Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
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Tese de doutorado

Diversidade e aplicações biotecnológicas de fungos endofíticos associados à espécies da sub-família Mirtoideae (Myrtaceae) presentes em ecossistemas do Brasil, Argentina e Espanha

Aline Bruna Martins Vaz

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Tese de Doutorado apresentado ao programa de Pós-gradução em Microbiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito para a obtenção do título de Doutor em Microbiologia

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Pequeno príncipe. Saint Exupéry

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Sumário

Resumo

Fungos endofíticos são aqueles que, pelo menos durante parte do ciclo de vida, habitam tecidos vegetais vivos sem causar sintomas aparentes de doença ou efeitos negativos ao hospedeiro. Nos últimos anos, plantas com histórico etnobotânico vêm sendo alvos constantes de estudos de bioprospecção, pois acredita-se que algumas propriedades medicinais atribuídas ao vegetal também possam estar relacionadas à produção de metabólitos secundários por seus fungos endofíticos. O primeiro capítulo deste trabalho teve o objetivo de caracterizar a diversidade de fungos endofíticos associados às plantas medicinais *Myrciaria floribunda*, *Eugenia* aff. *neomyrtifolia* e *Alchornea castaneifolia* e avaliar seu potencial quanto à produção de metabólitos bioativos. Um total de 93 fungos filamentosos foram isolados e identificados por meio do sequenciamento da região espaçadora transcrita interna (ITS) do rRNA. Trinta e oito extratos fúngicos apresentaram atividade contra pelo menos um dos micro-organismos alvo avaliados. Estudos sobre a biogeografia de micro-organismos são escassos e para elucidar se os padrões de diversidade endofítica são correlacionados à filogenia dos seus hospedeiros ou são influenciados pelas variáveis ambientais ou distância foi avaliada a distribuição da comunidade de fungos endofíticos associados à hospedeiros filogeneticamente relacionados de plantas da tribo Myrtae. O estudo foi realizado seguindo-se três escalas espaciais: regional, 101-5.000 km; local, 0-100 km e microescala, 0-1 km. Um total de 960 isolados foi obtido, agrupados e identificados em 52 espécies distintas. A similaridade da comunidade de fungos endofíticos diminuiu com o aumento da distância geográfica, o que foi observado pela análise de decaimento da distância. A análise de escalonamento metrico não dimensional (NMDS) mostrou que os fungos endofíticos agruparam-se a um nível de escala espacial local. Além disso, a análise de regressão múltipla de matrizes (MRM) sugeriu que a similaridade da comunidade endofítica depende da escala espacial analisada. Em outro trabalho realizado foi mostrado que os fungos endofíticos também podem interagir com fungos micorrízicos arbusculares melhorando a adapatabilidade das plantas por meio da promoção do crescimento vegetal. Neste trabalho, cento e cinquenta isolados de fungos endofíticos foram isolados das raízes de *M. communis* e oito diferentes grupos foram selecionados para o estudo com *Sorghum vulgare*. Os fungos não foram capazes de colonizar as raízes de sorgo, porém apresentaram efeitos significativos no crescimento e colonização por micorrizas arbusculares. A quarta parte deste trabalho teve como finalidade avaliar a diversidade de fungos endofíticos associados à *Myrtus communis* utilizando-se técnicas tradicionais de isolamento e por meio de eletrofose em gel de gradiente desnaturante (DGGE). Um total de 113 isolados de fungos endofíticos foi obtido pela técnica de isolamento em meio de cultura em ágar batata dextrosado (BDA). A similaridade da comunidade endofítica não diminuiu com o

aumento da distância geográfica. Os resultados do presente trabalho sugerem uma elevada diversidade de fungos endofíticos associados a hospedeiros presentes em diversos ecossistemas. Além disso, nossos resultados também mostraram o potencial biotecnológico destes fungos como produtores de substâncias antimicrobianas e como promotores do crescimento vegetal.

Abstract

Endophytic fungi inhabit healthy plant tissues during at least one stage of their life cycle without causing any apparent symptom of disease or negative effects on the hosts. In the last years, the endophytic fungal mycota associated with popular medicinal plants have been perfomed because some medicinal properties have been associated to secondary metabolities production by their endophytic fungi associated. Fungal endophyte communities associated with leaves of *Myrciaria floribunda*, *Alchornea castaneifolia*, and *Eugenia* aff. *bimarginata* were examined, collected from Brazilian Cerrado ecosystems, and studied for their ability to produce antimicrobial activity. A total of 93 isolates of endophytic fungi were obtained and identified by sequencing of internal transcribed spacer (ITS) regions of the rRNA gene. Thirty-eight fungal extracts presented antimicrobial activity against at least one of the different target microorganisms tested. The biogeography of microorganisms is poorly understