves as endophytes and pathogens (Barradas et al. 2016). The genus *Neofusiccocum* is considered an aggressive pathogen of several plants, including eucalyptus species. *Botryosphaeria dothidea* (Moug.) Ces. & De Not., previously classified as a *Neofusiccocum* anamorph, causes severe damage to crops worldwide, and it is reported to cause latent endophytic infections (Smith et al. 1996). In addition, *Phyllosticta capitalensis* is associated with a wide range of hosts, causing symptoms such as leaf spots and fruit necrosis (Wulandari et al. 2009; Wang et al. 2012; Wikee et al. 2013).

Another new occurrence recovered for Brazil was *N. eucalypti* (Phaeomoniellales), previously reported by Rooney-Lath and Crous (Crous et al. 2015) on stems of *E. globulus* in California (USA). In this study, *N. eucalypti* was considered a rare species, because only one isolate was recovered by the LP technique.

Culture media also influence the recovering of endophytic fungal diversity. DRB (nutrient-rich) recovered a higher number of isolates and species diversity than SNA (poor in carbon sources). Fungi grow more slowly on SNA than on DRB, probably due to the low amount of nutrients present in the former. The efficiency of DRB may be attributed to the amount of simple sugars available. Other studies have demonstrated the effectiveness of the DRB for isolating saprobe fungi (Bills and Polishook 1994; Polishook et al. 1996; Costa et al. 2015). Although SNA was not efficient for recovering the endophytic fungi, Fakhrunnisa and Ghaffar (2006) had satisfactory results using this culture medium for the isolation of Fusarium. Apparently, the use of carbon-rich media, such as modified DRB and others commonly used (potato-dextrose agar and oatmeal agar), may be useful for the isolation of endophytic fungi.

The similar diversity indices and the high dissimilarity index of the communities recovered by the different techniques demonstrate they complement each other. For optimizing endophytic fungal isolation, we suggest that PF could be used as a complimentary method to LP to assess the communities of these fungi.

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Tables

Table 1. Diversity metrics describing the fungal communities from leaves of *Eucalyptus microcorys* obtained by two isolation techniques: leaf plating (LP) and particle filtration (PF).

Diversity metrics	**LP**	**PF**
Richness	48	27
Species/isolates ratio	0.09	0.12
Number of isolates	489	220
Shannon index (*H'*)	2.69	2.54
Simpson index (*1-D*)	0.86	0.88
Chao-1	69.3	49

Table 2. Fungi (in number of isolates) recovered from *Eucalyptus microcorys* using two isolation techniques: leaf plating (LP) and particle filtration (PF) and two culture media.

Fungi	**LP**		**PF**		**Total**
	DRB*	**SNA****	**DRB**	**SNA**	
Annulohypoxylon moriforme (Henn.) Ju, Rogers & Hsieh 2005	1	0	0	0	1
Apoharknessia insueta (B. Sutton) Crous & Lee 2004	3	0	0	0	3
Calonectria sp. De Not. 1867	1	0	1	0	2
Castanediella eucalypticola Crous & Wingf. 2016	0	1	4	5	10
Ceratobasidiaceae G.W. Martin 1948	1	0	0	0	1
Cladosporium cladosporioides species complex (Fresen.) Vries 1952	3	0	0	1	4
Colletotrichum sp.1 Corda 1831	9	8	0	1	18
Colletotrichum sp.2	6	1	0	0	7
Colletotrichum sp.3	20	1	0	0	21
Cytospora austromontana Adams & Wingf. 2005	1	0	0	0	1
C. eucalypticola Van der Westh. 1965	0	0	2	3	5

Continua

Fungi	LP		PF		Total
	DRB*	SNA**	DRB	SNA	
C. variostromatica Adams & Wingf. 2005	5	0	0	0	5
Daldinia sp.1 Ces. & De Not. 1863	19	2	4	0	25
Daldinia sp.2	3	0	0	0	3
Diaporthe phaseolorum (Cooke & Ellis) Sacc. 1882	25	7	0	0	32
Dothistroma septosporum (Dorogin) M. Morelet 1968	0	0	2	0	2
Epicoccum sp. Link 1816	4	2	0	0	6
Fusicladium amoenum (R.F. Castañeda & Dugan) Crous, K. Schub. & U. Braun 2007	0	0	1	0	1
Geomyces sp.1 Traaen 1914	0	1	0	0	1
Geomyces sp.2	0	1	0	0	1
Gliocephalotrichum bacillisporum Decock & Huret 2006	0	0	0	2	2
Hypoxylon sp.1 Bull. 1791	2	0	0	0	2
Hypoxylon sp.2	1	0	0	0	1
Marssonina californica (Ellis & Everh.) Magnus 1906	1	0	0	0	1
Nemania sp. Gray 1821	12	6	1	0	19
N. abortiva Rogers, Ju & Hemmes 2006	1	0	0	0	1
N. difusa (Sowerby) Gray 1821	8	3	12	0	23
Neofusicoccum sp. Crous, Slippers & Phillips 2006	10	0	0	0	10
N. parvum (Pennycook & Samuels) Crous, Slippers & Phillips 2006	24	0	6	19	49
Neophaeomoniella eucalypti Rooney-Lath. & Crous 2015	1	0	0	0	1
Neurospora discreta Perkins & Raju 1986	1	0	4	2	7
Nigrospora oryzae (Berk. & Broome) Petch 1924	12	0	1	0	13
Pantospora guazumae Cif. 1938	2	0	0	0	2
Phlebia floridensis Nakasone & Burds. 1995	0	0	1	0	1
Phyllosticta capitalensis Henn. 1908	8	0	30	16	54
Preussia minimoides (S.I. Ahmed & Cain) Valldos. & Guarro 1990	2	0	0	0	2

Continua

Conclusão

Fungi	LP		PF		Total
	DRB*	SNA**	DRB	SNA	
Pseudocercospora norchiensis Crous 2007	0	0	2	5	7
Pseudoplagiostoma eucalypti Cheew., Wingf. & Crous 2010	18	1	41	3	63
Pseudoplagiostoma sp. Cheew., Wingf. & Crous 2010	0	0	1	0	1
Readeriella eucalypti (Gonz. Frag.) Crous 2006	0	0	4	9	13
Satchmopsis brasiliensis Sutton & Hodges 1975	0	0	3	9	12
Skeletocutis diluta (Rajchenb.) David & Rajchenb. 1992	0	1	1	0	2
Teratosphaeria ohnowa (Crous & Wingf.) Crous & Braun 2007	0	0	1	0	1
Xylaria sp.1 Hill ex Schrank 1789	2	1	0	0	3
Xylaria sp.2	1	0	0	0	1
Xylaria sp.3	6	2	1	0	9
Xylaria sp.4	1	0	0	0	1
Xylaria sp.5	2	0	0	0	2
Xylaria sp.6	1	1	0	0	2
X. globosa (Pers.) Mont. 1855	109	49	7	1	166
X. berteroi (Mont.) Cooke ex Rogers & Ju 2012;	2	0	0	0	2
X. apiculata Cooke 1879	46	18	0	0	64
FNI 1***	0	0	1	0	1
FNI 2	1	0	0	0	1
FNI 3	1	0	0	0	1
FNI 4	1	0	0	0	1
FNI 5	1	0	0	0	1
FNI 6 (*Microcylindroseptoria eucalypti*, ver Capítulo 3)	1	1	1	12	15
FNI 7	2	1	0	0	3
Total	381	108	132	88	709

* Dicloran rose bengal / ** Synthetic nutrient agar / *** FNI: non-identified fungi.

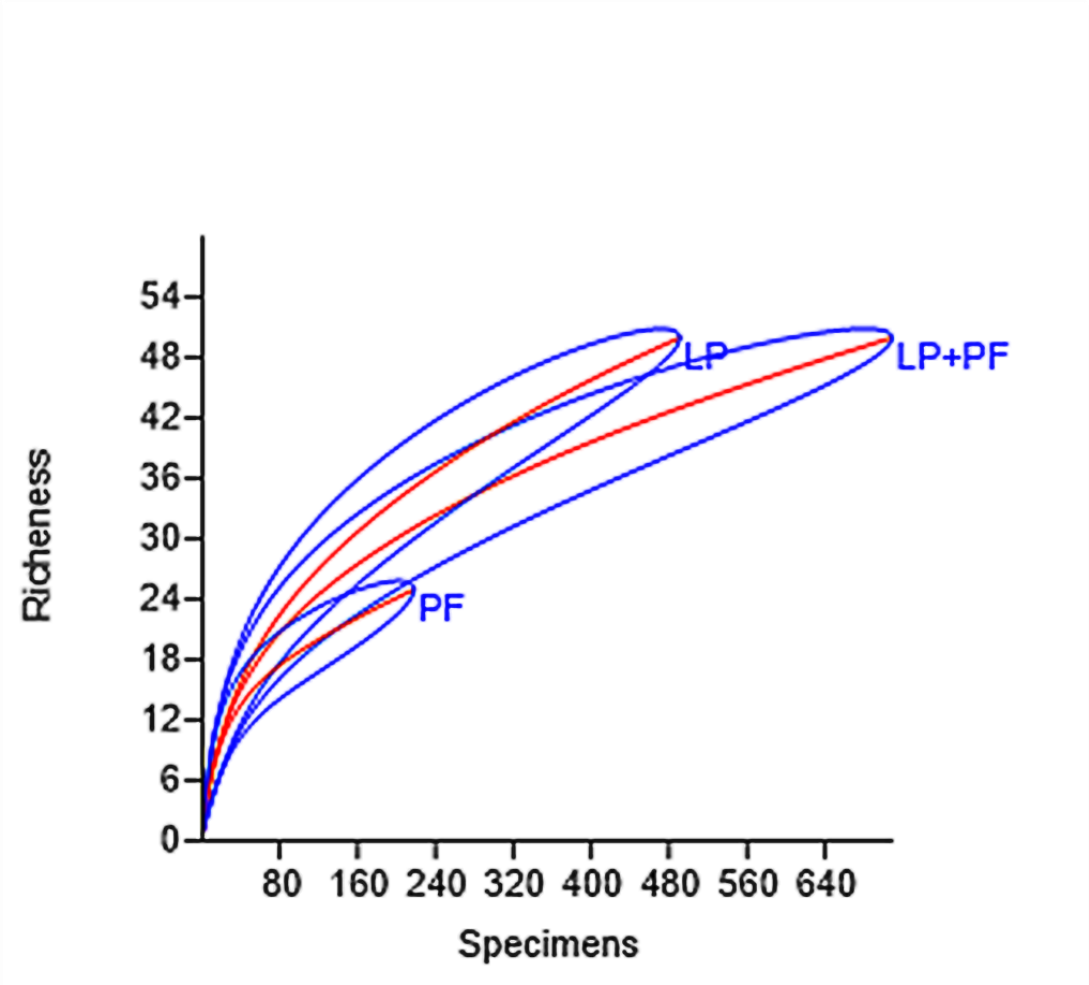

Fig.1 Rarefaction curves of endophytic fungi associated with *Eucalyptus microcorys* leaves recovered by the different isolation techniques. LP: leaf plating technique; PF: particle filtration technique. LP + PF: combination of the two techniques. Blue lines: Confidence interval at 95%.

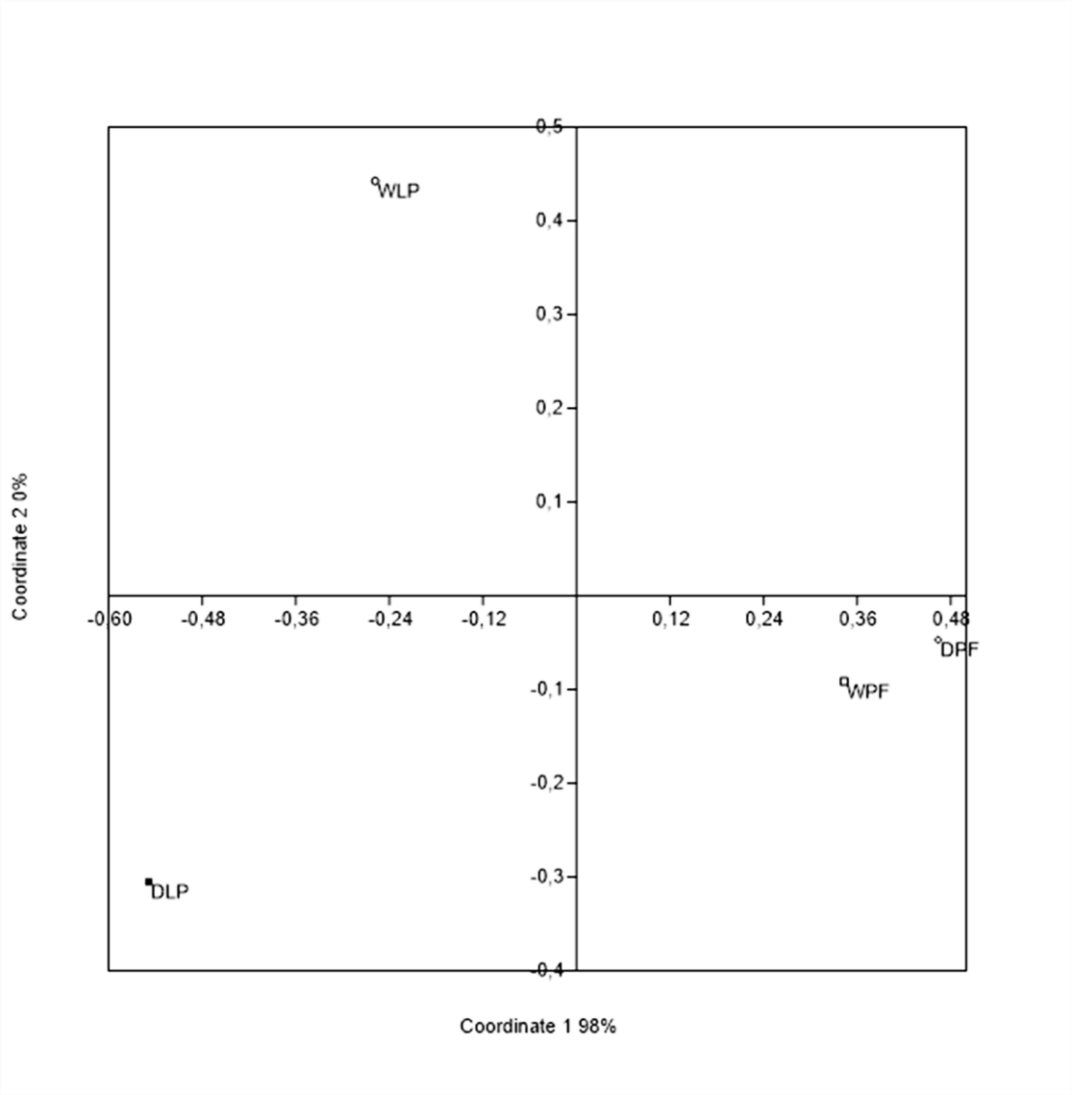

Fig.2 Nonmetric multidimensional scaling analysis (NMDS) demonstrating the distribution of the endophytic fungal community in leaves of *Eucalyptus microcorys*. W: wet; D: dry; LP: leaf plating technique; PF: particle filtration technique. Stress: 0.

5 FUNGAL COMMUNITY FROM DIFFERENT *Eucalyptus microcorys* LEAF STAGES

(Texto elaborado segundo as normas da revista Forest Pathology).

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Abstract

Brazil is the largest producer of charcoal worldwide and *Eucalyptus microcorys* can be used with as one raw material for charcoal production in the country. Despite its economic importance, the fungal community associated with this plant species is largely unknown. Herein, we evaluated the diversity of saprobic fungi in three different stages of *E. microcorys* leaf litter and compared such diversity to the endophytic fungal community of *E. microcorys* from a previously published dataset. We investigated fungal communities using the particle filtration technique coupled with isolation on two different culture media. The obtained isolates were grouped into morphospecies and identified by morphology and DNA sequencing. We recovered a total of 2,558 fungal colonies, represented by 48 taxa. We report the following five species for the first time in Brazil: *Castanediella eucalypti*, *Harknessia pseudohawaiiensi*, *Neophaeomoniella niveniae*, *Xenogliocladiopsis cypellocarpa* and *Xyladictyochaeta lusitanica*. In addition, we found putative new species and well-known fungal pathogens of eucalyptus. The fungal diversity decreases, while dominance of certain fungal species increased across the healthy-leaf litter continuum. The fungal community of healthy leaves differed significantly from the communities in the leaf litter. This can be seen in the results of the multivariate analyses and the few shared taxa between leaf types. *E. microcorys* hosts a fairly diverse saprobic fungal community on its leaves, which varies with leaf maturity. Studies exploring this large microbiota are essential for discovering new taxa and new occurrences.

Keywords Diversity, litter, particle filtration, saprobes.

Introduction

Eucalyptus L'Hér is considered one of the most important plants for the global economy (Turnbull 2000), due to its role in reforestation of degraded areas and production of medicine, furniture, charcoal, paper, honey , and ornamental foliage (Barber et al. 2003; Khaekhum et al. 2017). Brazil is the largest producer of eucalyptus worldwide (IBGE 2018), with over 5 million hectares of this plant in the country (IBGE 2018). The Floresta Estadual Edmundo Navarro de Andrade (FEENA) is considered the "cradle of eucalyptus" in Brazil and has the largest diversity of *Eucalyptus* species outside its place of origin that is Australia and adjacent islands of Oceania. However, knowledge about the microorganisms presents in *Eucalyptus* spp. of FEENA, and especially for *E. microcorys*, is still elusive (Lacerda et al. 2018).

The entire *Eucalyptus* genus hosts a wide variety of fungi (Summerell et al. 2006), with approximately 700 fungal species found on plants of this genus in the past years (Silva et al. 2012). Sankaran et al. (1995) listed 313 fungal species from *E. globulus* Labill. alone. From the late nineteenth to the early twenty-first century, 180 new fungal taxa were found on *Eucalyptus* (Hyde et al. 2007), including several *Mycosphaerella* species (currently named *Ramularia*, Rossman et al. 2015). In just eight years, about 80 new fungus species were discovered from *Eucalyptus* (Crous 1998; Crous et al. 2004ab; 2006ab; Summerell et al. 2006). In addition, several other taxa have been described from different *Eucalyptus* species: *Castanediella*, *Calonectria*, *Harknessia*, *Neofusicoccum*, *Neophaeomoniella*, *Pseudoplagiostoma*, *Teratosphaeria Xenogliocladiopsis*, *Xyladictyochaeta* (Crous and Kendrick 1994; Summerell et al. 2006; Andjic et al. 2010; Cheewangkoon et al. 2010; Crous et al. 2012; 2015; Lombard et al. 2015; Crous et al. 2016; Hernández-Restrepo et al. 2017). Most of these fungi were collected from symptomatic leaves or the leaf litter and can cause severe diseases in eucalyptus. Such plant pathogens represent a significant threat to the productivity and sustainability of this crop.

Plant pathogens use multiple strategies to infect their hosts. One such strategy involves using the leaf litter as part of the pathogen’s life cycles (Monteil et al. 2012). Spores of eucalyptus pathogens, like *Calonectria*, *Ramullaria* and *Neofusicoccum*, remain on senescent leaves in the leaf litter and can infect healthy leaves (Alfenas et al. 2009). *Neofusicoccum eucalyptorum* (Crous, H. Sm. ter & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips 2006 is considered a severe pathogen, causing cancer in several *Eucalyptus* species (Pérez et al.

2009a). However, *N. eucalyptorum* has been found sporulating on woody debris of *E. maidenii* F. Muell. (Pérez et al. 2008). These necrotrophic pathogens are frequently found in the leaf litter since they decompose the host biomass to build mycelium and spores in this habitat (Kan 2006). In order to obtain an agile and effective control, it is important to understand the epidemiology of several diseases and to study the senescence of eucalyptus leaves. One alternative for reducing the impact of such diseases is biological control through endophytic microorganisms from eucalyptus (Sbravatti Júnior et al. 2013; 2016).

Besides their importance in agriculture, saprobic fungi associated with eucalyptus can be used in several other areas. According to Fillat et al. (2016), these microorganisms have potential biotechnological application in the lignocellulosic industry. For example, *Hormonema* sp. provides high brightness in a biobleaching sequence with the natural mediator acetosyringone (Fillat et al. 2017). Recently, fungi in the order Helotiales were associated with the roots of *E. urophylla* S.T. Blake and were able to degrade phenolic allelochemicals produced by this plant species (Liu et al. 2018). In addition, Kharwar et al. (2010) discovered fungal isolates from *Corymbia citriodora* (Hook.) K.D. Hill & L.A.S. Johnson capable of inhibiting human and plant pathogenic fungi *in vitro*. Thus, studies that explore the saprobic microbiota associated with *Eucalyptus* are essential for discovering new species, which could improve industrial processes (Cheewangkoon et al. 2009; Fillat et al 2016). Considering the importance of FEENA as a bank of diversity of eucalyptus species and the lack of studies on the microbiota associated with *E. microcorys*, this study assessed the saprobic fungal community present in three different leaf stages of *E. microcorys* in the leaf litter and compared the fungal community with the endophytic fungal community found in a previous study by Lacerda et al. (2018).

Material and methods

Leaf sampling

The leaves from the leaf litter were collected at the "Edmundo Navarro de Andrade State Forest" (FEENA), from the same three *E. microcorys* trees and in the same collection period studied by Lacerda et al. (2018): Specimen #1 (22°24.876'S 47°32.616'W), Specimen #2 (22°24.891'S 47°32.622'W) and Specimen #3 (22°24.891'S 47°32.648'W).

To evaluate the fungal diversity in the leaf litter, we categorized leaves into three leaf stages, according to the degradation (color) of chlorophyll a and b present in the leaves: S2 =

Second foliar stage, leaves were still green; S3 = third foliar stage, yellowish leaves; S4 = fourth foliar stage, brown leaves (Figure 1). We collected ten leaves of each litter stage, of each plant in two distinct periods (wet and dry seasons) in 2015 and 2016. At the end of the experiment we analyzed a total 180 leaves from three different leaf stages. The collection of fresh leaves (S1) for the endophytic fungi isolation is described in Lacerda et al. (2018), using the same methods.

Fungi isolation

The plant material was rinsed in running tap water to remove debris. Leaf disinfection was carried out according to Crous et al. (1997). The leaf surface was disinfected through immersion for 1 minute in 2% NaOCl, 30 seconds in 70% ethanol, and 1 minute in sterile distilled water (SDW). After this process, we performed the particle filtration isolation technique proposed by Bills and Polishook (1994) and modified by Lacerda et al. (2018). We briefly triturated two grams of leaves in a blender for one minute with 50 mL of SDW. Then, these fragments were washed with SDW and filtered by sieves with descending mesh openings (1.0, 0.7, 0.25 and 0.18 mm). Particles smaller than 0.25 mm were transferred to disposable tubes and re-suspended in SDW (up to 50 mL). Then, this suspension was shaken for one minute and was left to rest for 10 minutes so fragments could settle. Then, the supernatant was discarded, and the tube was again filled with SDW (up to 50 mL). We repeated this procedure four times and, finally, the fragments were re-suspended in 20 mL of SDW. Aliquots of 100 μL of this suspension were inoculated on two culture media: dichloran rose bengal (without dichloran, Paulus et al. 2003) and synthetic nutritive agar (Nirenberg 1976), both supplemented with 150 μg mL^{-1} chloramphenicol and 25 mg L^{-1} bengal rose. To verify the asepsis efficiency (control), 100 μL of the last wash water was spread on both culture media.

The plates were incubated in the dark at 25 °C for 14 days and observed daily. Hyphal tips or conidia of emerging colonies were transferred onto potato carrot agar (PCA, Himedia) plates to obtain axenic cultures for further identification.

Morphological and molecular identification

The isolates were grouped into morphospecies and identified by their macro and microscopic characters using several articles (Barber et al. 2005; Cheewangkoon et al. 2008;

Lombard et al. 2010; Bensch et al. 2012) and standard manuals (Carmichael et al. 1980; Sutton 1980; Von Arx 1981; Seifert and Gams 2001). Representative isolates of each morphospecies were submitted to sequencing of the internal transcribed spacer region (ITS). Thus, a total of 444 isolates was sequenced. For isolates where identification was not possible with the barcode, other genome regions were sequenced (Table S1). In addition, we generated phylogenetic trees (Figures S1–S19) of all orders, to more accurately identify the isolates. The trees were generated in MEGA v.7 (Kumar et al. 2016), using the neighbor-joining algorithm, Kimura 2-parameter as nucleotide substitution model and 1000 bootstrap pseudoreplications. More details about the genomic DNA extraction, sequencing, and phylogeny of the isolates are described in Lacerda et al. (2018). The sequences generated in this study were deposited into the NCBI—GenBank under accessions: ITS (MH329677–MH329696), LSU (MH323446–MH323447), SSU (MH323445), Btub (MF686432–MF686434) and Actin (MH397721–MH397724).

Community structure

To compare the saprobic fungal communities of *E. microcorys* from the leaf litter to the endophytic fungal communities from fresh leaves, we used the endophytic fungal data from Lacerda et al. (2018). In Excel, we built a matrix with taxa and samples displayed in columns and rows, respectively. To compare leaf stages, we calculated the Shannon (H') diversity index for the different *E. microcorys* leaf stages. For a direct statistical comparison between the richness and data sets, we constructed a matrix with the ecological metrics of each leaf stage and created rarefaction curves using the 'Mao tau' index to calculate the expected richness of fungal taxa. To determine the prevalence of each taxon compared to the total prevalence of taxa observed, we calculated the relative frequency of each species.

To observe the taxa shared between communities, we constructed a Venn diagram. Similarity percentage (SIMPER) was used to investigate differences in the fungal composition between the leaf stages and to identify which species contributed most to the dissimilarities observed. The differences in community composition among leaf stages were performed using a combination of multivariate exploratory analyses based on Bray–Curtis similarities: permutational multivariate analysis of variance (PerMANOVA) and Non-metric multidimensional scaling (NMDS). To evaluate patterns of fungal orders observed at all stages, the relative frequency of each order was calculated and a dispersion graph was constructed. All analyzes were performed in PAST v. 2.17c. (Hammer et al. 2001).

Results

A total of 2,558 colonies were obtained from three different *E. microcorys* leaf types. The isolates comprised 48 taxa, distributed in 13 orders and two phyla: Basidiomycota and Ascomycota (Table 1 and Table S2). Ascomycota was the most abundant, with 99.9% of the taxa observed. Dothideales, Hypocreales, Xylariales, and Capnodiales were the most abundant ascomycetous orders, representing more than 95.6% of the isolates (Figure 2). The only five isolates found from the phylum Basidiomycota belong to the Cantharellales and Polyporales orders. Herein, the taxa *Castanediella eucalypti*, *Harknessia pseudohawaiiensi*, *Neophaeomoniella niveniae*, *Xenogliocladiopsis cypellocarpa,* and *Xyladictyochaeta lusitanica* are reported for the first time in leaf litter from Brazil. Regarding the culture media, DRB presented higher taxa richness (43) than SNA (26), independent from the leaf type.

The taxa richness of the leaf stages never approached the estimated values of fungal richness (Table 2), thus rarefaction curves did not reach an asymptote (Figure S20). This implies that taxa richness would continue to increase with increased sampling effort, independent from the leaf stage. However, comparisons between the different leaf types showed that the endophytic fungal community in healthy leaves (data extracted from Lacerda et al. 2018) had greater richness and diversity of fungi than the leaf litter communities (Table 2). Fungal diversity decreased across the different stages of leaf decomposition. On the other hand, the dominance of particular fungi increased with the different stages of leaf decomposition (Table 2). The most abundant taxa, FNI 6 (n= 1,837 isolates), *Calonectria* sp. (n= 193) and *Castanediella eucalypti* (n= 114) increased their abundance along the leaf decomposition gradient. Interestingly, FNI6 was a putative new fungal species associated with eucalyptus in all leaf stages and collections. We used four different markers (LSU, ITS, SSU and Actin), which all showed low similarities with known sequences deposited in the database. Preliminary morphological and phylogenetic analyses suggest this taxon is a lineage between the families Aureobasidiaceae and Dothideaceae. This taxon will be described elsewhere.

When we compared the fungal community of the fresh leaves to the leaf litter communities, only 8 taxa (13.3%) were shared between all leaf types (Figure 3). FNI 6, *Calonectria* sp., *Cladosporium cladosporioides* (species complex), *Phyllosticta capitalensis*, *Pseudocercospora norchiensis*, *Pseudoplagiostoma eucalypti*, *Readeriella eucalypti,* and *Satchmopsis brasiliensis* were the only taxa shared across all leaf stages. However, these taxa

showed variations in their abundances. For instance, FNI 6, *Calonectria* sp., *C. cladosporioides,* and *R. eucalypti* increased in abundance as leaves decomposed. On the other hand, the opposite was observed for *P. capitalensis*, *P. norchiensis*, *P. eucalypti* and *S. brasiliensis*. This dissimilarity between the fungal communities was also observed by the SIMPER analysis, with 74.7% of dissimilarity between all leaf stages. The species that contributed most to this dissimilarity were FNI 6 (55%), *Pseudosydowia eucalypti* (5.9%), *Calonectria* sp. (5.5%), and *P. eucalypti* (4.7%).

The NMDS revealed a clustering in the fungal communities by leaf stages (Figure 4). Coordinate 1 separated the fresh leaves (S1) from the last decomposition stage (S4). This coordinate also split the dry and wet period, with exception of samples S1 and S2 (wet) and S4 (dry). On the other hand, coordinate 2 separated the endophytic community (S1) from the litter community (S2, S3 and S4), with the exception of sample S2 (wet) and S4 (dry). There was no clear distinction between leaf types S2 and S3, demonstrating that the fungal communities at these stages were similar. This was also observed by the permutational multivariate analysis of variance (P= 0.0001). However, the remaining stages showed significant differences between one another (P= 0.0001).

Discussion

This is the first study to address the diversity of fungal communities in different leaf stages of *E. microcorys*. Herein, we found that the leaf litter fungal communities differed distinctly from the community in fresh leaves. Our results indicate that this plant species hosts a wide variety of fungal species, which are apparently recurrent and specific to the genus *Eucalyptus*. Among the fungal species observed, we found new species occurrences for Brazil and putative new species.

The fungal community associated with *E. microcorys* leaves experiences major changes throughout leaf decomposition. The observed variations in diversity and abundance of fungi may be associated with the nutritional properties present at each leaf stage. According to Begon et al. (2006), such decline in richness is expected due to the assimilation of simple sugars by fast-growing fungi, proteins, and amino acids which diffuse freely in the early stages of decomposition. Since the most recalcitrant biomass remains in the substrate, only the organisms capable of decomposing such biomass will prevail (Begon et al. 2006). Such changes in the fungal community throughout leaf stages were also observed in other plant species (Promputtha et al. 2002; Das et al. 2007; Vorıskova and Baldrian 2013). Our findings

support the distinction of the endophytic fungal community between decomposition stages observed by other researchers (Koide et al. 2005; Duong et al. 2008; Unterseher et al. 2013; Szink et al. 2016), and is demonstrated herein for the first time for *E. microcorys*. However, eight taxa were found in all leaf stages and, with the exception of FNI 6 and *S. brasiliensis*, all taxa were previously reported causing diseases in eucalyptus and other plant species (Pérez et al. 2009b; Chungu et al. 2010; Lombard et al. 2010; Lueangpraplut and Unartngam 2011; Sánchez Márquez et al. 2011; Wickert et al. 2014). These fungi are probably necrotrophic pathogens of eucalyptus, and after host cell death they can act in the decomposition of plant tissue and transform leaves into fungal biomass (Newton et al. 2010).

The phylum Ascomycota was extensively observed on leaves of *E. microcorys* in the different stages of senescence. According to Voříšková and Baldrian (2013), this group dominates the initial stages of leaf litter decomposition. Some fungal orders, such as Xylariales, increased in dominance according to leaf senescence. Xylariaceous endophytes are known to decompose lignin and carbohydrate polymers such as cellulose (Lumyong et al. 2002; Osono et al. 2002), which can indicate that these microorganisms could be acting in the decomposition of the leaf. Nevertheless, fungi in the orders Hypocreales and Dothideales, which also increased in abundance in advanced stages of leaf decomposition, are known to cause several diseases in eucalyptus (Wingfield et al. 2008; Lombard et al. 2010; Chen et al. 2011; Huang et al. 2012). For example, *Calonectria* is associated with several eucalyptus diseases, such as leaf blight, damping off, cutting rot, and root rot (Alfenas 1986). Such diseases present a great challenge for plantation development worldwide. In Brazil, *Calonectria* spp. causes severe defoliation in eucalyptus plantations, leading to considerable economic losses (Alfenas et al. 2015). Other taxa found in this study are also known to cause severe diseases in eucalyptus, as *Neofusicoccum parvum*, *Pseudocercospora norchiensis*, and *Pseudoteratosphaeria* spp. Productivity of eucalyptus plantations is affected by defoliation levels higher than 25% in the presence of *Pseudoteratosphaeria* spp. (Smith et al. 2018; Lundquist 1987), reaching up to 75% in some cases (Hunter et al. 2009). Consequently, this plant material serves as an inoculation source to infect healthy leaves. Thus, it is necessary to understand the biology of such pathogens to develop effective management strategies (Hunter et al. 2011). Therefore, studying the different foliar stages is necessary to understand the life cycle of these microorganisms and to implement measures that prevent great economic damages.

Overall, the genus *Eucalyptus* harbors a unique and diverse fungal community (Hyde et al. 2007). All taxa, except for *Neophaeomoniella niveniae*, have been previously reported for

Eucalyptus spp. However, *N. eucalypti* Rooney-Lath. & Crous 2015 was found in *E. globulus* (Crous et al. 2015). Most of the taxa reported in this study have been found in other *Eucalyptus* species. For example, at least five *Castanediella* species are associated with leaf spots in *Eucalyptus*, as *C. communis* Crous & M.J. Wingf. 2016, *C. eucalypti*, *C. eucalypticola*, *C. eucalyptigena* Crous 2017 and *C. malaysiana* Hern.-Restr., M.J. Wingf. & Crous 2016 (Crous et al. 2016 ; Hernández-Restrepo et al. 2017 ; Crous et al. 2017). The genus *Xenogliocladiopsis* also has high specificity with eucalyptus leaves and *X. cypellocarpae* and *X. eucalyptorum* have been previously reported in *E. cypellocarpa* and *Eucalyptus* sp. The exclusivity and recurrence of such fungal taxa with eucalyptus make this plant a possible reservoir of new species and fungal diversity (Cheewangkoon et al. 2009).

The new occurrences of saprobic fungi in Brazil and the putative new species from studying only one eucalyptus species show the importance of research related to fungi diversity for this plant. In addition, the endophytic fungi we found in different leaf stages of this important forest species is an essential step towards understanding the life cycle of plant pathogens. With this knowledge we can gain access to genetic resources, as fungal metabolites, and improve management processes for *E. microcorys*.

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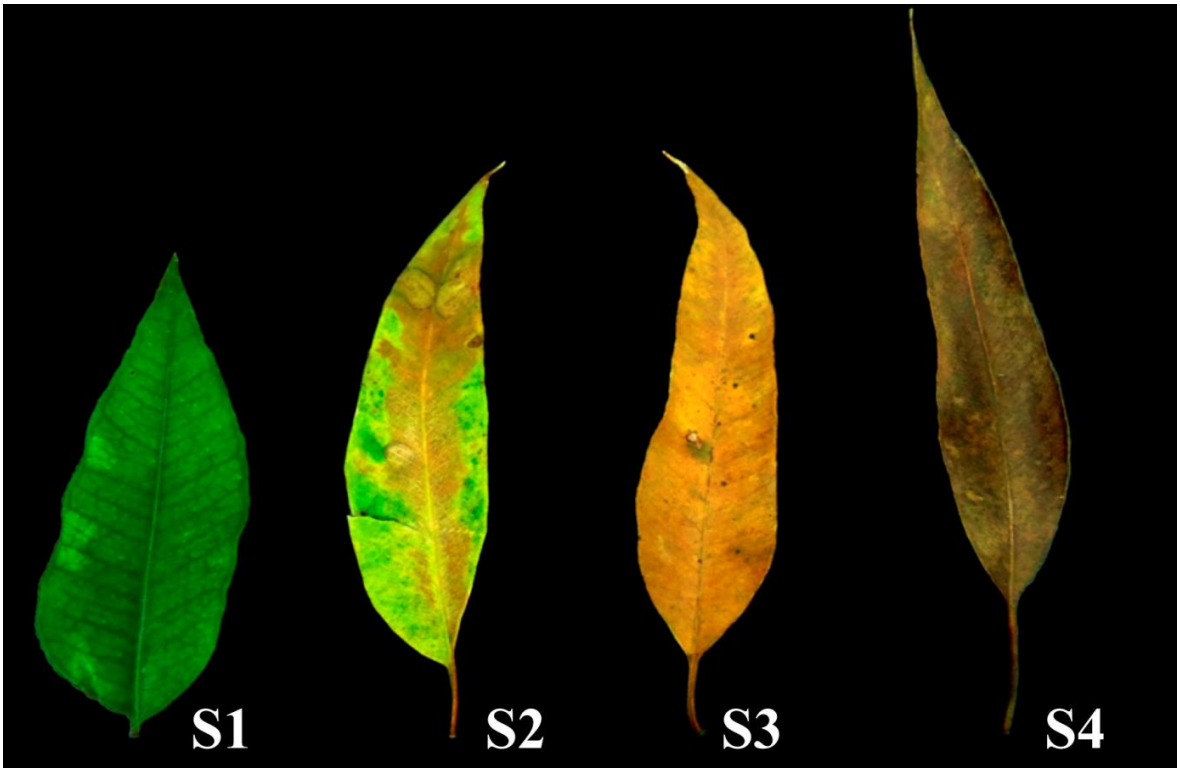

Figure 1. Different *Eucalyptus microcorys* leaf types evaluated in this study. S1= healthy leaves. S2 = Second stage of decomposition, leaves still green. S3 = third stage of decomposition, yellowish leaves. S4 = fourth stage of decomposition, brown leaves.

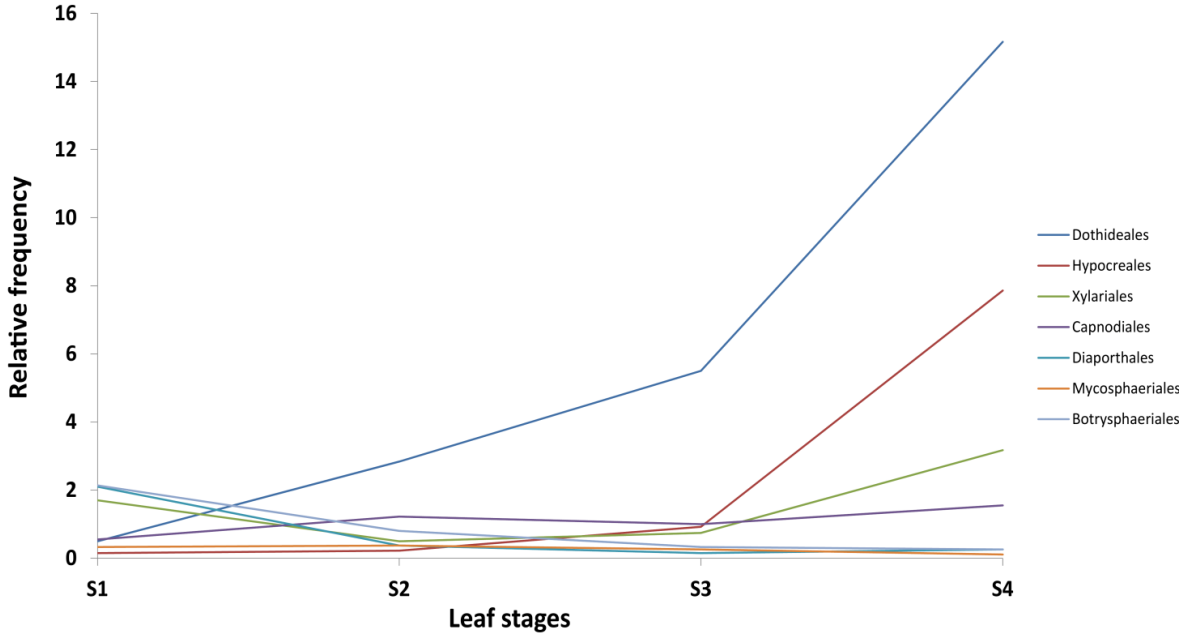

Figure 2. Abundance of fungal orders recovered from different leaf types found in the leaf litter of *Eucalyptus microcorys*. S1= healthy leaves. S2 = Second foliar stage. S3 = third foliar stage. S4 = fourth foliar stage. Since Dothideales was over abundant, the relative frequency was divided by 3 for better visualization.

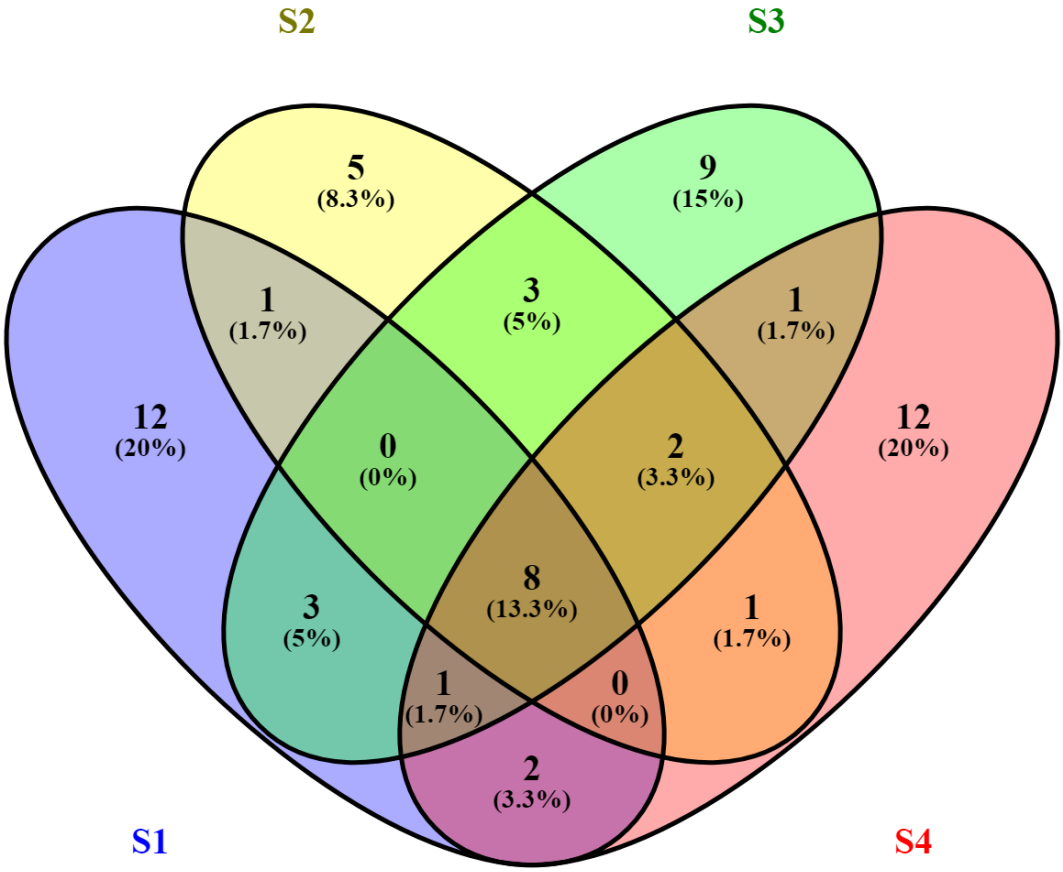

Figure 3. Number of shared fungal taxa from the different *Eucalyptus microcorys* leaf stages. S1= healthy leaves. S2 = Second foliar stage. S3 = third foliar stage. S4 = fourth foliar stage.

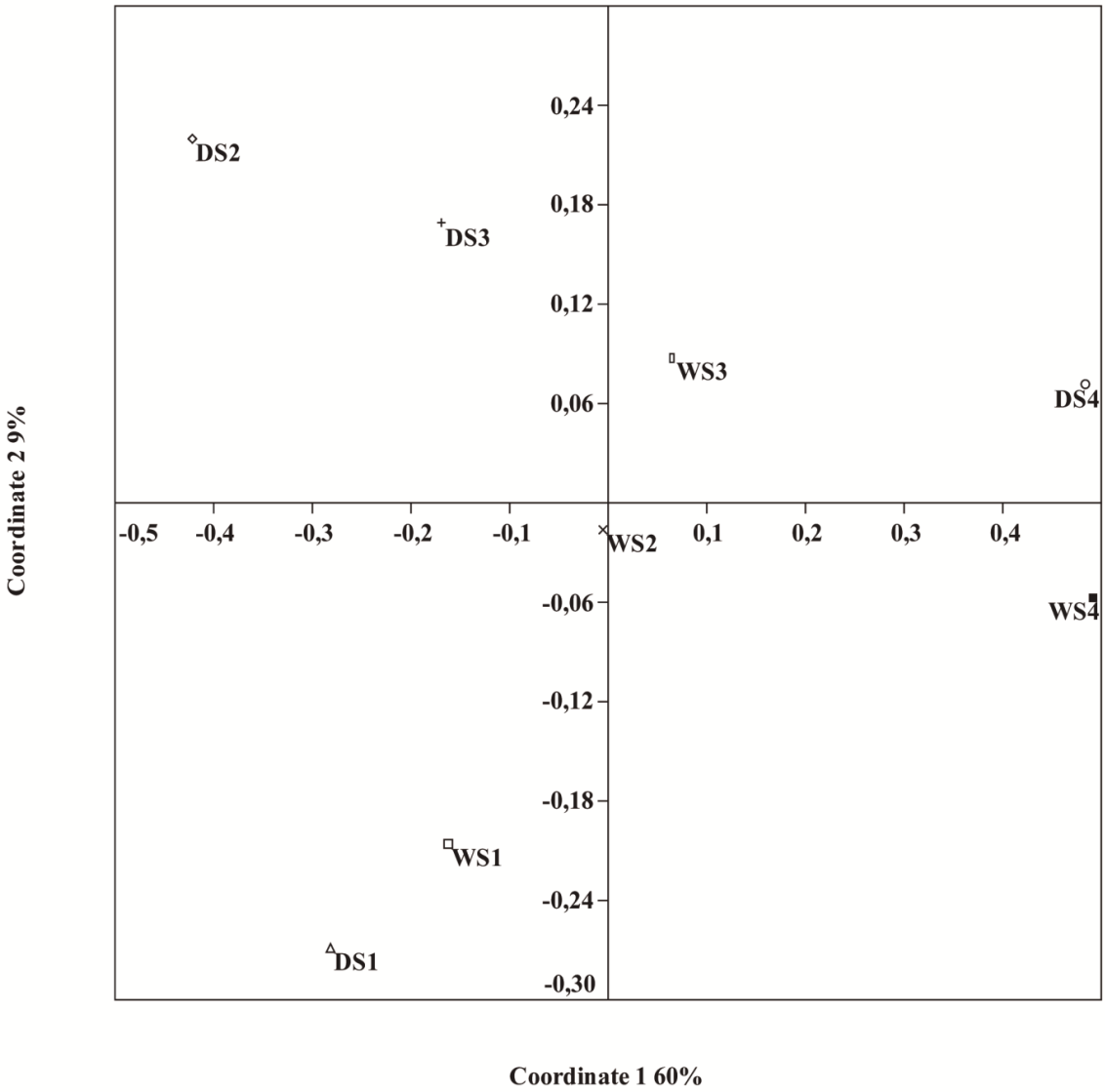

Figure 4. Structuring of the fungal communities found in *Eucalyptus microcorys* leaves depicted by non-metric multidimensional scaling analysis. S1= healthy leaves. S2 = Second foliar stage. S3 = third foliar stage. S4 = fourth foliar stage. D = dry and W = wet.

Table 1. Number of isolates and relative abundance of fungi recovered from different *Eucalyptus microcorys* leaf stages.

Taxa	Leaf types				Abd
	S1*	S2	S3	S4	
Alternaria alternata (Fr.) Keissl. 1912	0	1	0	0	0.036
Calonectria sp. De Not. 1867	1	5	4	183	6.96
Castanediella eucalypti Crous, Hern.-Restr. & M.J. Wingf. 2015	0	13	15	86	4.1
C. eucalypticola Crous & M.J. Wingf. 2016	9	0	0	0	0.33
Ceratobasidiaceae G.W. Martin 1948	0	0	0	1	0.036
Ceriporia lacerata N. Maek., Suhara & R. Kondo 2003	0	1	0	0	0.036
Cladosporium cladosporioides (Fresen.) G.A. de Vries 1952 (species complex)	1	2	1	6	0.36
Colletotrichum sp.1 Corda 1831	1	0	0	0	0.036
Colletotrichum sp.2	0	0	2	0	0.071
Coniella diplodiella (Speg.) Petr. & Syd. 1927	0	0	1	0	0.036
Cytospora eucalypticola Van der Westh. 1965	5	0	2	0	0.25
Daldinia sp. Ces. & De Not. 1863	4	0	0	0	0.14
Diaporthe sp. Nitschke 1870 (*Diaporthe phaseolorum* **)	0	0	0	6	0.22
Dothistroma septosporum (Dorogin) M. Morelet 1968	2	0	3	0	0.18
Epicoccum sp. Link 1816	0	0	1	0	0.036
Eutypella sp. (Nitschke) Sacc. 1875	0	0	2	0	0.071
Flavodon sp. Ryvarden 1973	0	0	1	0	0.036
Fusicladium amoenum (R.F. Castañeda & Dugan) Crous, K. Schub. & U. Braun 2007	1	0	0	0	0.036
cf. *Geomyces* sp. Traaen 1914 (*Geomyces* sp.2**)	0	0	0	1	0.036
Gliocephalotrichum sp. J.J. Ellis & Hesselt. 1962 (*G. bacillisporum***)	2	0	19	25	1.65
Harknessia pseudohawaiiensi Crous & Carnegie 2012	0	3	0	0	0.11
Nemania sp. Gray 1821	1	0	0	0	0.036
N. diffusa (Sowerby) Gray 1821	12	0	0	0	0.43
Neofusicoccum parvum (Pennycook & Samuels) Crous. Slippers & A.J.L. Phillips 2006	25	1	0	0	0.94

Continua

Taxa	Leaf types				Abd
	S1*	S2	S3	S4	
Neophaeomoniella eucalypti Rooney-Lath. & Crous 2015	0	1	1	0	0.071
N. niveniae (Crous) Crous 2015	0	0	0	1	0.036
Neurospora sp. Shear & B.O. Dodge 1927	6	0	0	0	0.22
Nigrospora oryzae (Berk. & Broome) Petch 1924	1	0	0	0	0.036
Pantospora guazumae Cif. 1938	0	0	1	0	0.036
Passalora loranthincola (Petr.) U. Braun 1995	0	7	0	0	0.25
Penicillium citrinum Thom 1910	0	0	1	1	0.071
P. herquei Bainier & Sartory 1912	0	0	0	7	0.25
P. rubens Biourge 1923	0	0	10	0	0.36
Phlebia cf. *floridensis* Nakasone & Burds. 1995	1	0	0	0	0.036
Phyllosticta capitalensis Henn. 1908	46	21	9	7	2.99
Pseudocercospora norchiensis Crous 2007	7	2	2	3	0.5
Pseudoplagiostoma eucalypti Cheew. M.J. Wingf. & Crous 2010	44	7	1	1	1.91
Pseudoplagiostoma sp. Cheew. M.J. Wingf. & Crous 2010	1	0	0	0	0.036
Pseudosydowia eucalypti (Verwoerd & du Plessis) Thambug. & K.D. Hyde 2014	0	41	52	6	3.56
Pseudoteratosphaeria flexuosa (Crous & M.J. Wingf.) Quaedvl. & Crous 2014	0	4	2	0	0.22
P. ohnowa (Crous & M.J. Wingf.) Quaedvl. & Crous 2014 (*Teratosphaeria ohnowa***)	1	0	0	9	0.36
Readeriella eucalypti (Gonz. Frag.) Crous 2006	13	27	24	27	3.27
Satchmopsis brasiliensis B. Sutton & Hodges 1975	12	16	21	2	1.83
Skeletocutis diluta (Rajchenb.) A. David & Rajchenb. 1992	1	0	0	0	0.036
Trichoderma sp. Pers. 1794	0	1	0	5	0.22
Xenogliocladiopsis cypellocarpa L. Lombard & Crous 2015	0	0	0	4	0.14
Xyladictyochaeta lusitanica Hern.-Restr.. R.F. Castañeda & Gené 2017	0	0	0	10	0.36
Xylaria sp.1 Hill ex Schrank 1789 (*Xylaria globosa***)	8	0	3	0	0.4

Continua

Conclusão

Taxa	Leaf types				Abd
	S1*	S2	S3	S4	
Xylaria sp.2 (*Xylaria* sp.3)	1	0	0	0	0.036
FNI 1	0	0	0	1	0.036
FNI 2	0	0	1	0	0.036
FNI 3	0	0	0	2	0.071
FNI 4	0	0	1	0	0.04
FNI 5	0	1	1	0	0.071
FNI 6 (*Microcylindroseptoria eucalypti*)	13	36	97	1,691	66.13
FNI 7 (FNI 1**)	1	0	0	1	0.071
FNI 8	0	1	0	0	0.036
FNI 9	0	0	0	1	0.036
FNI 10	0	0	0	1	0.036
FNI 11	0	0	0	1	0.036
Total	220	191	278	2,089	100

S1 = first foliar stage. S2 = second foliar stage. S3 = third foliar stage. S4 = fourth foliar stage. Abd = Abundance. * Data from Lacerda et al. (2018) was used for comparisons between the endophytic and saprobic communities. FNI= non-identified fungi. (**name mentioned in the table 2 of chapter 1, Lacerda et al. 2018).

Table 2. Diversity metrics describing the fungal communities from different *Eucalyptus microcorys* leaf stages.

Diversity metrics	S1*	S2	S3	S4
Richness	27	20	27	27
Number of isolates	220	191	278	2.089
Dominance (D)	0.117	0.123	0.181	0.665
Shannon (H')	2.546	2.327	2.218	0.848
Chao-1	49	27	36.17	42

S1 = first foliar stage. S2 = second foliar stage. S3 = third foliar stage. S4 = fourth foliar stage. * Data from Lacerda et al. (2018) was used for comparisons between the endophytic and saprobic communities.

6 *Microcylindroseptoria*, A NEW FUNGAL GENUS FOUND IN EUCALYPTUS PLANTATIONS

(Texto elaborado segundo as normas da revista Antonie van Leeuwenhoek).

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Abstract

Eucalyptus plants harbor a great fungal diversity and can be considered a hotspot for the discovery of new species. Herein, we propose a new genus, *Microcylindroseptoria*, and a new species, *M. eucalypti*, found in leaves of *Eucalyptus microcorys* and in a leafcutter ant colony from an eucalyptus plantation in the state of São Paulo, Brazil. Five isolates were identified by using macro and microscopic characters, as well as three molecular markers (ITS, LSU and SSU). To verify the relationships between fungal taxa, we estimated phylogenetic trees by Bayesian inference (BI) and Maximum likelihood (ML) estimation. According to the phylogenetic analyses, the new genus belongs to the family Dothideaceae (Dothideales). The isolates showed close relationship with *Neocylindroseptoria pistaciae* with high Monte Carlo posterior probability (PP= 1). Morevover, the sequence similarity was 99% (SSU) with *Diplodia pyrenophora* (Botryosphaeriaceae), 97% (LSU) and less than 90% (ITS) with *N. pistaciae.* Morphologically, these fungi differ by the presence and shape of the conidiogenous cells and conidia type and size. *Microcylindroseptoria eucalypti* differs mainly from the other asexual genera in Dothideaceae due to the presence of sterile pycinidial conidiomata and for has two types conidia. The discovery of a new fungal genus found in eucalyptus plantations demonstrates the importance of this plant, not only in the world's economy, but also as a source of fungal species diversity.

Keywords: phylogeny, systematic, taxonomy, tropical fungi

Introduction

Brazil has one of the largest diversity of species in the world. It occupies the top rank in megadiversity of vascular plant species, with more than 54% of endemism (Giam et al. 2010). This plant richness allows associations to be established with various organisms like bacteria, nematodes, fungi, viruses, other plants, insects, and animals (Abd-Elgawad and Omer 1995; van Rhijn and Vanderleyden 1995; Armesto et al. 1996; Goodell 2008; Hardoim et al. 2015; Kaiser et al. 2015; Truyens et al. 2014; Goldbach and van Kammen 2018). The study of these symbiotic interactions is essential for understanding the complexity of the ecosystem and discovering of new species.

The plant-fungus symbiosis promotes plant biodiversity (van der Heijden et al. 2016) and it is considered essential to promote ecosystem services. Eucalyptus plants harbor a great fungal diversity and are a cache of new species (Cheewangkoon et al. 2009). Dothideaceae Chevall. was introduced by Chevallier (1826) with *Dothidea* Fr. as the type genus and *D. gibberulosa* (Fr.) Fr. as the type species. This family is characterized by locules embedded in ascostromata without definite perithecia (Thambugala et al. 2014). Several authors have discussed the inclusion and exclusion of taxa within the Dothideaceae (Luttrell 1973; Arx and Müller 1975; Hawksworth et al. 1995; Thambugala et al. 2014). However, we consider here Wijayawardene and collaborators (2018). Currently this group of fungi comprehends approximately 300 species distributed in 17 described genera: *Asteromellopsis* H.E. Hess & E. Müll., *Delphinella* (Sacc.) Kuntze, *Dictyodothis* Theiss. & Syd., *Dothidea* Fr., *Dothiora* Fr., *Endoconidioma* Tsuneda, *Endodothiora* Petr., *Hormonema* Lagerb. & Melin, *Kabatina* R. Schneid. & Arx, *Neocylindroseptoria* Thambug. & K.D. Hyde, *Neophaeocryptopus* Wanas., Camporesi, E.B.G. Jones & K.D. Hyde, *Phaeocryptopus* Naumov, *Plowrightia* Sacc., *Pringsheimia* Schulzer, *Rhizosphaera* L. Mangin & Har., *Stylodothis* Arx & E. Müll. and *Sydowia* Bres (Crous et al. 2004; Wijayawardene et al. 2018). Some of these genera were recently described, such as *Neocylindroseptoria*, that is a monotypic genus, *N. pistaceae* (Quaedvl., Verkley & Crous) Thambug. & K.D. Hyde. This fungus was placed in the ancient genus *Cylindroseptoria* for presenting pycnidial rather than cupulate conidiomata and exhibits genetic synapomorphies with *Cylindroseptoria* (Quaedvlieg et al. 2013; Thambugala et al. 2014). Even though presenting phylogenetic distance and morphological differences, *Neocylindroseptoria* is the closest relative of the proposed genus *Microcylindroseptoria*. The latter differs morphologically from *Neocylindroseptoria* by presenting another type of conidiogenic blastic formation, with conidia arising directly from the hypha. Phylogenetic

analyses using the concatenated molecular markers separate the isolates found in this study as a distinct clade in Dothideaceae. Thus, here we describe one new genus, *Microcylindroseptoria*, based on both morphological and molecular analyses.

Materials and methods

Sample collection and fungi isolation

A total of four isolates were obtained from healthy and litter leaves from three *Eucalyptus microcorys* F. Muell trees (Coordinates: 22°24'52.6"S 47°32'37.0"W; 22°24'53.5"S 47°32'37.3"W; 22°24'53.5"S 47°32'38.9"W) at the Edmundo Navarro de Andrade State Forest, Rio Claro, SP. An additional isolate was obtained from a *Atta sexdens* Linnaeus leafcutter ant colony found at an eucalyptus plantation in the municipality of Botucatu, SP (Coordinate: 22°54'32.3"S 48°18'47.3"W).

After sampling, asepsis of the leaves was carried out according to Crous and Wingfield (1997). Then, fungal isolation was performed by the particle filtration method described by Bills and Polishook (1994) and modified by Lacerda et al. (2018), using two culture media: Dichloran Rose Bengal (Paulus et al. 2003) and Synthetic Nutrient Agar (Nirenberg 1976), both supplemented with 150 μg mL^{-1} of both chloramphenicol and rose Bengal (Table S1). For the isolation of fungi present in the leafcutter ant colony, fragments of fungus garden with approximately 3 mm^3 were removed and transferred to potato dextrose agar (PDA, Acumedia), 2% malt agar (MEA, Acumedia) and synthetic nutrient agar (SNA), all supplemented with 150 μg mL^{-1} of chloramphenicol (Pereira et al. 2016, Table S1). All plates were incubated in the dark at 25 °C during 14 days. Plates were observed daily for fungal growth.

Regardless of the isolation source, all fungi obtained were confirmed as axenic cultures. To do so, we prepared monosporic cultures in water agar and pure cultures were transferred to MEA (Table S1) for further identification.

Morphological characterization

Morphological characters of the isolates were analyzed on PDA, potato carrot agar (PCA, Himedia), corn meal agar (CMA, Acumedia) and oatmeal agar (OA, 15g QUAKER® Fine Flakes Oats, 15g Agar Acumedia, Table S1). Colony diameters and appearance were

recorded. The plates were maintained at 15 °C, 28 °C and 45 °C in darkness for 14 days. For the visualization of internal structures, we prepared sections of 5 μm thick cut using Leica VT1000S Vibratome. In addition, we performed scanning electron microscopy for visualization of other smaller structures. For this, the isolates were transferred to PDA plates and incubated at 25 °C for 3 to 7 days. The cultures were fixed with vapor of osmium tetroxide. After 4 days, mycelium fragments were extracted and transferred to an aluminum holder. Then, the samples were dehydrated in acetone baths with increasing concentrations of 50, 75, 90, 95 and 100%. After dehydration at critical point (Balzers CPD030), the material was immobilized in stubs and metallized by gold sputtering (Balzers SCD050). Finally, the material was examined in a scanning electron microscope (Hitachi TM3000). The reproductive structures were then analyzed using the identification keys (Orton 1924; Barr and Hanlin 1990; Barr 2001; Thambugala et al. 2014).

Molecular identification and phylogenetic analyses

Axenic cultures from each isolate were grown for 7 days on PDA at 25 °C for genomic DNA extraction, according to Lacerda et al. (2018). Fragments of three DNA loci were amplified using the following primers and PCR conditions: LSU (LR0/LR5) and SSU (NS1/NSU) described by Thambugala et al. (2014) and ITS (ITS4/ITS5) according to Schoch et al. (2012). Amplicons were visualized on 1% agarose gel and purified using Wizard® SV Gel and PCR Clean-Up System kit (Promega), according to the manufacturer's instructions. Cycle sequencing reactions were set up using the BigDye™ Terminator v.3.1 Cycle Sequencing Kit (ThermoFisher), following the manufacturer's instructions and applied in an ABI 3500 sequencer. Consensus sequences were assembled in BioEdit v. 7.0.5.3 (Hall 1999). DNA sequences generated in this study were lodged in GenBank: ITS (MH777079–MH777082), LSU (MH777074–MH777078) and SSU (MH777092–MH777096).

Searches for homologous sequences were performed in the NCBI-GenBank database. The alignment of sequences generated in this study along with additional sequences from published Dothideales species was carried out in MAFFT 7 (Katoh et al. 2017), for each marker separately. All sequences used in this study are listed in Table 1. The SSU sequences generated in this study were removed from the phylogenetic analysis, due to the low number of Dothideaceae sequences deposited in GenBank. The alignments were curated in the online version of Gblocks (http://phylogeny.lirmm.fr/phylo_cgi/index.cgi) and then were concatenated in WinClada (Nixon 2002). Bayesian inference (BI) phylogenetic analyses were

performed with the LSU and ITS concatenated datasets in MrBayes v.3.2.1 (Ronquist and Huelsenbeck 2003). The final dataset comprised a total of 555 bp. The best model selected was K80+I+G for both LSU and ITS alignments. This mathematical model of nucleotide substitution was selected based on the Bayesian information criterion, calculated in jModelTest2 (Darriba et al. 2012), with a 95% confidence interval. The first 25% of trees were removed as burn-in and the final consensus tree was inferred using the 50% majority rule with posterior probabilities for each node. The tree was rooted to *Celosporium laricicola* Tsuneda & M.L. Davey (UAMH 11008). In addition, we inferred phylogenetic trees with Maximum Likelihood (ML) using the same mathematical model calculated by jModelTest2. The ML analysis was carried out in MEGA v.5.2 (Tamura et al. 2011). The obtained trees were edited in FigTree v1.3.1 (Rambaut 2012) and then in Adobe® Illustrator® CC (Adobe Systems, San Jose, CA, USA).

Results

Taxonomy

Microcylindroseptoria **Lacerda L.T., A. Rodrigues & Gusmão, gen. nov.,** Figs. 1A–I.

MycoBank: XXXX

Generic description:

Asexual state: Colonies on PDA with hyaline young somatic hyphae, thin-walled, branched, older hyphae melanized, dark, thick-walled, branched, often developing into chlamydospores. Initially colony with yeast-like characteristics and conidia with also yeast-like budding (Fig. D). After approximately 7 days we observed conidiomata in culture. Conidiomata pycnidial, sterile, superficial to semi-immersed, globose to irregular, black, solitary to gregarious, up to 100 µm diam, pycnidial walls of textura angularis, covered with brown hyphae, with or without ostiole, when present central and papillate (Fig. 1E–F). Blastic conidiogenous cells, conidiophores reduced to conidiogenous cells. Conidia slightly irregular. Conidia are hyaline, cylindrical, clavate to irregular, smooth-walled, sometimes with slightly truncate basis, born directly on hypha or in monophialidic, hyaline, smooth-walled conidiogenous cells, aseptate

(Fig. 1G). Chlamydospores like structures were observed in the submerged and superficial hyphae (Fig. 1I). S**exual state:** Not observed.

Etymology: Micro = for presenting conidia smaller than the other genera, cylindro = cylindrical conidia, septoria = septoria-like.

Type species: *Microcylindroseptoria eucalypti* Lacerda L.T., Gusmão & A. Rodrigues

Diagnosis: *Microcylindroseptoria* found as endophytic and saprobic on leaves of *E. microcorys*, characterized by the only genus of the Dothideacea family that has sterile conidioma and two types condia (Table S2).

***Microcylindroseptoria eucalypti* Lacerda L.T., Gusmão & A. Rodrigues, sp. nov.** Figs. 1A–I.

MycoBank: XXXX

Generic description:
Asexual state: Colonies on PDA with hyaline young somatic hyphae, thin-walled, branched, older hyphae melanized, dark, thick-walled, branched, often developing into chlamydospores. Conidia yeast-like, hyaline, smooth, ellipsoidal to irregular, budding common, polar, with 5–10 × 1.5–3 µm. (Fig. D). After approximately 7 days the production of conidiomata occurs. Conidiomata sterile pycnidial, superficial to semi-imersed, globose to irregular, black, solitary to gregarious, covered with brown hyphae, up to 100 µm diam, with or without ostiole, when present central ostiole, papillate (Fig. 1E–F). Blastic conidiogenous cells, conidiophores reduced to conidiogenous cells with 7–9 × 2–4 µm (Fig. 1G). Conidia slightly irregular. Conidia are hyaline, cylindrical, clavate to irregular, smooth-walled, sometimes with slightly truncate basis, born directly on hypha or in monophialidic, hyaline, smooth-walled conidiogenous cells, aseptate, (4) 5–11 (-12) × (1) 1.05–2.7 (-2.87) µm (Fig. 1G). Chlamydospores like structures were observed in the submerged and superficial hyphae (Fig. 1H). S**exual state:** Not observed.

Culture characteristics: Colonies on PDA at 28 °C: initially yeast-like, cream-colored, but after 7 days reached 8.5 mm in diameter, radial, olivaceous-black, black, filamentous,

sometimes wrinkled with white borders (Fig. 1A); on PCA at 28 °C: filamentous, black to brown, circular, lacking aerial mycelium, after 7 days reached 7 mm in diameter (Fig. 1B); on CMA at 28 °C: flat, radial, olivaceous-black in the center, with sepia borders, after 7 days reached 8 mm (Fig. 1C); on PDA at 15 °C: initially yeast-like, cream-colored, but after 7 days filamentous, black to olivaceous-black, irregular to circular, after 7 days reached 4 mm (Fig. S1A); on PCA at 15 °C: circular, filamentous, olivaceous-black, grey to black, olivaceous, with reddish borders, reddish soluble pigment release in the culture medium, after 7 days reached 4 mm (Fig. S1B); on CMA at 15 °C: flat, circular to radial, black to olivaceous-black, with sepia borders, after 7 days reached 4 mm (Fig. S1C). No growth at 45 °C in any culture medium tested.

Etymology: Named after the host genus on which it occurs, *Eucalyptus.*

Habitat and host plant: Found in eucalyptus plantations.

Type: BRAZIL. Rio Claro, São Paulo, 22°24'52.6"S 47°32'37.0"W, litter leaves of *E. microcorys*, April 2015. *L. T. Lacerda* LTL15 (=LESF 1141).

Additional specimens examined: BRAZIL. Botucatu, São Paulo, 22°54'32.3"S 48°18'47.3"W, fungus garden, April 2012. *J. S. Pereira and A. Rodrigues*, JSP 06 B 5.2 (= LESF 1144). BRAZIL. São Paulo, Rio Claro, 22°24'53.5"S 47°32'37.3"W, healthy leaves, October 2015. *L. T. Lacerda*, LTL 114 (= LESF 1142), LTL 5.13 (= LESF 1143). BRAZIL. São Paulo, Rio Claro, 22°24'53.5"S 47°32'38.9"W, leaflitter, April 2016. *L. T. Lacerda*, LTL 6.8 (= LESF 1145).

Comments: According to morphological characters and phylogenetic analyses *Microcylindrospetoria* is a new genus in the family Dothideaceae (Dothideales). The closest relative to *Microcylindroseptoria* is *Neocylindroseptoria*. However, morphologically, *Microcylindroseptoria* differs mainly by presenting sterile pycinidial conidiomata and two types conidia. *Microcylindroseptoria* has conidia yeast-like and cylindrical conidia varying (4) 5–11 (-12) × (1) 1.05–2.7 (-2.87) µm, whereas *N. pistaciae* has conidia size between 11–13 × 2.5–3.5 µm.

Based on our phylogenetic analysis using concatenated ITS and LSU sequences, *Microcylindroseptoria* is closely related to *Neocylindroseptoria.* However, the similarity between sequences was 97% for LSU and less than 90% for ITS. There are no SSU sequences deposited for this species in GenBank, nevertheless the closest sequence to *M. eucalypti* is *Diplodia pyrenophora* (Berk. ex Sacc.) Crous & M.E. Palm, with 99% similarity. More importantly, *Microcylindroseptoria* separates from *Neocylindroseptoria* in different clades with high Monte Carlo posterior probability (PP= 1).

Key proposed for pycnidial and acervular-producing genera in Dothideaceae[abc]

1. conidiogenesis usually synchronous with yeast-like conidia formed basipetally……...……..……*Hormonema*
1. conidiomata often present……..……..…….……..2

2. conidiomata sporodochial or acervular (blastospores presente rarely) ……....*Kabatina*
2. conidiomata pycnidial..……..…….....….……...…3

3. cylindrical conidia……..…….…....……...…..….……4
3. conidia in other shapes.....5

4. one type of conidia, greater than 11 µm lining the inner cavity of the conidiomata……...……...…… *Neocylindroseptoria*
4. two types conidia, conidia with yeast-like and conidia formed by blastic conidiogenous less than or equal to 11 µm produced externally of the conidiomata……..…….……...*Mycrocylindroseptoria*

5. fusiform conidia to falcate with narrowed ends, initially hyaline, becoming pale brown at maturity ……..……..…….…...*Neophaeocryptopus*
5. two-celled or two type conidia, being an endoconidia type..... *Endoconidioma*

[a] based on the original description of the genera

[b] We cannot find the original *Asteromellopsis* and *Pringsheimia*

[c] The other genera of Dothideaceae present the asexual unknown form.

Discussion

We propose a new taxa belonging to the family Dothideaceae. *Microcylindroseptoria* was found in monoculture of eucalyptus and probably is endophytic in leaves of this plant. The discovery of a new genus of fungus in eucalyptus plantations demonstrates the importance of continuing studies on this plant species to find taxonomic fungal novelties.

Our analyses showed that *Neocylindroseptoria* is the closest genus to *Microcylindroseptoria*. However, *Microcylindroseptoria* differs morphologically and genetically from *Neocylindroseptoria*. They resemble by having globose pycnidial, conidiogenous cells monophialidic and cylindrical conidia. However, the new taxon has sterile pycinidial conidiomata and two types conidia. Furthermore, *Mycocylindroseptoria* separates from *Neocylindroseptoria* and formed a strong clade in Dothideaceae with with high Monte Carlo posterior probability (PP= 1).

Eucalyptus is a hotspot for the discovery of new microbial species (Cheewangkoon et al. 2009). Recently, more than 30 fungal species belonging to the genus *Calonectria* were discovered from *Eucalyptus* species (Lombard et al. 2010; Chen et al. 2011; Crous et al. 2012, 2013; Alfenas et al. 2015; Lombard et al. 2015, 2016; Li et al. 2017; Marin-Felix et al. 2017). Crous et al. (2017) have also discovered several new genera and species associated with eucalyptus, demonstrating the importance of this plant in the discovery of new fungal species. *Dothidea eucalypti* Crous is the most recently described species in the order Dothideales associated with eucalyptus (Crous et al. 2017). However, it differs from *Microcylindroseptoria* by the morphology of conidia and that of the conidiogenous cells. Thus, on the basis of phylogenetic and taxonomic data, *M. eucalypti* represent a new taxon in Dothideaceae.

Regarding the isolation of *M. eucalypti* from ant colonies, this event sheds some lights in the ecology of the fungus. Leafcutter ant species in the genus *Atta* have the habit of foraging on fresh leaves to nourish their mutualistic fungus, *Leucoagaricus gongylophorus* (Möller) Singer (Silva et al. 2006). Thus, we assume that *Microcylindrosepetoria* probably was present in eucalyptus leaves, which were foraged by ant workers and transported to the colony.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1. Taxa used in the phylogenetic analysis and their corresponding culture collection and NCBI-GenBank accession numbers.

Taxa	Strains	GenBank accessions	
		LSU	ITS
Aureobasidium leucospermi	CPC 15180	JN712555	JN712489
Aureobasidium leucospermi	CPC 15081	JN712553	JN712487
Aureobasidium mangrovei	IBRC-M-30265	KY089084	KY089085
Aureobasidium mangrovei	IBRC-M-30266	KY089086	KY089087
Aureobasidium iranianum	CCTU 268	NG057049	NR_137598
Aureobasidium proteae	CPC 2824	JN712557	JN712491
Aureobasidium proteae	CPC 2825	JN712558	JN712492
Aureobasidium pullulans	CBS 584.75	DQ470956	FJ150906
Aureobasidium pullulans	MFLUCC 14-0288	KM461701	KM388542
Aureobasidium thailandense	NRRL 58543	JX462675	JX462675
Aureobasidium thailandense	NRRL 58539	JX462674	JX462674
Celosporium larixicola	UAMH 11008	FJ997288	FJ997287
Delphinella strobiligena	CBS 735.71	DQ470977	
Dothidea berberidis	CBS 186.58	EU167601	EU167601
Dothidea insculpta	CBS 189.58	DQ247802	AF027764
Dothidea insculpta	MFUCC 13−0686	KM388551	KM388543
Dothidea hippophaeos	CBS 188.58	DQ678048	KY929139
Dothidea muelleri	CBS 191.58	EU167593	EU167593
Dothidea ribesia	MFLUCC 13−0670	KM388553	KM388545
Dothidea sambuci	DAOM 231303	AY544681	AY883094
Dothidella periclymeni	178096	FJ215702	
Dothiora cannabinae	CBS 737.71	DQ470984	AJ244243
Dothiora ceratoniae	CBS 477.69	KF251151	KF251151
Dothiora elliptica	CBS 736.71	GU301811	
Endoconidioma populi	UAMH 10902	HM185488	HM185487
Elsinoe phaseoli	CBS 165.31	DQ678095	KX887263

Continua

Conclusão

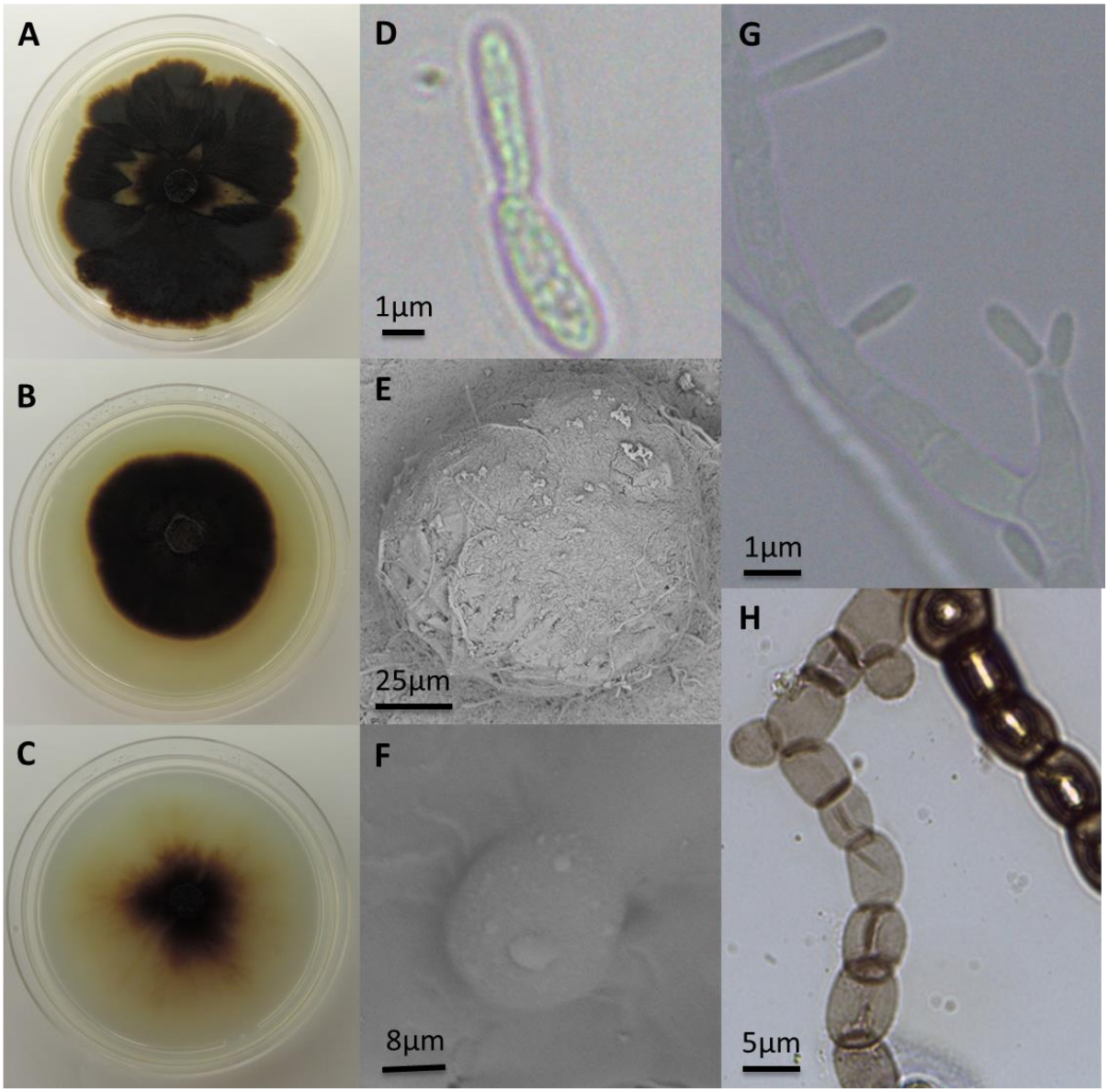
Taxa	**Strains**	**GenBank accessions**	
		LSU	**ITS**
Elsinoe veneta	CBS 150.27	DQ767658	KX887282
Hormonema viticola	L9D-11	KF201297	KF201300
Kabatiella caulivora	CBS 242.64	EU167576	EU167576
Kabatiella microsticta	CBS 114.64	FJ150940	FJ150873
Neocylindroseptoria pistaciae	CBS 471.69	KF251656	KF251152
Microcylindroseptoria eucalypti	LESF1141^{T}	MH777080	MH777077
Microcylindroseptoria eucalypti	LESF1142	MH777079	MH777076
Microcylindroseptoria eucalypti	LESF1143	MH777082	MH777075
Microcylindroseptoria eucalypti	LESF1144	KR093869	MH777078
Microcylindroseptoria eucalypti	LESF1145	MH777081	MH777074
Neophaeocryptopus cytisi	MFLUCC:14-0970	NG_059643	NR_148094
Neophaeocryptopus spartii	MFLU 15.3470	MF443254	MF443251
Phaeocryptopus nudus	CBS 268.37	GU301856	EU700371
Pringsheimia smilacis	CBS 873.71	FJ150970	AJ244257
Pseudosydowia eucalypti	CPC:14028	GQ303327	GQ303296
Pseudosydowia eucalypti	CPC:14927	GQ303328	GQ303297
Rhizosphaera kalkhoffii	ATCC 26605	EF114706	
Rhizosphaera oudemansii	184813	EF114707	
Rhizosphaera pini	64367	EF114708	
Selenophoma australiensis	CBS 124776	GQ303324	GQ303293
Selenophoma linicola	CBS 468.48	EU754212	
Stylodothis puccinioides	CBS 193.58	AY004342	
Sydowia polyspora	CBS 116.29	DQ678058	

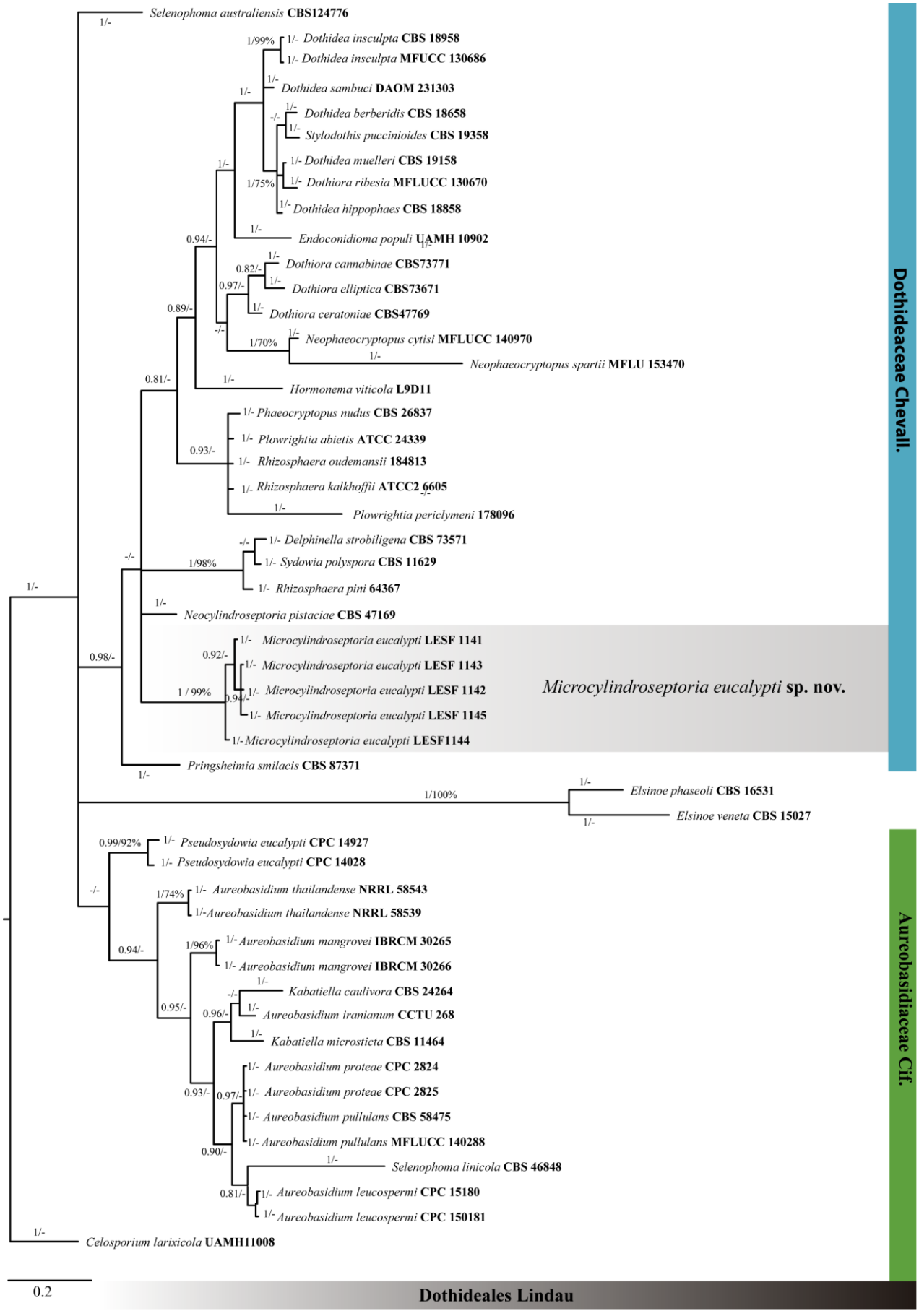
^{T} Type species.

Figures legends

Fig. 1. *Microcylindroseptoria eucalypti*. **A–C.** Culture characteristics at 28 °C. **A.** On potato-dextrose agar (PDA). **B.** On potato-carrot agar (PCA). **C.** On corn meal agar (CMA). **D.** Conidia with some yeast-like budding occurring. **E.** Conidiomata globose pycnidial. **F.** Papillate ostiole. **G.** Blastic conidiogenous monophialidic and conidia**. H**. Chlamydospore-like structures.

Fig. 2. Phylogenetic tree based on LSU and ITS showing the relationship of *Mycrocylindroseptoria eucalypti* with allied taxa in Dothideales. The tree was estimated using Bayesian Inference (BI) and Maximum Likelihood (ML) algorithms. Numbers on branches are Bayesian posterior probabilities and ML bootstrap values, respectively. Only values higher than 0.75 for BI and 70% for ML are shown. The tree was rooted with *Celosporium laricicola* (UAMH 11008) as outgroup.


A
B
C
D
1µm
E
25µm
F
8µm
G
1µm
H
5µm


Selenophoma australiensis CBS124776
Dothidea insculpta CBS 18958
Dothidea insculpta MFUCC 130686
Dothidea sambuci DAOM 231303
Dothidea berberidis CBS 18658
Stylodothis puccinioides CBS 19358
Dothidea muelleri CBS 19158
Dothiora ribesia MFLUCC 130670
Dothidea hippophaes CBS 18858
Endoconidioma populi UAMH 10902
Dothiora cannabinae CBS73771
Dothiora elliptica CBS73671
Dothiora ceratoniae CBS47769
Neophaeocryptopus cytisi MFLUCC 140970
Neophaeocryptopus spartii MFLU 153470
Hormonema viticola L9D11
Phaeocryptopus nudus CBS 26837
Plowrightia abietis ATCC 24339
Rhizosphaera oudemansii 184813
Rhizosphaera kalkhoffii ATCC2 6605
Plowrightia periclymeni 178096
Delphinella strobiligena CBS 73571
Sydowia polyspora CBS 11629
Rhizosphaera pini 64367
Neocylindroseptoria pistaciae CBS 47169
Microcylindroseptoria eucalypti LESF 1141
Microcylindroseptoria eucalypti LESF 1143
Microcylindroseptoria eucalypti LESF 1142
Microcylindroseptoria eucalypti LESF 1145
Microcylindroseptoria eucalypti LESF1144
Pringsheimia smilacis CBS 87371
Elsinoe phaseoli CBS 16531
Elsinoe veneta CBS 15027
Pseudosydowia eucalypti CPC 14927
Pseudosydowia eucalypti CPC 14028
Aureobasidium thailandense NRRL 58543
Aureobasidium thailandense NRRL 58539
Aureobasidium mangrovei IBRCM 30265
Aureobasidium mangrovei IBRCM 30266
Kabatiella caulivora CBS 24264
Aureobasidium iranianum CCTU 268
Kabatiella microsticta CBS 11464
Aureobasidium proteae CPC 2824
Aureobasidium proteae CPC 2825
Aureobasidium pullulans CBS 58475
Aureobasidium pullulans MFLUCC 140288
Selenophoma linicola CBS 46848
Aureobasidium leucospermi CPC 15180
Aureobasidium leucospermi CPC 150181
Celosporium larixicola UAMH11008
Microcylindroseptoria eucalypti sp. nov.
Dothideaceae Chevall.
Aureobasidiaceae Cif.
0.2
Dothideales Lindau

7 CONSIDERAÇÕES

- Em conjunto, os métodos de fragmentos foliares e de filtração de partículas recuperaram um maior número de taxa endofíticos. Tendo em vista os elevados índices de diversidade que cada técnica recuperou, além da alta dissimilaridade observada entre elas, sugerimos a utilização como métodos complementares no estudo das comunidades de fungos endofíticos.

- *Eucalytpus microcorys* abriga uma variedade de espécies fúngicas, sendo um *hotspot* para a descoberta de novos taxa. Além da descoberta de um novo gênero, constamos a presença de vários fungos, os quais não foi possível a identificação, mesmo avaliando as características morfológicas e diferentes marcadores moleculares. Tais fungos podem ser novos taxa.

- A composição da comunidade fúngica presente nas folhas de *E. microcorys* varia conforme a maturidade foliar. Tal mudança provavelmente ocorre devido à degradação da biomassa vegetal e liberação de macro e micronutrientes durante esse processo.

8 MATERIAL SUPLEMENTAR

Capítulo 1 - DIVERSITY OF ENDOPHYTIC FUNGI IN *Eucalyptus microcorys* ASSESSED BY COMPLEMENTARY ISOLATION METHODS

Table S1. Taxa identification by ITS barcodes.

Fungi*	**Molecular identification**						
		Genbank			**Mycobank**		
	bp	**%**	**Accession**	**Taxa**	**%**	**Accession**	**Taxa**
Annulohypoxylon moriforme	947	99	KX376321	*A. moriforme*	99	KU683751	*A.moriforme* CBS 123579
Fusicladium amoenum	546	90	EU035425	*F. amoenum*	100	AF393682	*F. amoenum* ATCC 200947
Apoharknessia insueta	581	100	JQ706083	*A. insueta*	100	JQ706083	*A. insueta* CPC 1451
Calonectria sp.	529	100	GQ280557	*Calonectria scoparia*	100		*C. pseudoscoporia* CBS125255
Castanediella eucalypticola	553	100	NR145254	*C. eucalypticola*	100	NR145254	*C. eucalypticola* CPC 26539
Cladosporium cladosporioides	531	99	KX664415	*C. cladosporioides*	79		*P. neopyrolae* CBS 134750
Ceratobasidiaceae	690	100	AB449172	Ceratobasidiaceae	100	AB449186	Ceratobasidiaceae
Colletotrichum sp.	493	100	KU529859	*C. gloeosporioides*	100	JN050242	*C. gigasporum*
Colletotrichum sp.	575	100	MG693784	*C. karstii*	100	KC293576	*C. citricola*
Colletotrichum sp.	556	100	KX620324	*C. siamense*	100		*C. ti* ICMP4832
Cytospora austromontana	596	97	JN693510	*C. austromontana*	84		*Phaeocytostroma ambiguum* CPC 17079
Cytospora eucalypticola	582	97	KR093883	*C. eucalypticola*	95		*C. eucalypticola* CBS 116851
Cytospora variostromatica	592	99	KR093848	*C. variostromatica*	97	NR_136967	*C. variostromatica* CBS 116858
Daldinia sp. **	557	97	JQ761035	*Daldinia* sp.	96	JQ761035	Daldinia sp.
Daldinia sp. **	565	97	JN198510	*Daldinia* sp.	97	JN198510	*Daldinia* sp.
Diaporthe phaseolorum	560	99	HM855215	*D. phaseolorum*	99	KX020564	*D. phaseolorum*
Dothistroma sp.	451	98	KR995128	*D. septosporum*	98	AY808304	*D. pini* CMW 14820
Epicoccum sp.	527	100	KX928749	*Epicoccum* sp.	100	KR012895	*E. nigrum* CIAT536
Geomyces sp.	531	99	KR093875	*Geomyces* sp.	99	KR093875	*Geomyces* sp.
Geomyces sp.	527	97	KR093875	*Geomyces* sp.	---	---------	----------
Gliocephalotrichum bacillisporum	536	99	KF513251	*G. bacillisporum*	99	KF513251	*G. bacillisporum* CBS 25091
Hypoxylon sp. **	542	97	JQ009321	*H. vinosopulvinatum*	70		*Colletotrichum parsonsiae* CBS 128525
*Hypoxylon monticulosum***	563	99	KU604572	*H. monticulosum*	98	KU604572	*H. monticulosum* STMA 16000
*Marssonina californica**/***	628	100	MF663546	*Mytilinidion californicum*	88	FR682335	Dothideomycetes
Nemania sp.**	534	99	KU747844	*Xylariaceae* sp.	99	KF435438	*Nemania* sp.
*Nemania bipapillata***	554	99	C	*N. bipapillata*	100	AJ390429	*N. bipapillata*
*Nemania diffusa***	561	99	MF185340	*N. diffusa*	100	KP133219	*N. diffusa*
Neophaeomoniella eucalypti	593	99	NR138001	*N. eucalypti*	99	KR476749	*N. eucalypti* CPC 25161
Neofusicoccum sp.	655	96	NR_137026	*Neofusicoccum* sp.	---	--------	----------
Neofusicoccum parvum	562	99	JQ619650	*N. parvum*	99	FJ790823	*N. parvum*
Neurospora discreta	560	99	GU327632	*N. discreta*	99	GU327632	*N. discreta* ATCC MYA-4616
Nigrospora oryzae	540	99	KT966518	*N. oryzae*	99	KT966518	*N. oryzae*
Pantospora guazumae	516	99	JN190956	*P. guazumae*	99	NR119971	*P. guazumae* CBS 130299
Phlebia floridensis	628	99	KP135385	*P. floridensis*	--	----------	----------
Phyllosticta capitalensis	621	100	KU204436	*P. capitalensis*	100	JQ743587	*P. capitalensis* CBS 123373

Continua

Conclusão

Fungi*	Molecular identification						
		Genbank			Mycobank		
	bp	%	Accession	Taxa	%	Accession	Taxa
Preussia minimoides	519	99	KF55765	*P. minimoides*	86		*Sporormiella minima* CBS 52450
Pseudocercospora norchiensis	521	99	EF394859	*P. norchiensis*	100	JX901785	*P. norchiensis* CBS 120738
Pseudoplagiostoma eucalypti	632	100	AB978371	*P. eucalypti*	100	NR120156	*P. eucalypti* CBS 132529
Readeriella eucalypti	526	100	GQ852781	*R. eucalypti*	100		*R. eucalypti* CBS120079
Satchmopsis brasiliensis	542	100	DQ195784	*S. brasiliensis*	100	DQ195784	*S. brasiliensis* CBS 42093
Skeletocutis diluta	631	100	JF692198	*S. diluta*	100	JF692197	*S. diluta*
Teratosphaeria ohnowa	530	99	EF394845	*T. ohnowa*	84		*Septoria malagutii* CBS 106.80
Xylaria apiculata.	565	100	KY031972	*X. apiculata*	100	KY031972	*X. apiculata*
Xylaria berteri.	530	99	MF185353	*X. berteri*	99	GU324749	*X. berteri*
Xylaria sp.	590	99	JX624265	*Xylaria* sp.	99	JX624265	*Xylaria* sp.
Xylaria sp.	538	99	EU009989	*Xylaria* sp.	92	JQ814313	*X. multiplex*
Xylaria sp.	568	99	JN418794	*Xylaria* sp.	99	JQ760675	Xylariales sp.
Xylaria sp.	563	100	FJ799949	*Xylaria* sp.	100	FJ799949	*Xylaria* sp.
Xylaria sp.	578	99	KF025951	*Xylaria* sp.	86		*Chaetoconis polygoni* CBS 405.95
Ascomycota(FNI)	576	98	KR093869	Ascomycota	98	KR093869	Ascomycota
Ascomycota (FNI)	527	90	KU747723	*Physalospora* sp.	92	KX988295	*Obolarina* sp.
Basidiomycota (FNI)	675	98	KT585731	Basidiomycota	98	JF449872	Agaricomycetes

* The final identification was made based on the morphological and molecular identification;
** Taxa identified using other markers than ITS;
*** Current name;
--Similarity under 70%.

Capítulo 2 - FUNGAL COMMUNITY FROM DIFFERENT *Eucalyptus microcorys* LEAF STAGES

Table S1. Primers and amplification parameters used in this study.

Locus	Primer	Primer sequence	Primer reference	PCR parameters
ITS	ITS5 (F)	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)	94 °C/3min, 35 cycles of 94 °C/1min, 55 °C/1min, 72 °C/2min (Schoch et al. 2012)
	ITS4 (R)	TCCTCCGCTTATTGATATGC		
β-tubulin	bt2a (F)	GGTAACCAAATCGGTGCTGCTTTC	Glass and Donaldson (1995)	5 cycles of 94 °C/1min, 68 °C/90s, 72 ºC/2min, with a decrease in annealing temperature of 1 °C /cycle. 25 cycles of 94 °C/1min, 64 °C/90s, 72 °C/2 min (Wang et al. 2004)
	bt2b (R)	ACCCTCAGTGTAGTGACCCTTGGC		
LSU	LR0 (F)	ACCCGC TGAACTTAA GC	Vilgalys and Hester (1990)	94 °C/4 min, 35 cycles of 94 °C/45s, 56 °C/45s, 72 °C/1min (Thambugala et al. 2014)
	LR5 (R)	TCCTGAGGGAAACTT CG		
SSU	NS1 (F)	GTAGTCATATGCTTGTCTC	White et al. (1990)	94 °C/4 min, 35 cycles of 94 °C/ 45s, 56 °C/45s, 72 °C/1min (Thambugala et al. 2014)
	NS4 (R)	CTTCCGTCAATTCCTTTAAG		
Actin	ACT512F	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn (1999)	95 °C/3min, 61 °C/90s, 72 °C/2min, 4 cycles of 95 °C/1min, 61 °C/90s, 72 °C/2min, with a decrease in annealing temperature of 1 °C/cycle. 25 cycles of 95 °C/1min, 57 °C/90s, 72 °C/2min (This study)
	ACT783R	TAGGAGTCCTCCTGGCCCAT		

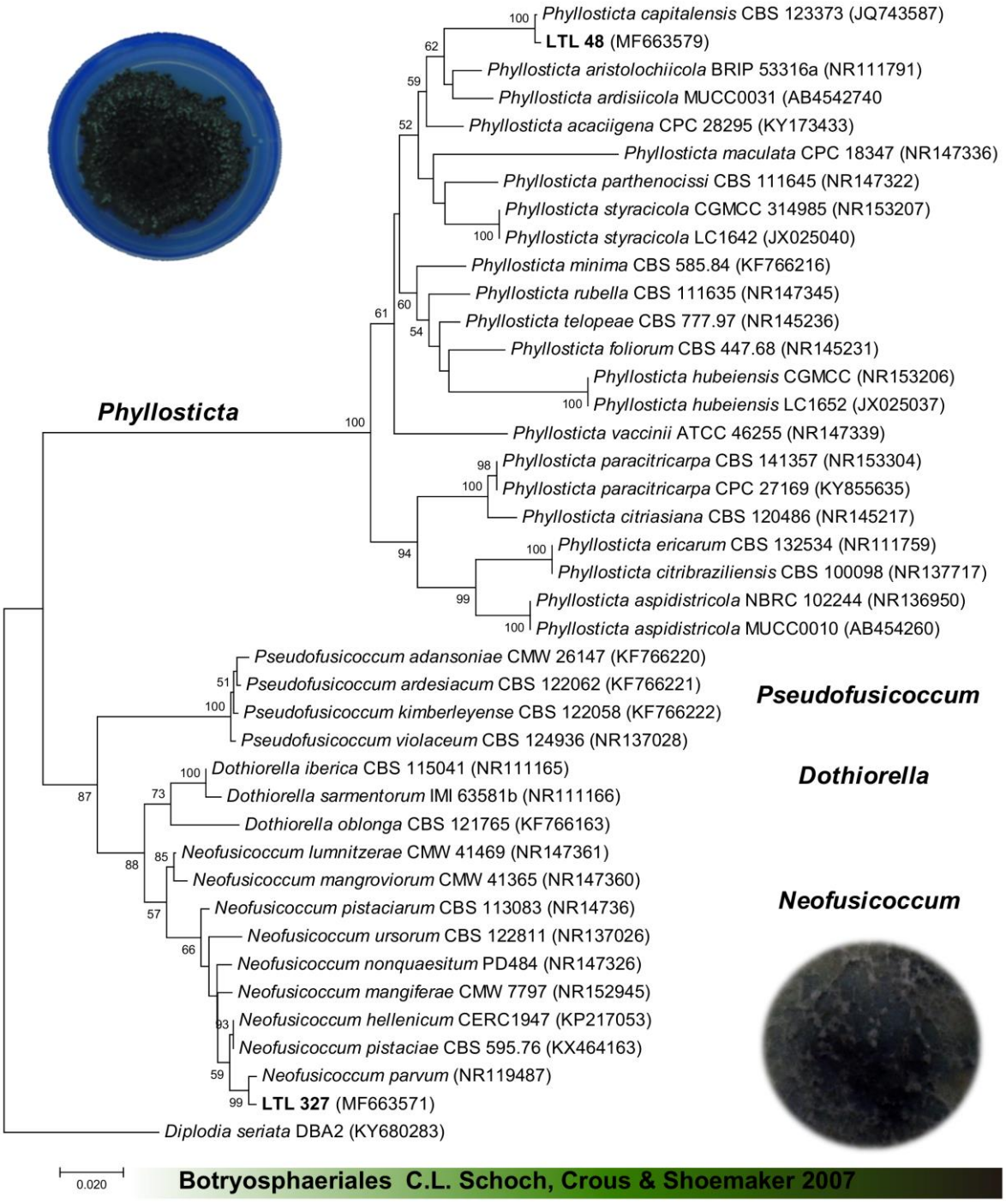


Figure S1. Phylogeny of fungi in the order Botryosphaeriales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Diplodia seriata* De Not. 1845.

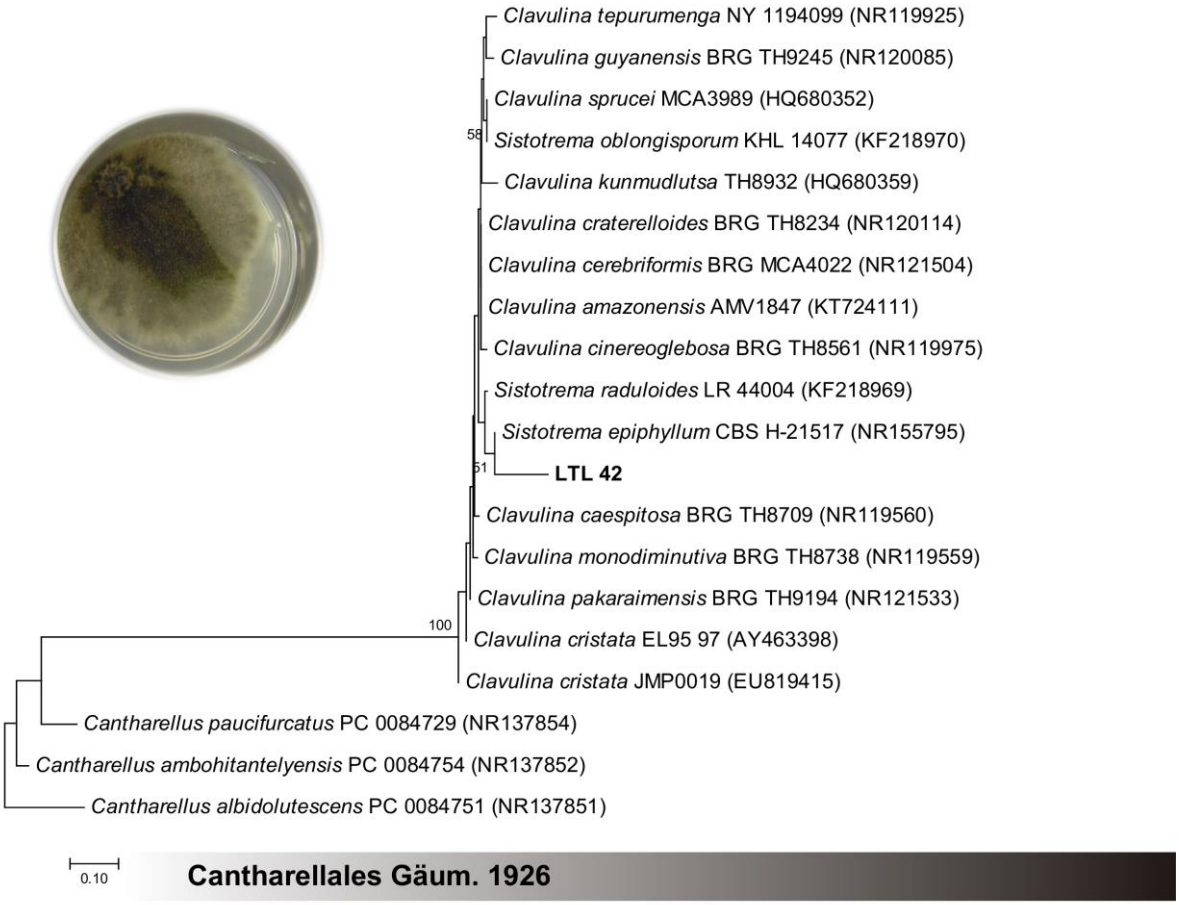


Figure S2. Phylogeny of fungi in the order Cantharellales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Cantharellus albidolutescens* Buyck, Eyssart. & V. Hofst. 2014.

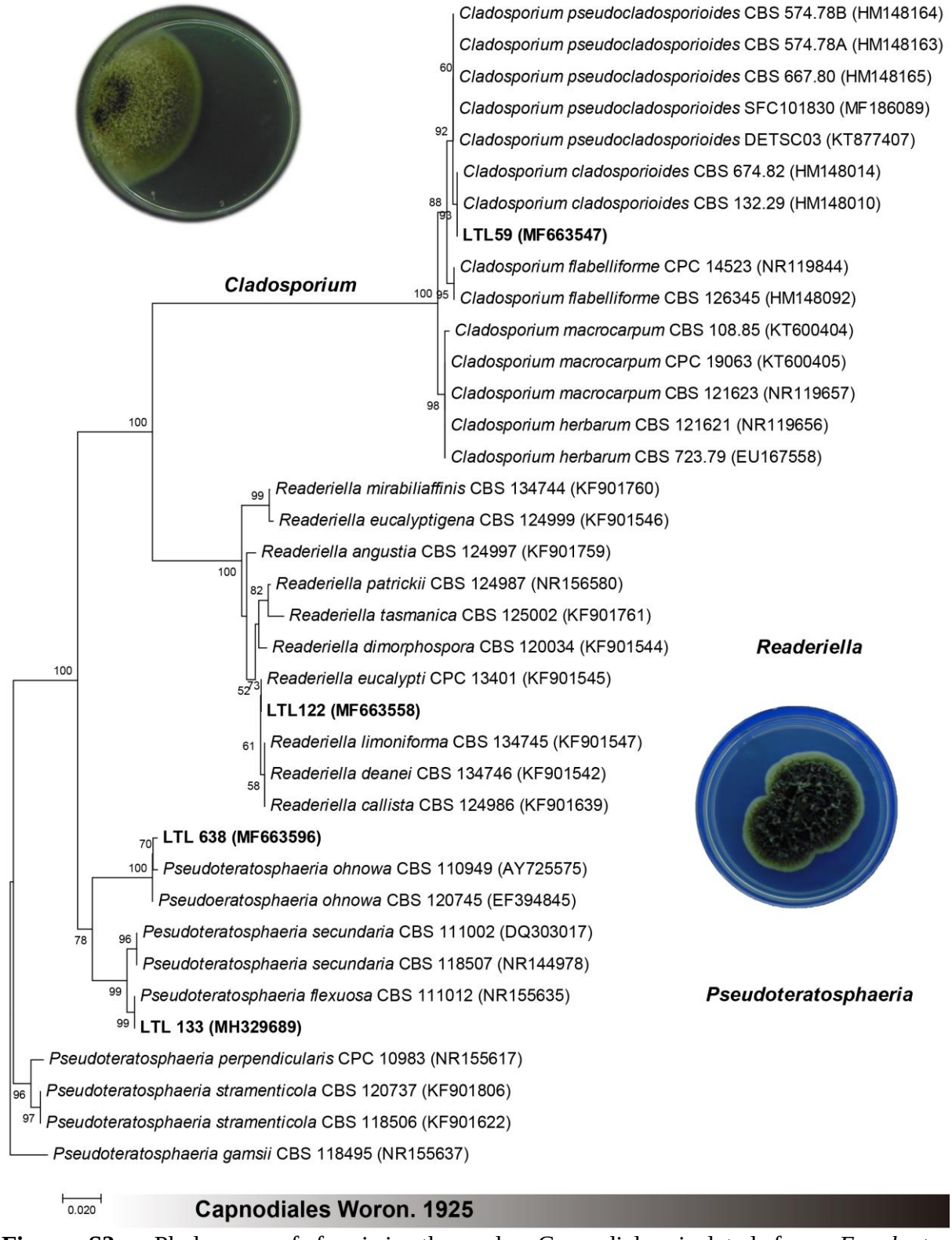


Figure S3. Phylogeny of fungi in the order Capnodiales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Pseudoteratosphaeria gamsii (Crous)* Quaedvl. & Crous 2014.

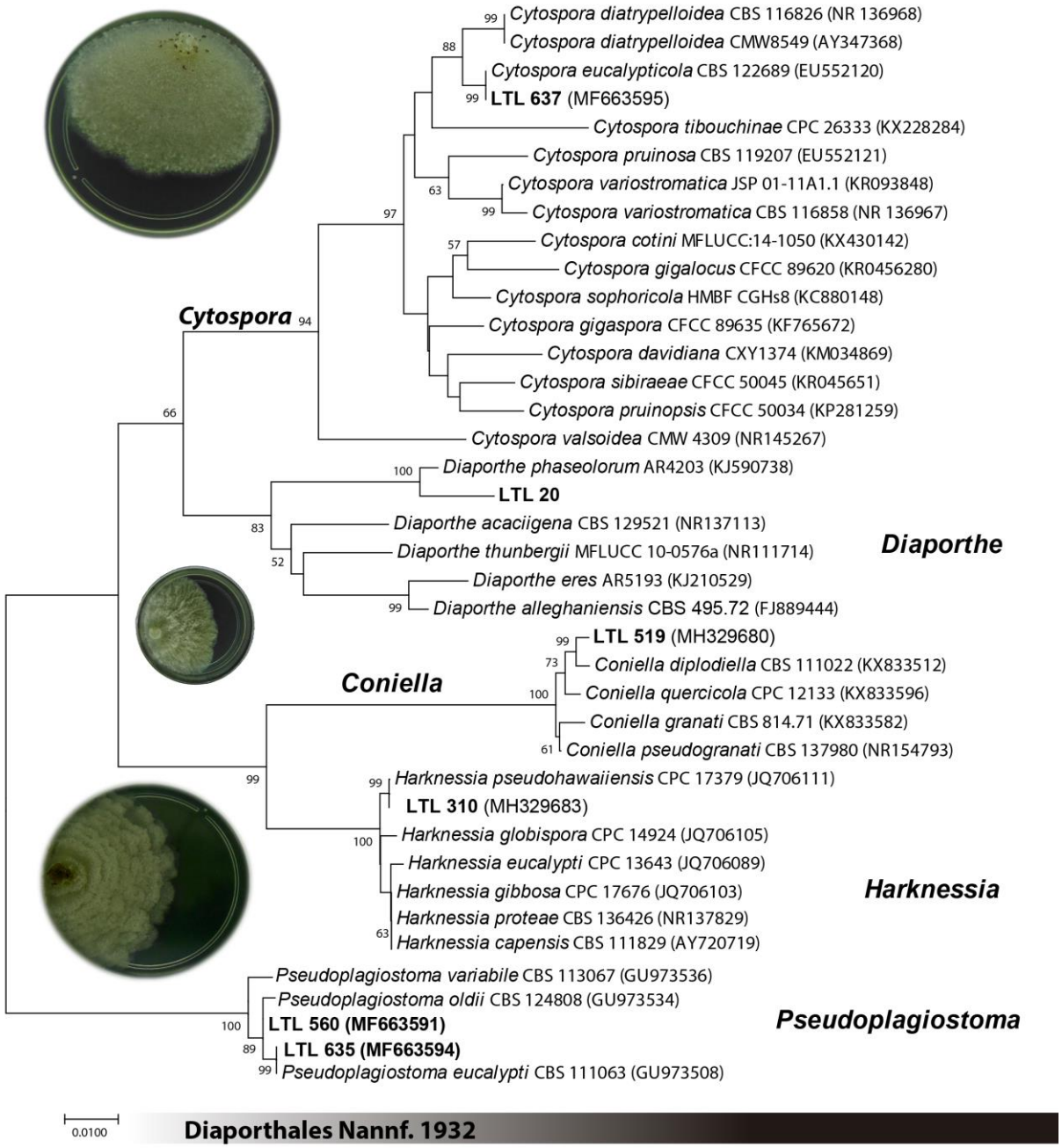


Figure S4. Phylogeny of fungi in the order Diaporthales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Pseudoplagiostoma* Cheew., M.J. Wingf. & Crous 2010.

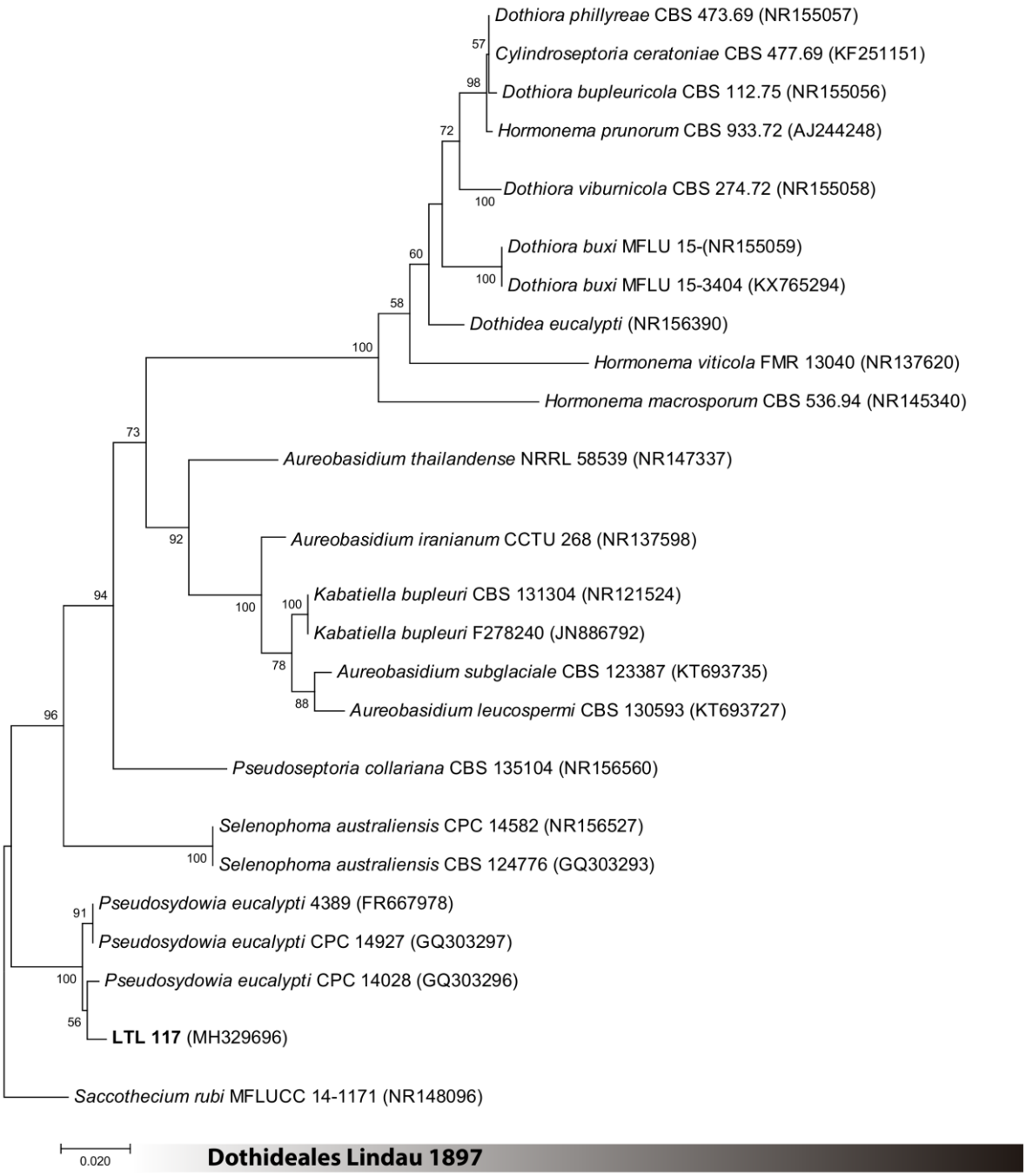


Figure S5. Phylogeny of fungi in the order Dothideales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Saccothecium rubi* Jayasiri, Wanas., Camporesi & K.D. Hyde 2016.

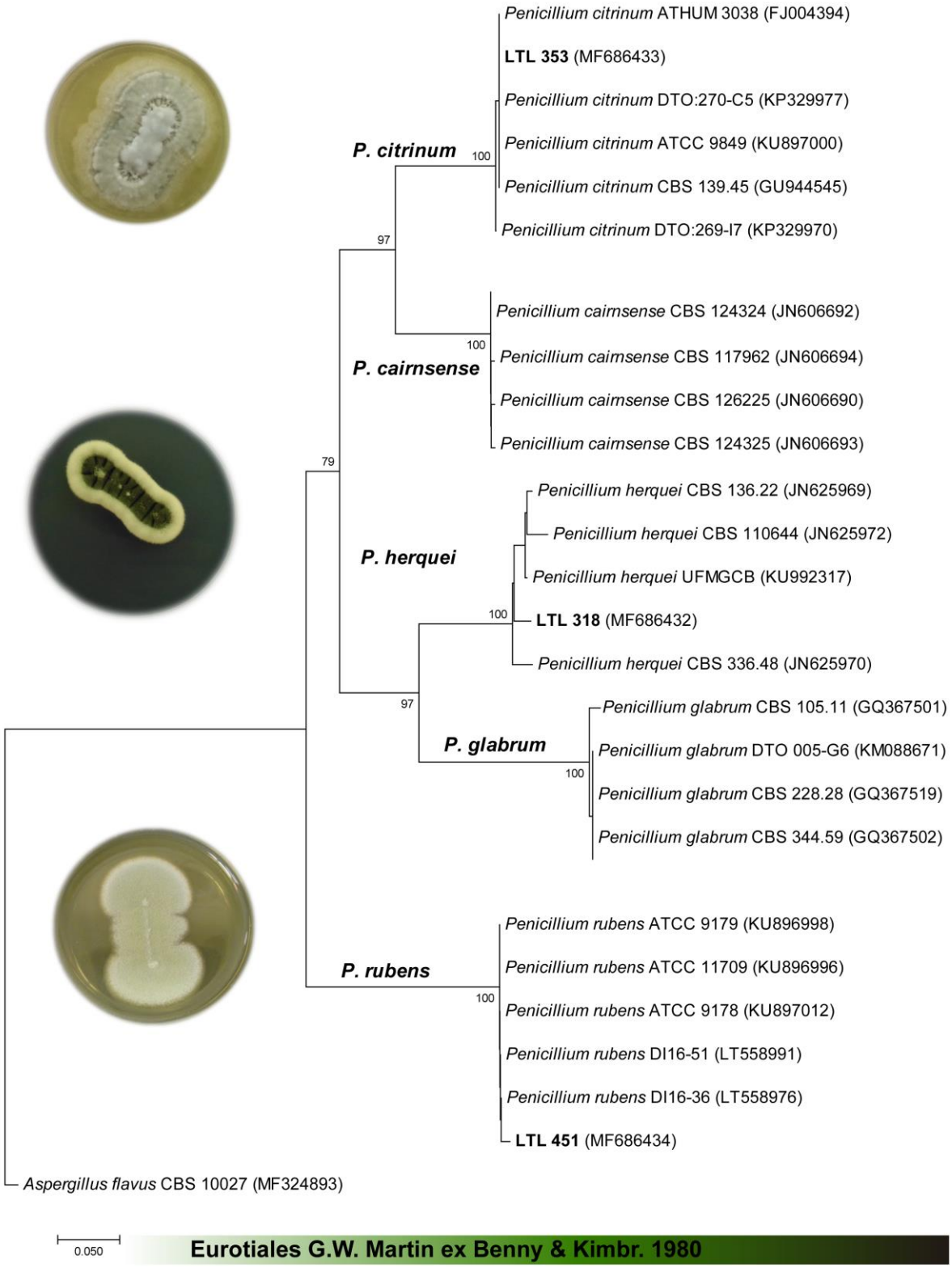


Figure S6. Phylogeny of fungi in the order Eurotiales isolated from *Eucalyptus microcorys*. The tree is based on Btub sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Aspergillus flavus* Link 1809.

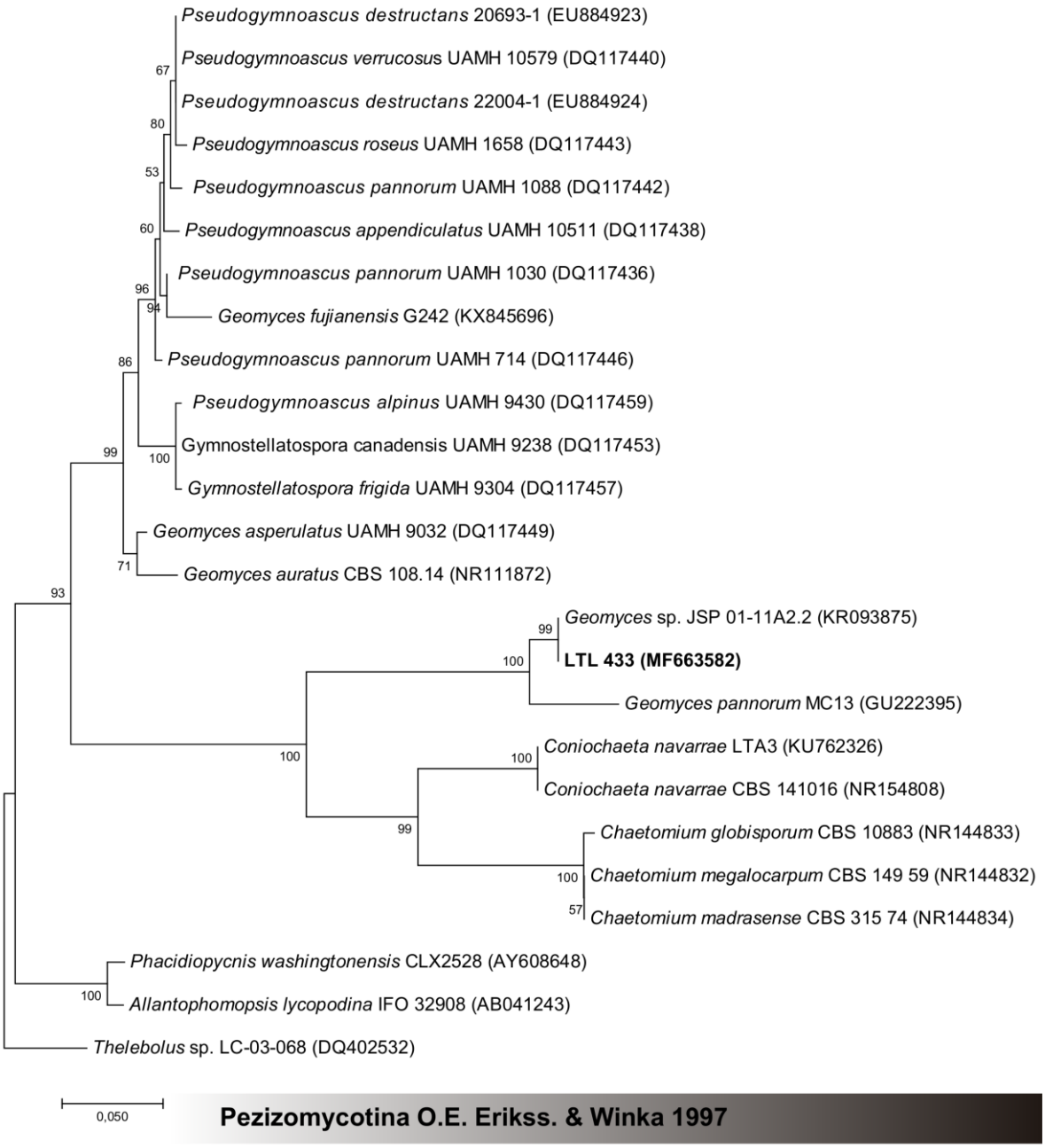


Figure S7. Phylogeny of fungi in the *Geomyces* isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. *Thelebolus* Tode 1790 was used as outgroup.

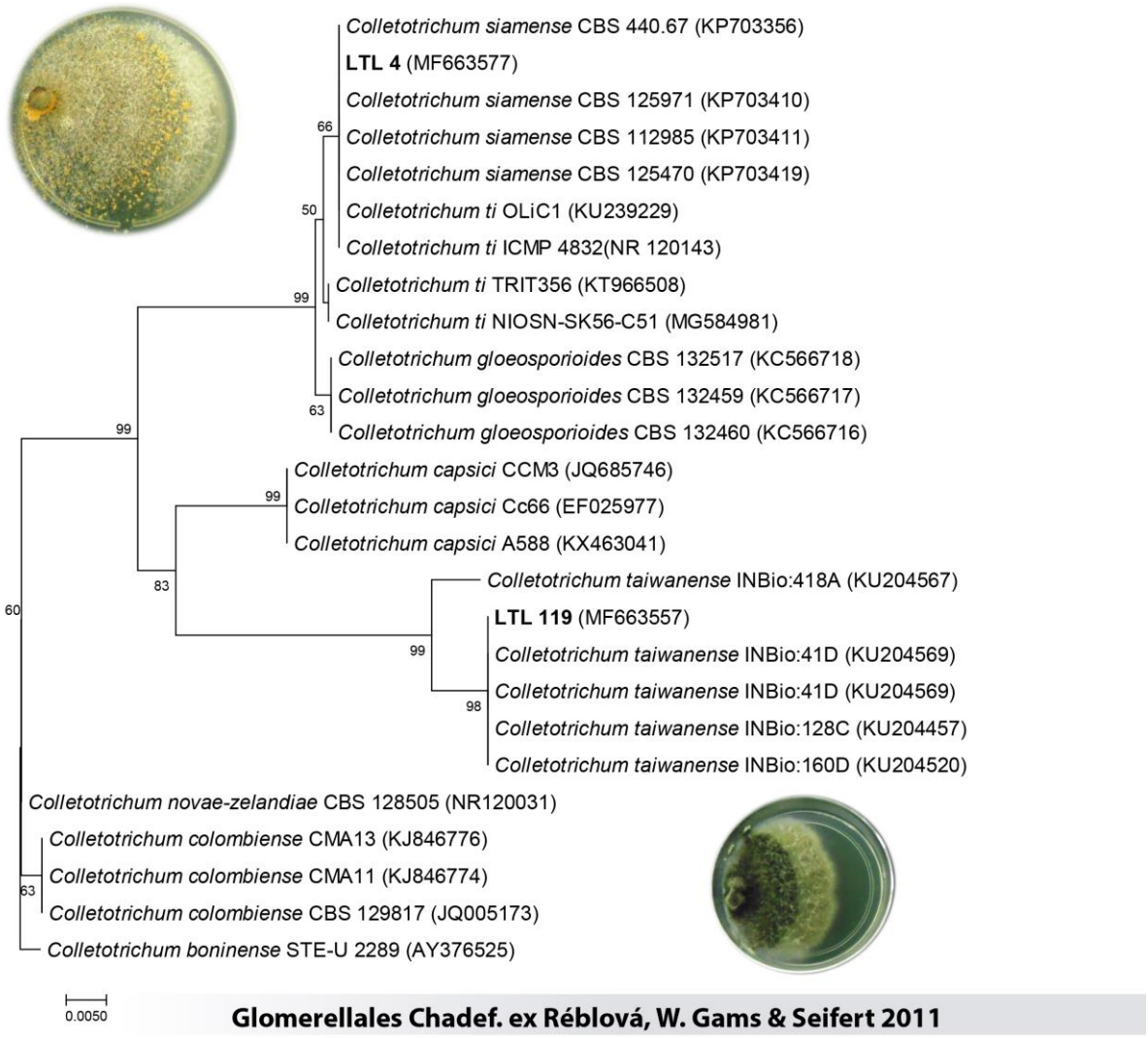


Figure S8. Phylogeny of fungi in the order Glomerellales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Colletotrichum boninense* Moriwaki, Toy. Sato & Tsukib. 2003.

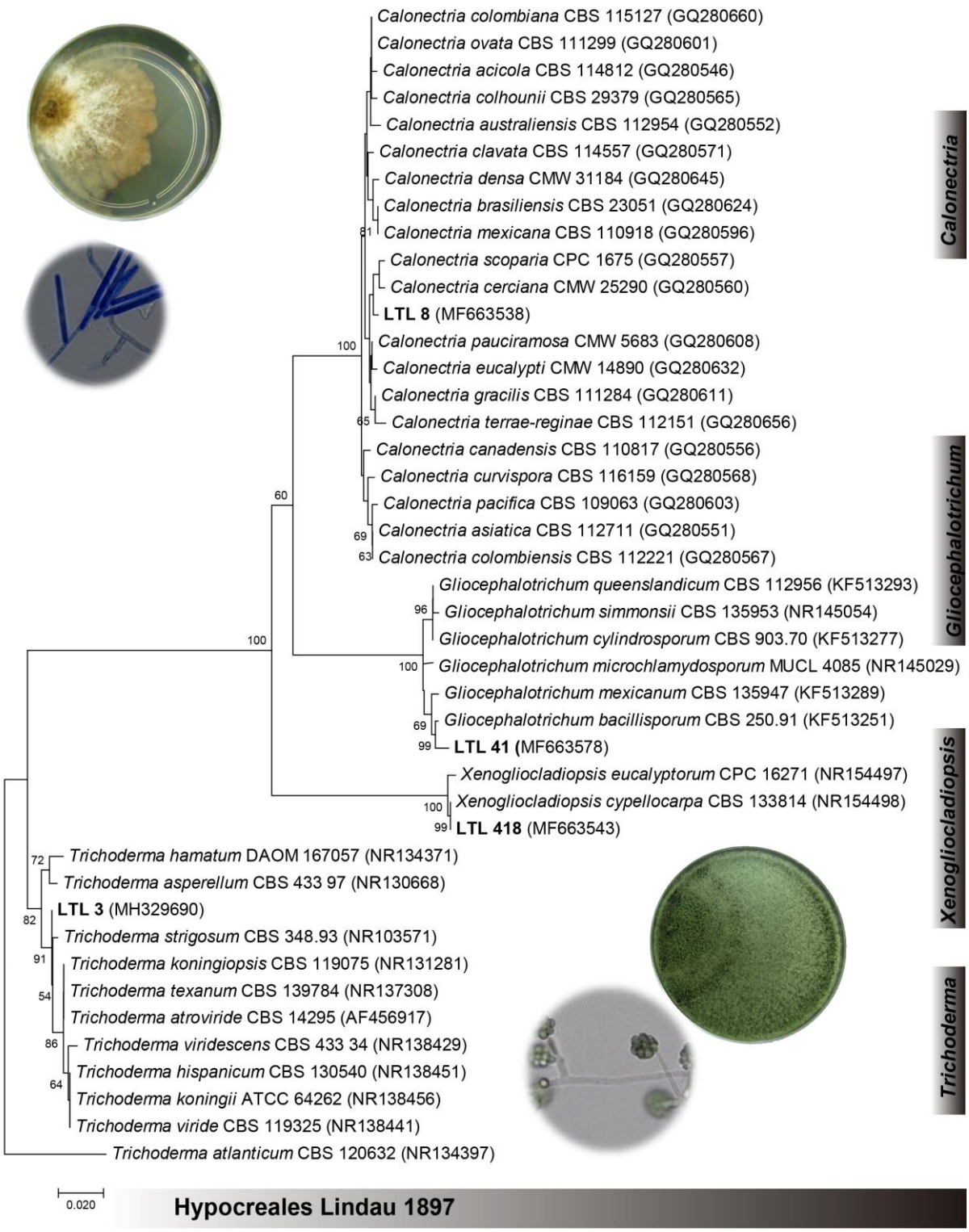


Figure S9. Phylogeny of fungi in the order Hypocreales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Trichoderma atlanticum* Jaklitsch 2011.

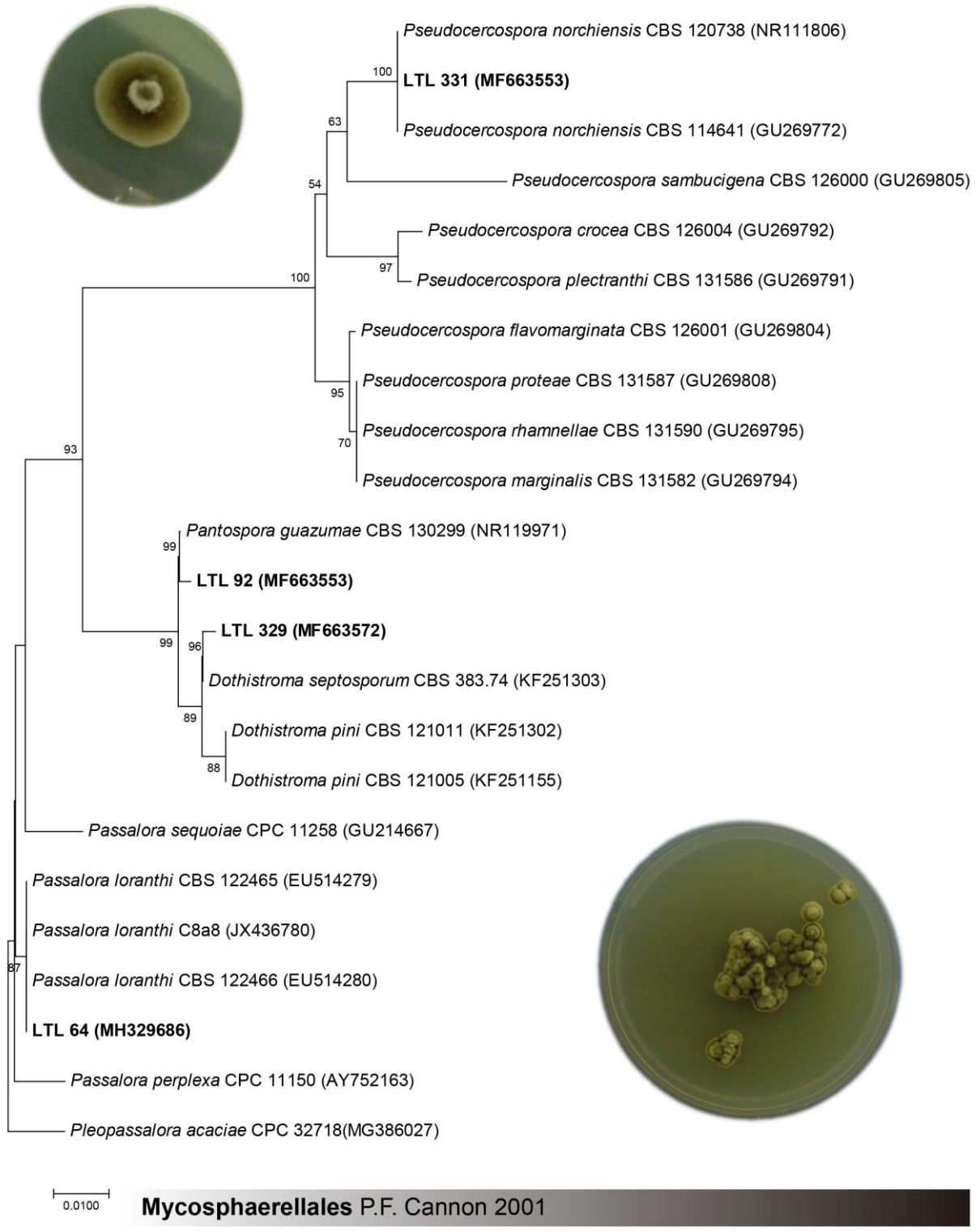


Figure S10. Phylogeny of fungi in the order Mycosphaerellales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Pleopassalora acaciae* Crous & Jacq. Edwards 2017.

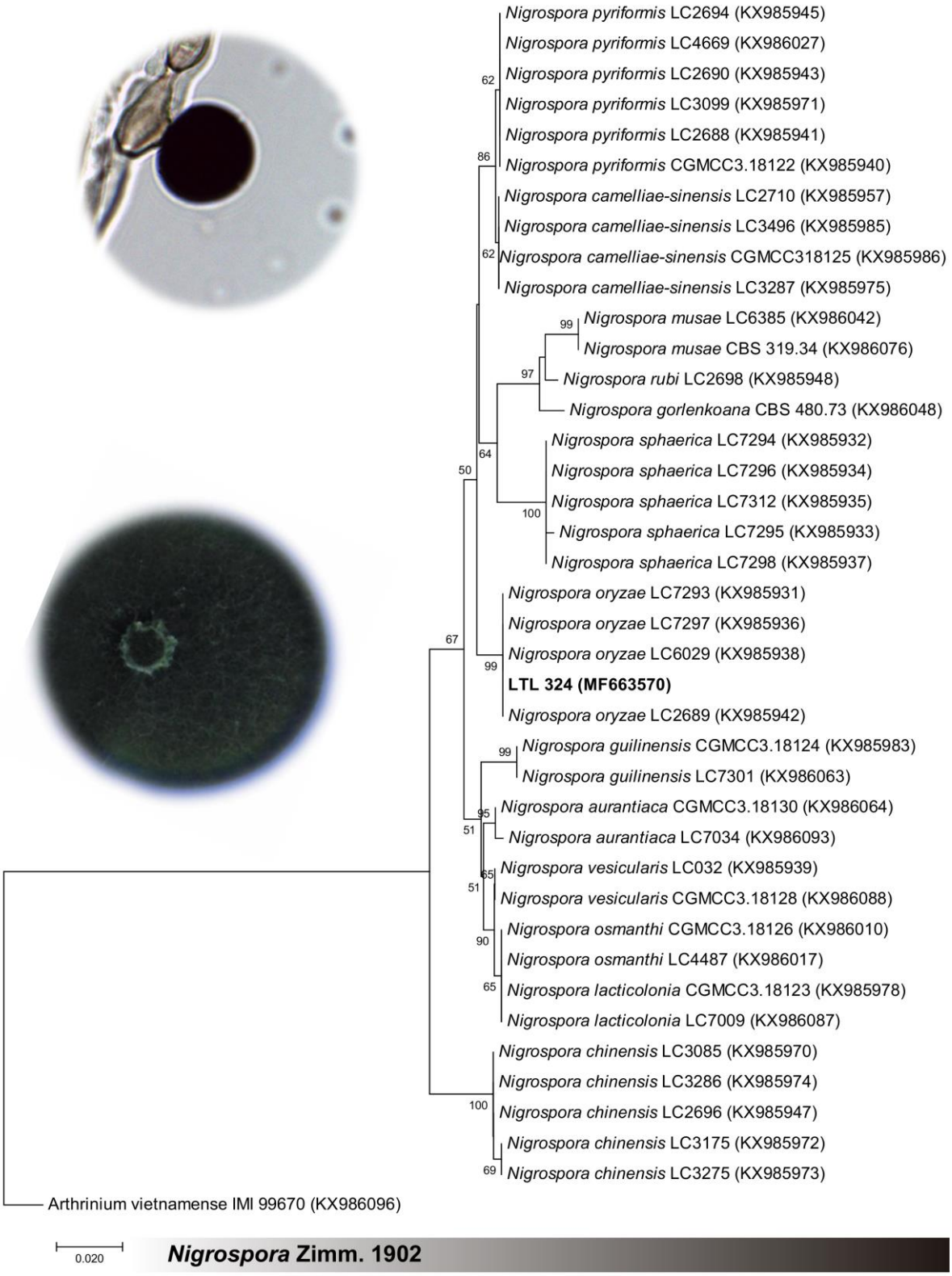


Figure S11. Phylogeny of fungi in the genus *Nigrospora* isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Arthrinium Kunze* 1817.

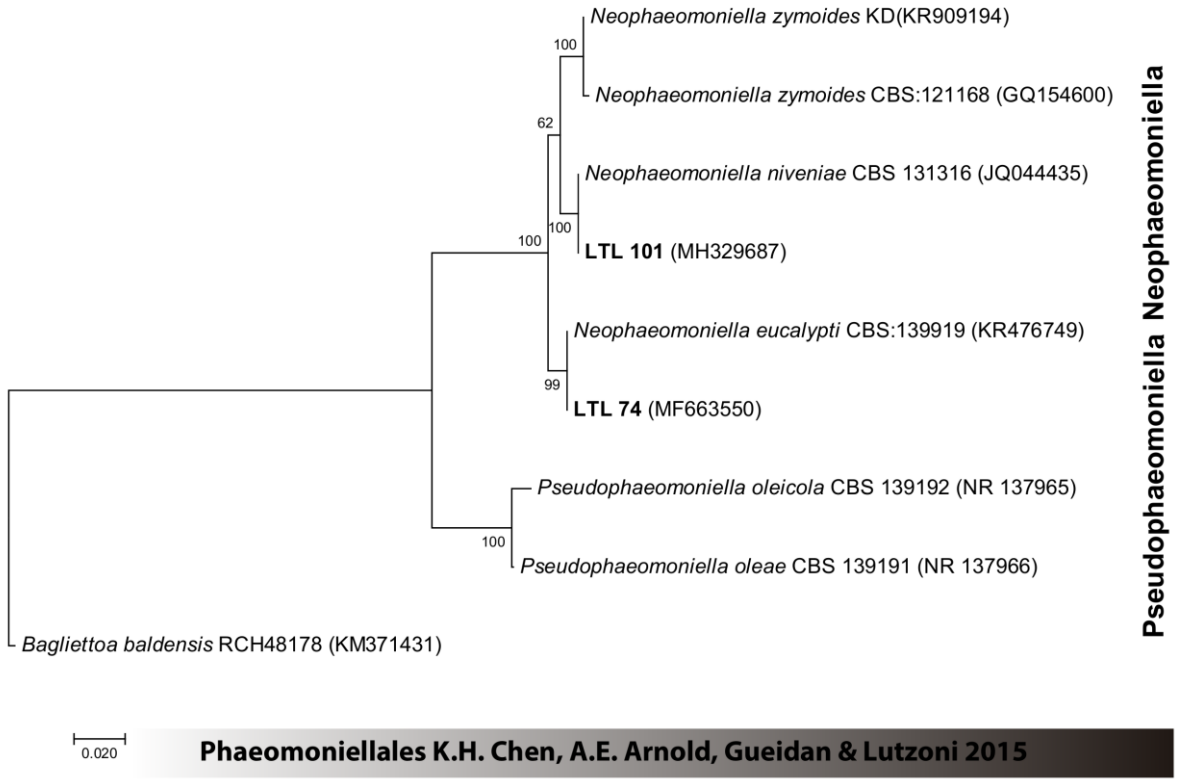


Figure S12. Phylogeny of the ITS region of the order Phaeomoniellales of fungi isolated from *Eucalyptus microcorys*, based on the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Bagliettoa baldensis* (A. Massal.) Vězda 1981.

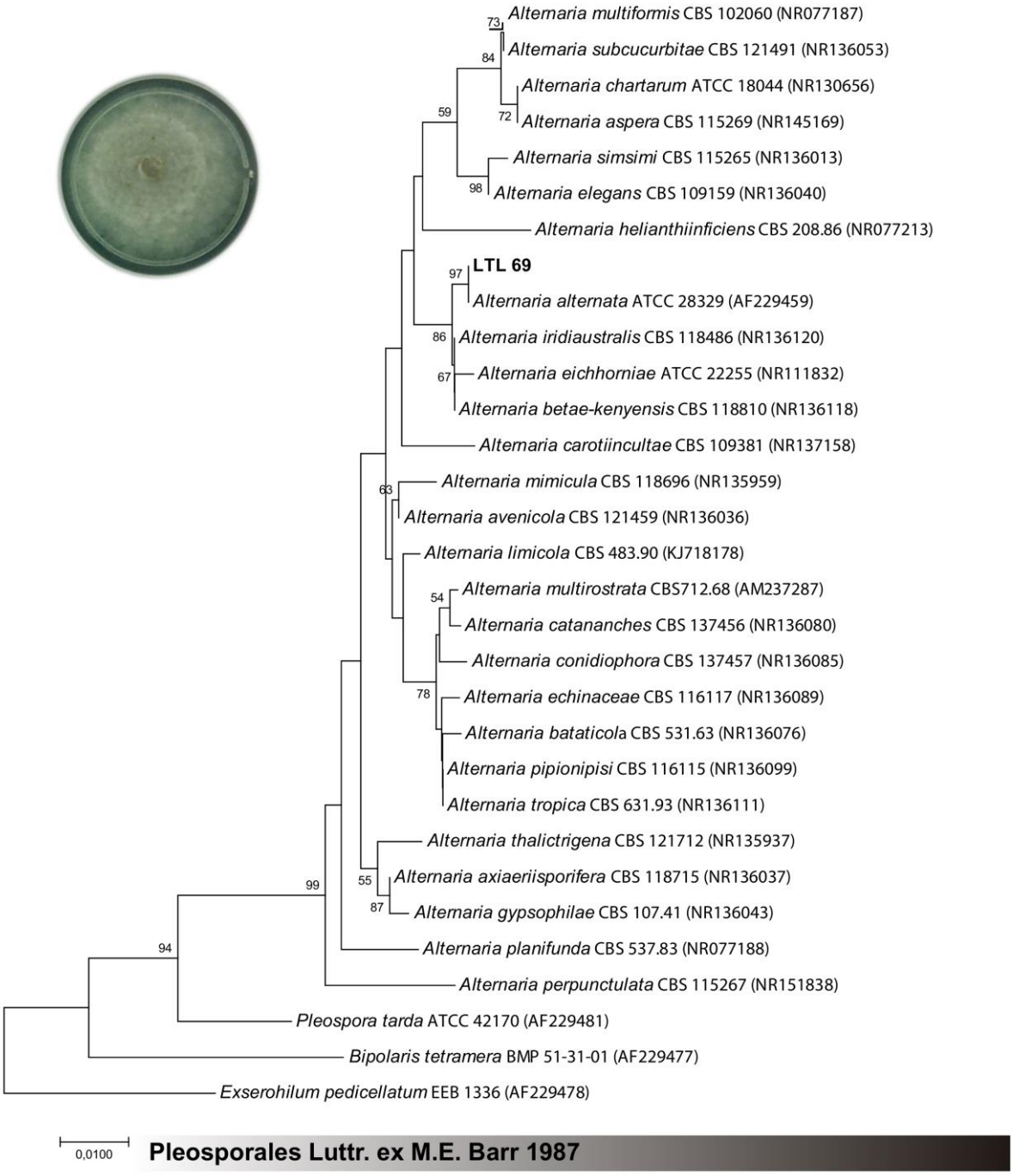


Figure S13. Phylogeny of fungi in the order Pleosporales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Exserohilum pedicellatum* (A.W. Henry) K.J. Leonard & Suggs 1974.

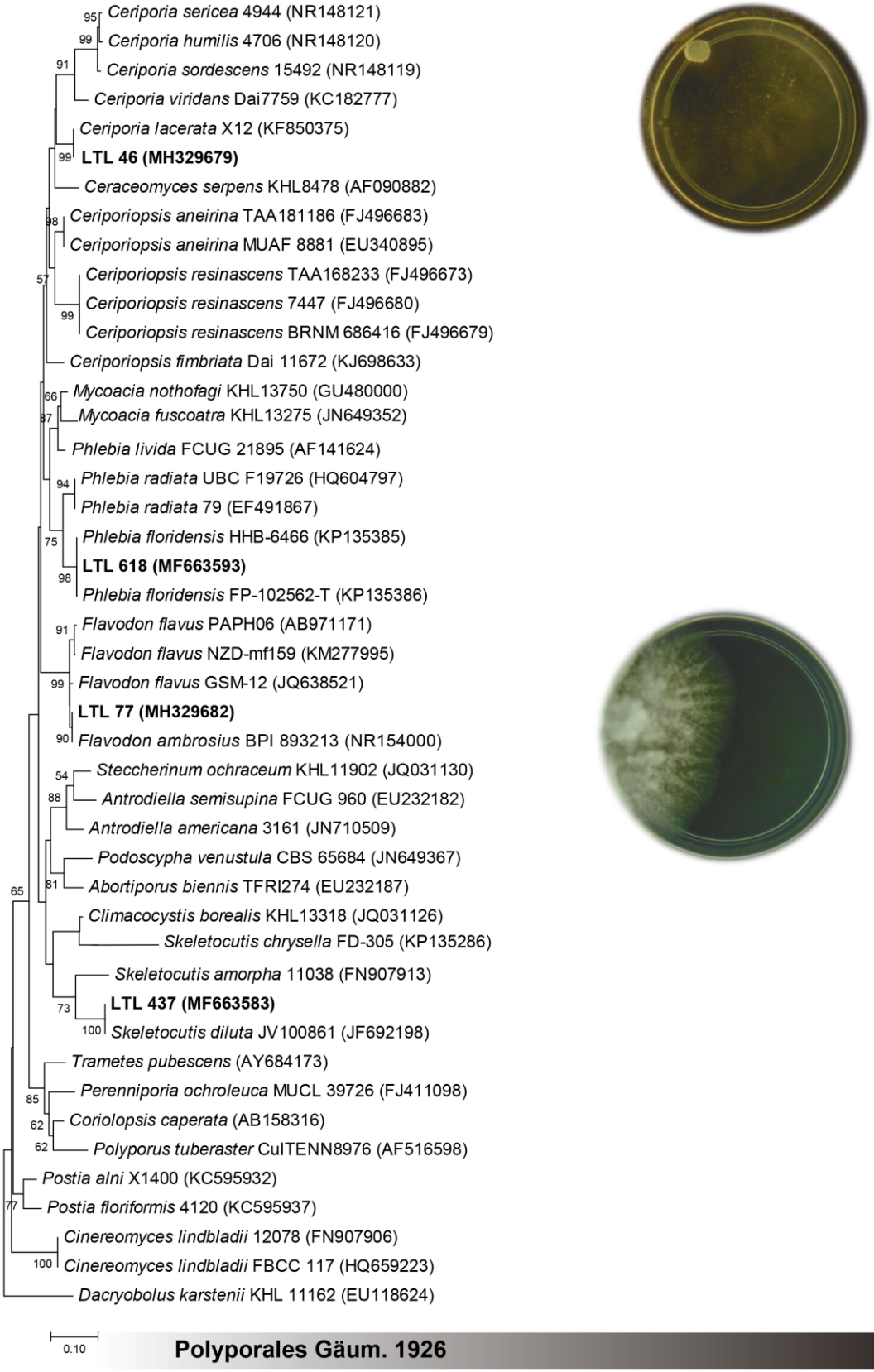


Figure S14. Phylogeny of fungi in the order Polyporales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Dacryobolus karstenii* (Bres.) Oberw. ex Parmasto 1968.

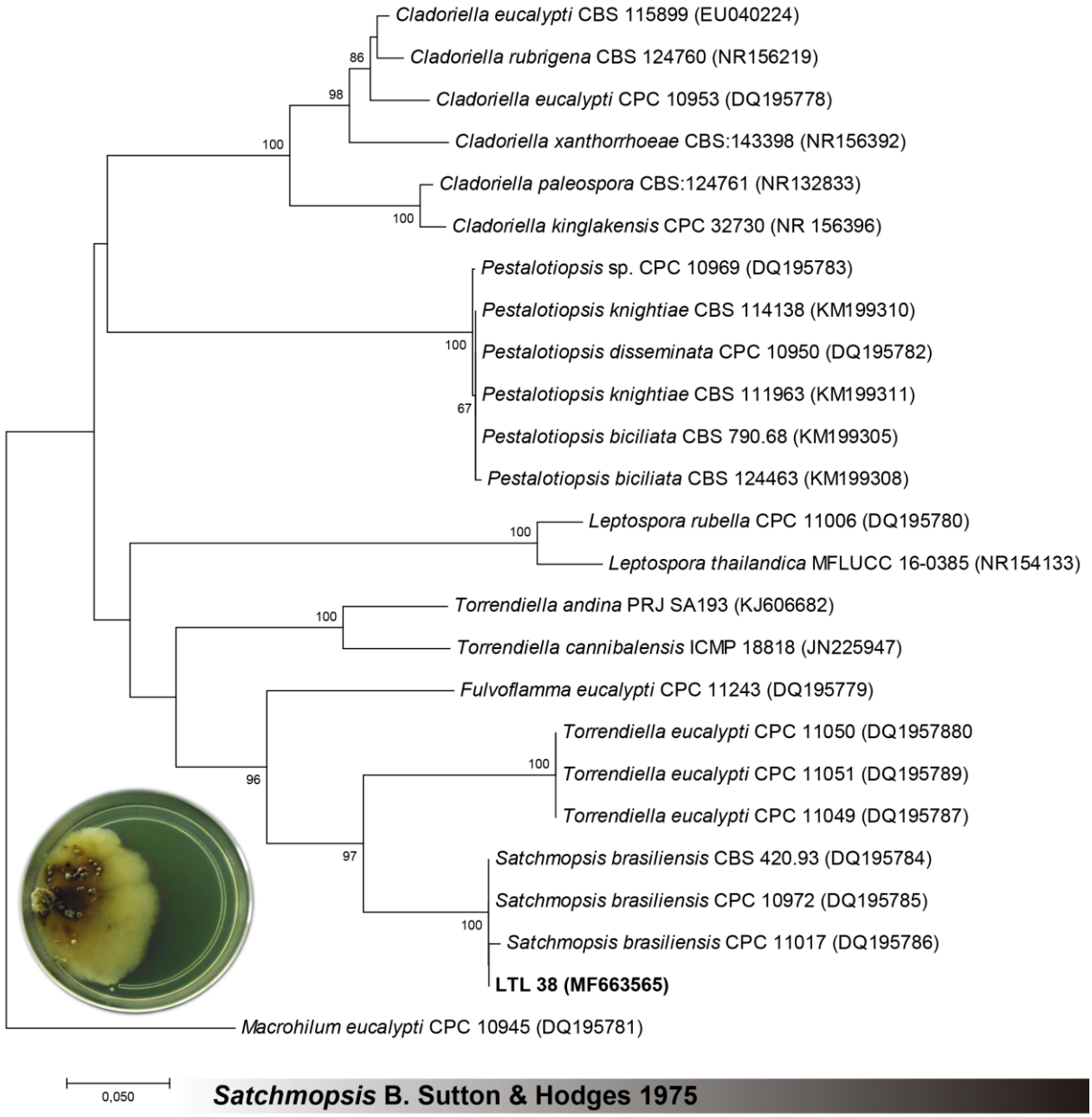


Figure S15. Phylogeny of fungi in the genus *Satchmopsis* isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Dacryobolus karstenii* (Bres.) Oberw. ex Parmasto 1968.

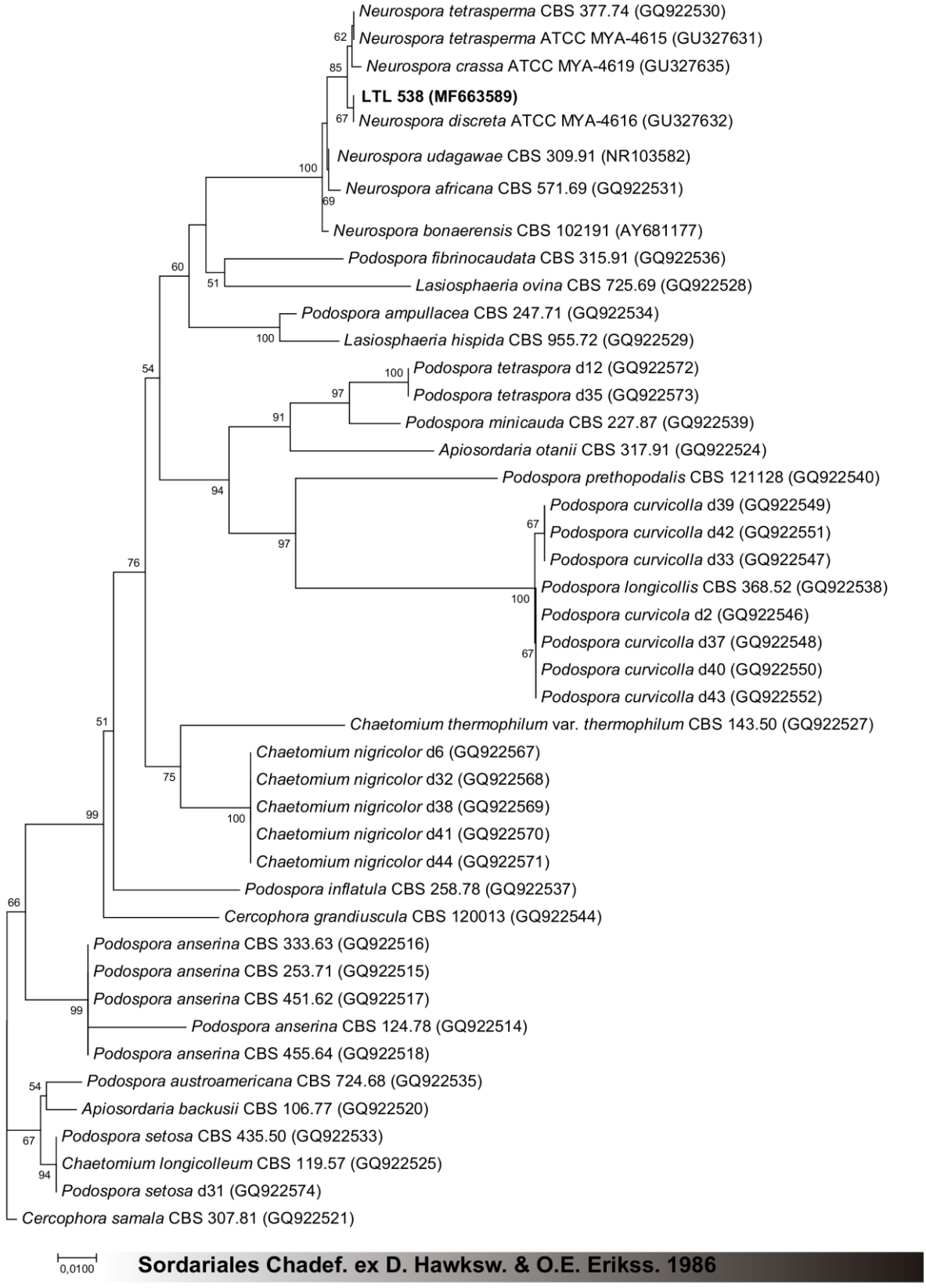


Figure S16. Phylogeny of fungi in the order Sordariales isolated from *Eucalyptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Cercophora samala* Udagawa & T. Muroi 1979.

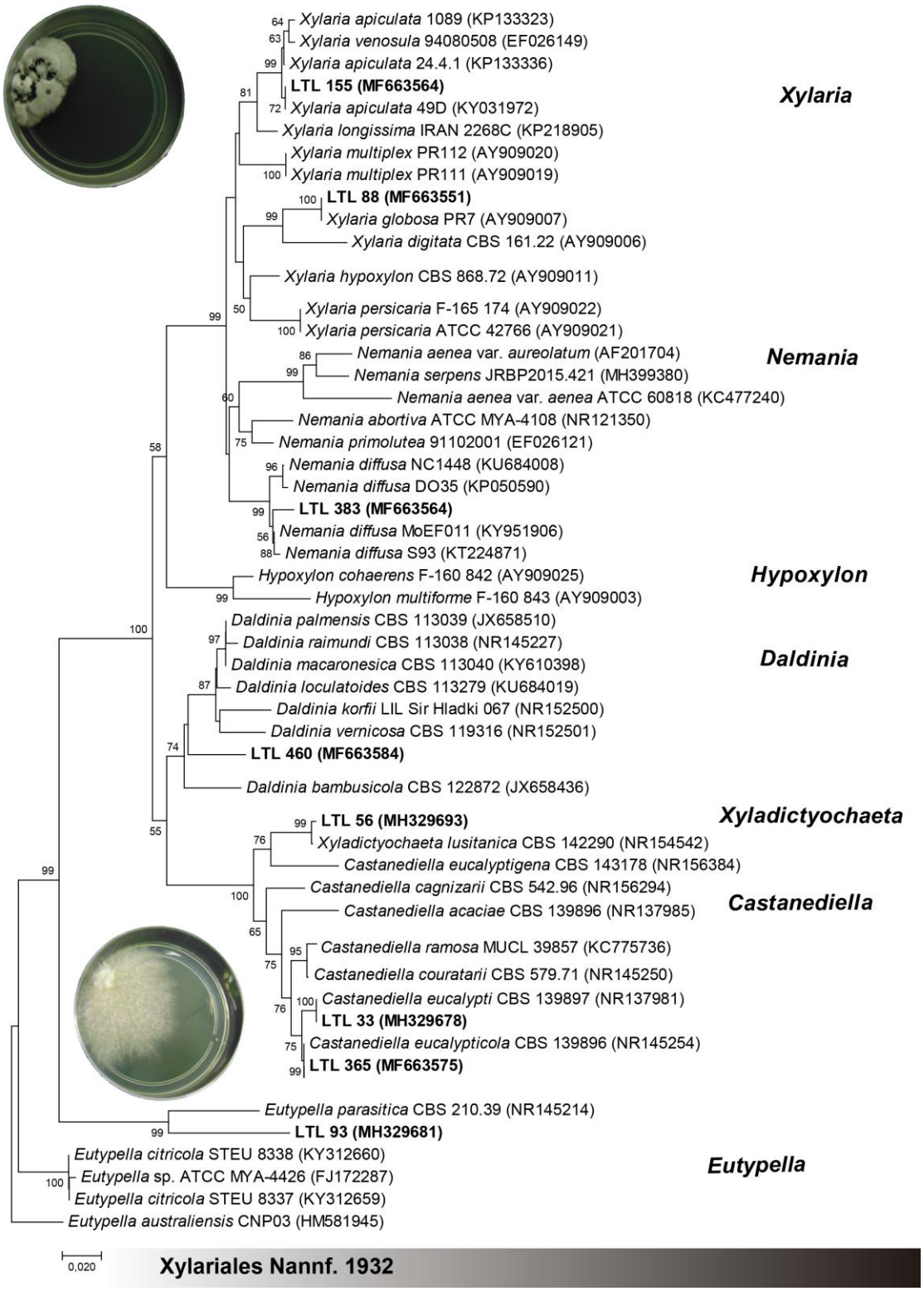


Figure S17. Phylogeny of fungi in the order Xylariales isolated from *Eucalyptus microcorys*. The tree is based on LSU sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Eutypella australiensis* Trouillas, Sosnowski & Gubler 2010.

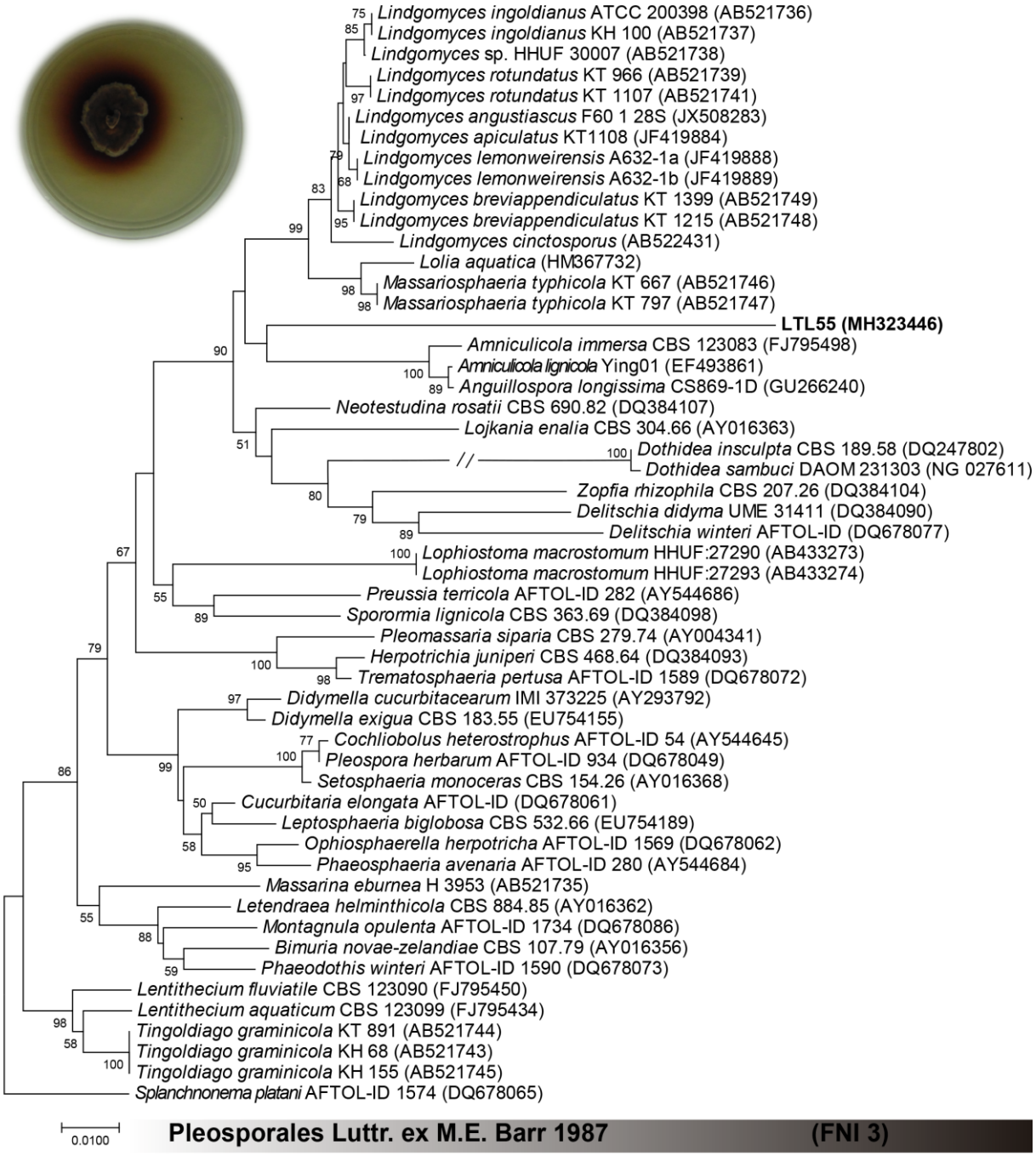


Figure S18. Phylogeny of fungi in the order Pleosporales of isolated FNI3 from *Eucalyptus microcorys*. The tree is based on LSU sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Splanchnonema platani* M.E. Barr 1982.

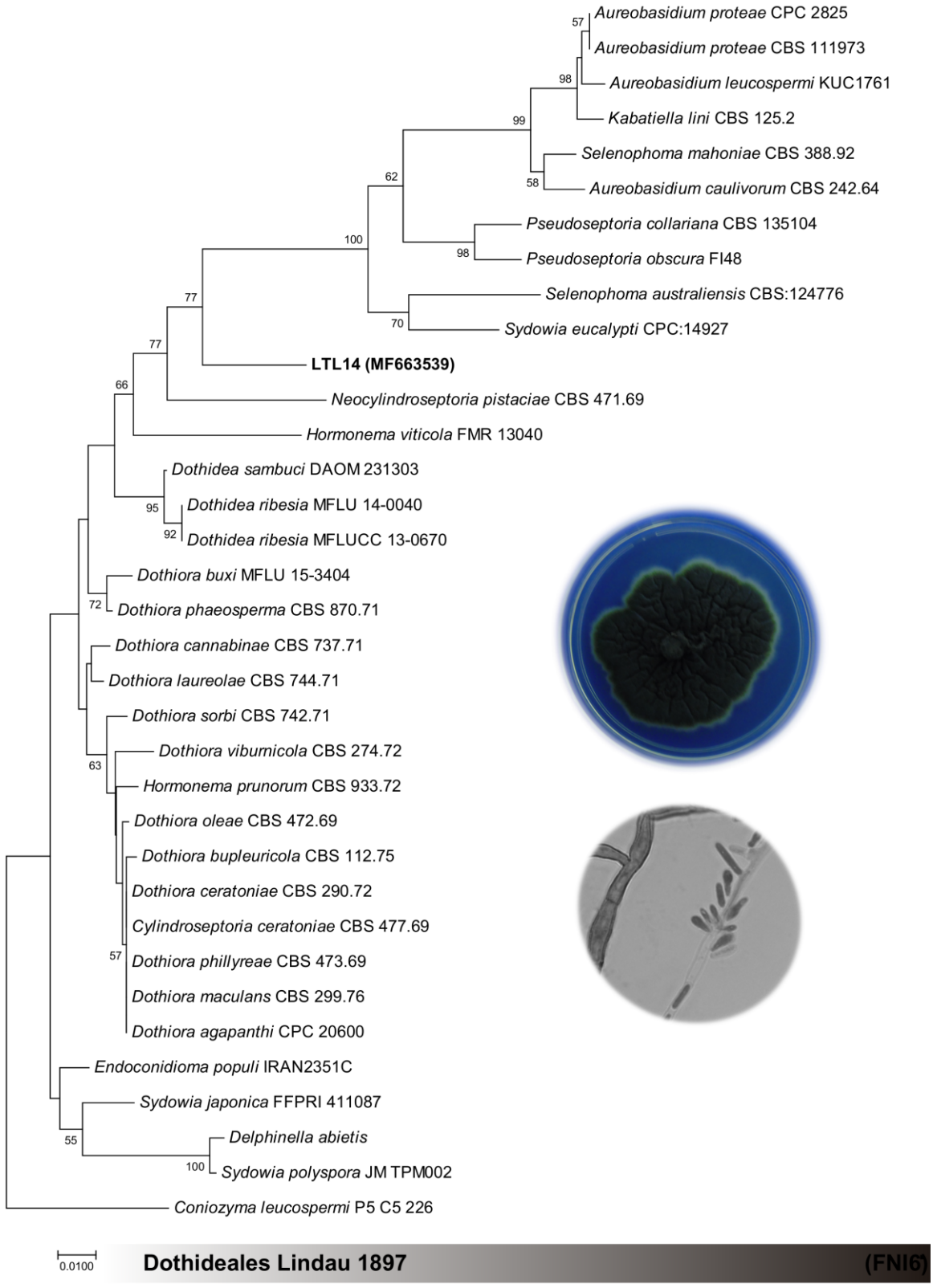


Figure S19. Phylogeny of fungi in the order Dothideales of isolated FNI6 from *Eucaliptus microcorys*. The tree is based on ITS sequences and inferred by the Neighbor joining analysis with 1000 bootstrap pseudoreplicates. The tree is rooted with *Coniozyma leucospermi* (Crous & Denman) Crous 2008.

Table S2. Fungal identification using ITS barcode.

Fungi*	bp	Molecular identification					
		NCBI-Genbank			Mycobank		
		%	Accession	Taxa	%	Accession	Taxa
Alternaria alternata	552	100	MH02168	*A.alternata*	100		*A.alternata* CBS 102599
Calonectria sp.	529	100	GQ28055	*C. scoparia*	100	GQ28064	*C. pseudoscoporia* CBS 125255
Castanediella eucalypti	556	100	NR_13798	*C. eucalypti*	100		*C.eucalypti* CBS 139897
Castanediella eucalypticola	553	100	NR_14525	*C. eucalypticola*	100	NR_14525	*C.eucalypticola* CPC 26539
Ceratobasidiaceae	690	100	AB449172	Ceratobasidiaceae	100	AB449186	Ceratobasidiaceae
Ceriporia lacerata	643	99	KF850375	*C. lacerata*	100	AB091675	*C. lacerata*
Cladosporium cladosporioides	531	99	KX66441	*C. cladosporioides*	79		*P. neopyrolae* CBS 134750
Colletotrichum sp.	493	100	KU52985	*C. gloeosporioides*	100	JN050242	*C. gigasporum*
Colletotrichum sp.	556	100	KX62032	*C. siamense*	100		*C. ti* ICMP4832
Coniella diplodiella	557	99	KX83351	*C. diplodiella*	99	AY339334	*C. diplodiella* CBS 590.84
Cytospora eucalypticola	582	97	KR093883	*C. eucalypticola*	95		*C. eucalypticola* CBS 116851
Daldinia sp.**	565	97	JN198510	*Daldinia* sp.	97	JN198510	*Daldinia* sp.
Diaporthe sp.	560	99	HM85521	*D. phaseolorum*	99	KX02056	*D. phaseolorum*
Dothistroma sp.	451	98	KR995128	*D. septosporum*	98	AY808304	*D. pini* CMW 14820
Epicoccum sp.	527	100	KX92874	*Epicoccum* sp.	100	KR012895	*E. nigrum* CIAT536
Eutypella sp.	576	99	JQ717311	*Eutypella* sp.	99	KF019235	*Eutypella* sp.
Flavodon sp.	593	99	KP096363	*Flavodon* sp.	99	KP096363	*F. ryvarden*
cf. *Geomyces* sp.	531	99	KR093875	*Geomyces* sp.	99	KR093875	*Geomyces* sp.
Gliocephalotrichum sp.	536	99	KF513251	*G. bacillisporum*	99	KF513251	*G. bacillisporum* CBS 25091
Harknessia pseudohawaiiensi	568	98	JQ706109	*H. pseudohawaiiensi*	98	JQ706109	*H. pseudohawaiiensis* CPC17379
*Nemania diffusa***	561	99	MF18534	*N. diffusa*	100	KP133219	*N. difusa*
Neophaeomoniella eucalypti	593	99	NR_13800	*N. eucalypti*	99	KR476749	*N. eucalypti* CPC 25161
Neophaeomoniella niveniae	685	100	JQ044435	*N.niveniae*	100	JQ044435	*N. niveniae* CBS 131316
Neofusicoccum parvum	562	99	JQ619650	*N. parvum*	99	FJ790823	*N. parvum*
Neurospora sp.	560	99	GU32763	*N. discreta*	99	GU32763	*N. discreta* ATCC MYA-4616
Nigrospora oryzae	540	99	KT966518	*N. oryzae*	99	KT966518	*N. oryzae*
Pantospora guazumae	516	99	JN190956	*P. guazumae*	99	NR_11997	*P. guazumae* CBS 130299
*Penicillium cairnsense***	570	99	NR_12150	*P. cairnsense*	99	NR_12150	*P. cairnsense* CBS 124325
*Penicillium citrinum***	535	100	LT_55889	*P. citrinum*	99	AF033422	*P. citrinum* NRRL 1841
*Penicillium glabrum***	560	100	LC228685	*P. glabrum*	100		*P. glabrum* CBS 153.73
*Penicillium herquei***	590	100	JN246042	*P. herquei*	100	JN617703	*P. herquei* CBS 347.51
*Penicillium rubens***	567	100	MF27666	*P. rubens*	100	LT558874	*P. rubens*
Phlebia cf. *floridensis*	628	99	KP135385	*P. floridensis*			
Phyllosticta capitalensis	621	100	KU20443	*P. capitalensis*	100	JQ743587	*P. capitalensis* CBS 123373
Pseudocercospora norchiensis	521	99	EF394859	*P. norchiensis*	100	JX901785	*P. norchiensis* CBS 120738
Pseudoplagiostoma eucalypti	632	100	AB978371	*P. eucalypti*	100	NR_12015	*P. eucalypti* CBS 132529
Pseudoplagiostoma sp.	592	100	AB978371	*P. eucalypti*	99		*P. corymbiae* CBS 132529
Pseudosydowia eucalypti	580	99	GQ303296	*P. eucalypti*	99		*P. eucalypti* CPC 14028
*Pseudoteratosphaeria flexuosa****	507	100	FJ493194	*T. flexuosa*	100	FJ493194	*T. flexuosa* CBS 111048
*Pseudoteratosphaeria ohnowa****	481	100	EF394845	*T. ohnowa*	99	AF468881	*T. ohnowa* CBS 113291
Readeriella eucalypti	526	100	GQ852781	*R. eucalypti*	100		*R. eucalypti* CBS 120079
Satchmopsis brasiliensis	542	100	DQ195784	*S. brasiliensis*	100	DQ195784	*S. brasiliensis* CBS 42093
Skeletocutis diluta	631	100	JF692198	*S. diluta*	100	JF692197	*S. diluta*
Trichoderma sp.	590	99	KR085969	*T.strigosum*	99		*T. atroviride* NBRC 101776
Xenogliocladiopsis cypellocarpa	533	99	NR154498	*X. cypellocarpa*	99	KM23176	*X. cypellocarpa*
Xyladictyochaeta lusitanica	534	99	NR154542	*X.lusitanica*	99	NR154542	*X. lusitanica* CBS 142290
Xylaria sp.**	590	99	JX62426	*Xylaria* sp.	99	JX62426	*Xylaria* sp.
Xylaria sp.**	538	99	EU009989	*Xylaria* sp.	92	JQ814313	*X. multiplex*

Continua

Conclusão

Fungi*	Molecular identification						
		NCBI-Genbank			Mycobank		
	bp	%	Accession	Taxa	%	Accession	Taxa
Montagnulaceae (FNI 1)	803	82	KF796674	*Kalmusia ebuli*	82		*K. ebuli* CBS 123120
Coniothyriaceae (FNI 3)	527	91	JX496115	*C. cerealis*	90		*C. cerealis* CBS 824.84
Basidiomycota (FNI 4)	675	98	KT585731	Basidiomycota	98	JF449872	Agaricomycetes
Dothideomycetes (FNI 5)	487	89	KC731558	*Pseudocercospora sphaerellae-eugeniae*		AJ877190	Dothideomycetes
Helotiales (FNI 4)	534	94	KX100372	*Leptodontidium* sp.	94		*L. elatius* CBS 624.69
Ascomycota (FNI 6)	576	98	KR093869	Ascomycota	98	KR093869	Ascomycota

* The final identification was made based on the morphological and molecular identification;
** Taxa identified using additional molecular markers;
*** Current name.
FNI: non-identified fungi.

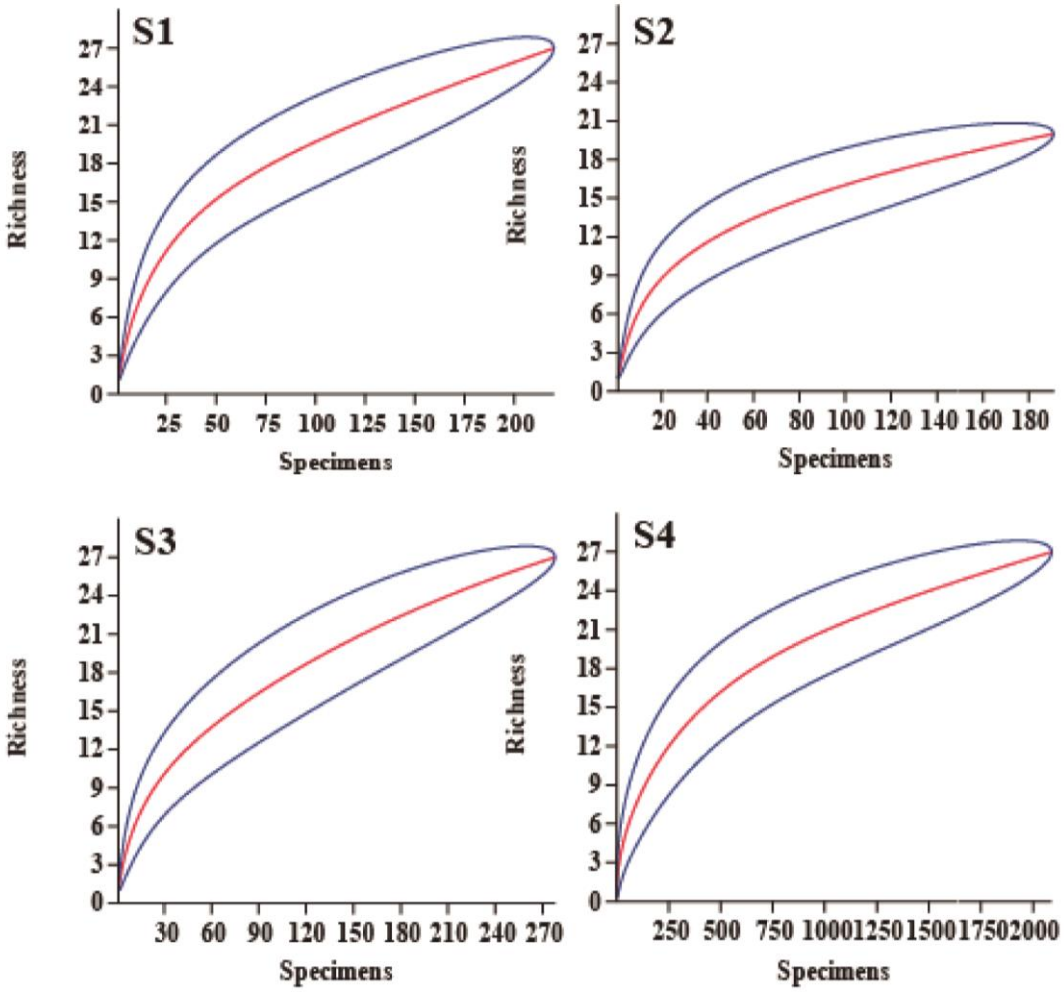


Figure S20. Fungal richness found in *Eucalyptus microcorys* leaves. Rarefaction curves (red lines) of S1 = first foliar stage. S2 = second foliar stage. S3 = third foliar stage. S4 = fourth foliar stage. * Data from Lacerda et al. (2008) was used for comparisons between the endophytic and saprobic communities. Blue lines: confidence interval at 95%.

Capítulo 3 - Microcylindroseptoria, A NEW FUNGAL GENUS FOUND IN EUCALYPTUS PLANTATIONS

Table S1. Culture media used for isolation* and identification of the new genus.

Culture media	**Abb.**	**Composition (g L^{-1})**
Dichloran Rose Bengal	DRB	5 Yeast extract 10 Dextrose 1 Potassium 0.5 Magnesium sulfate 0.025 Bengal rose 0.0015 Chloramphenicol 15 Agar
Malt Extract*	MEA	20 Malt extract 15 Agar
Synthetic Nutrient Agar	SNA	0.2 Glucose 0.2 Sucrose 1 Potassium dihydrogen phosphate 1 Potassium nitrate 0.25 Magnesium sulfate anhydrous 0.5 Potassium chloride 0.025 Bengal rose 0.0015 Chloramphenicol 15 Agar
Potato Dextrose Agar*	PDA	200 Potato infusion 20 Dextrose 15 Agar
Potato Carrot Agar	PCA	250 Potato infusion 200 Carrot 15 Agar
Corn Meal Agar	CMA	50 Corn meal extract 15 Agar
Oatmeal Agar	OA	15 Fine flakes oats 15 Agar

* These media were also used for isolation. In this case, we added 150 μg mL^{-1} of chloramphenicol.

Table S2. Genera from Dothideaceae family.

Taxa	Type species	Sexual state	Asexual state	Reference
Asteromellopsis	*A. insculpta*	Not found description	Not found description	Hess and Müller (1951)**
Delphinella	*D. strobiligena*	Ascostromata immersed on host, becoming erumpent at maturity, subglobose. Asci oblong, clavate, poly-sporous, bitunicate. Ascospores ellipsoid to fusiform, hyaline, 1-septate, smooth, surrounded by a thin sheath.	Unknown	Müller and Arx (1962)
Dictyodothis	*D. berberidis*	Ascostromata immersed, erumpent at maturity, aggregated, globose to subglobo. Asci eight-spored,bitunicate, clavate to sub-clavate or cylindrical. Ascospores oblong, ovoid to fusoid, overlapping uniseriate to biseriate, with septum.	Unknown	Theissen and Sydow (1915)
Dothidea	*D. sambuci*	Ascostromata erumpent to superficial, solitary or scattered, globose to subglobose. Asci eight-spored, bitunicate, fissitunicate, clavate to subcylindrica. Ascospores uniseriate, hyaline, becoming brown when mature, 1-septate, ellipsoidal.	Unknown	Fries (1823)
Dothiora	*D. pyrenophora*	Ascostromata immersed to host, erumpent at maturity. Asci eight-spored, bitunicate, fissitunicate, oblong to subclavate. Ascospores hyaline or pale grayish, muriform, 7−transverse septate, cylindrical to fusiform.	Unknown	Fries (1849)
Endoconidioma	*E. populi*	Unknown	Conidiomata picnidial, subglobose to flask- shaped. Endoconidia formed endogenously, hyaline conidia unicellular, cylindrical, mostly, ellipsoidal, occasionally constricted at the septum.	Tsuneda et al. (2004)

Continua

Taxa	Type species	Sexual state	Asexual state	Reference
Endodothiora	*E. sydowiana*	Ascostromata immersed, becoming erumpent, gregarious or solitary, subglobose. Asci bitunicate, cylindrical, 20−26−spored. Ascospores hyaline, oblong, 5–7 septate, smooth.	Unknown	Sydow et al. (1929)
Hormonema	*H. dematioides*	*Sydowia polyspora*	Conidiogenesis synchronous. Conidia formed on undifferentiated hyphae from inconspicuous scars, 1-2 conidial loci per cellhyaline, non-septate, smooth- and thin-walled, ellipsoidal. Chlamydospores like.	Lagerberg et al. (1927)
Kabatina	*K. thujae*	Unknown	Conidiomata sporodochial or acervular, partially immersed inside the host. Conidiophores stromatic or branched, hyaline to pale brown, smooth, septate. Conidiogenous cells enteroblastic, phialidic or percurrent, cylindrical to doliiform or subclavate. Conidia hyaline, cylindrical to ellipsoid, terminal in basipetal chains or singly.	Schneider and Arx (1966)
Microcylindroseptoria	*M. eucalypti*	Unknown	Conidiomata pycnidial, superficial to semi-imersed, globose to irregular, solitary to gregarious. Conidiophores reduced to conidiogenous cells ampuliform or cylindrical. Conidia are hyaline, clavate, cylindrical,to irregular, smooth. Chlamydospores like.	This paper
Neocylindrospetoria	*N. pistaciae*	Unknown	Conidiomata pycnidial, globose, erumpent, separate. Conidiophores reduced to conidiogenous cells. Conidiogenous cells phialidic, hyaline, smooth, ampulliform. Conidia cylindrical, hyaline, smooth, truncate, guttulate.	Thambugala et al. (2014)

Continua

Taxa	Type species	Sexual state	Asexual state	Reference
Neophaeocryptopus	*N. cytisi*	Ascostromata superficial, semi-immersed to erumpent, solitary, oblong. Asci bitunicate, fissitunicate, clavate to clavate, 8-spored. Ascospores hyaline,fusiform, 1-septate, smooth.	Conidiomata immersed to superficial, globose to subglobose, glabrous.Conidiophores reduced to conidiogenous cells. Conidiogenous cells holoblastic, phialidic, discrete, ampulliform to cylindric-clavate. Conidia solitary, fusiform to falcate, with narrowed ends, smooth.	Li et al. (2016)
Phaeocryptopus	*P. abietis**	Ascostromata superficial, globose to subglobose, gregarious, solitary. Asci eight-spored, bitunicate, fissitunicate, clavate, oblong. Ascospores hyaline,1-septate, guttulate, clavate, oblong.	Unknown	Petrak (1938)
Plowrightia	*P. ribesia*	Ascostromata immersed, becoming erumpent thorough bark at maturity, globose to subglobose. Asci bitunicate, cylindrical to subclavate, oblong, eight-spored. Ascospores fusiform, overlapping, uni to biseriate, hyaline, 1-septate, smooth.	Unknown	Saccardo (1883)
Pringsheimia	*P.rosarum*	Not found description	Not found description	Schulzer et al. (1866)**
Rhizosphaera	*R. abietis****	Not found description	Not found description	Smith and Zeller (1966)**
Stylodothis	*S. puccinioides*	Ascostromata gregarious, erumpent, globose to subglobose. Asci clavate to cylindrical, 2−4-spored, bitunicate. Ascospores uniseriate, 1-septate, ellipsoid to fusiform, smooth, yellowish when immature, becoming brown to dark brown at maturity.	Unknown	Arx and Müller (1975)

Continua

Conclusão

Taxa	Type species	Sexual state	Asexual state	Reference
Sydowia	*S.gregaria*	Ascostromata immersed to erumpent, solitary or gregarious, globose to subglobose. Asci clavate to oblong, 8-spored, bitunicate. Ascospores guttulate, hyaline, elliptic, obovate, smooth, transversely multiseptate.	*Hormonema*	Schmidle (1895)

*Current Name: *Phaeocryptopus nudus* (Peck) Petr. **Not found. *** Current Name: *Rhizosphaera pini*.

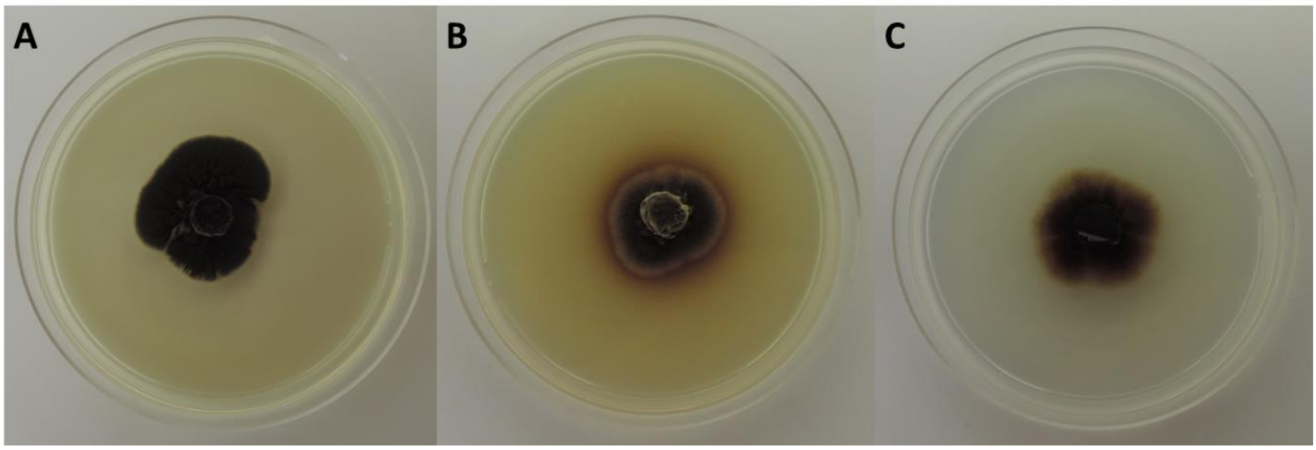


Fig. S1. *Microcylindroseptoria eucalypti* morphology. **A–C.** Culture characteristics at 15 °C. **A.** on potato-dextrose agar (PDA). **B.** on potato-carrot agar (PCA). **C.** on corn meal agar (CMA).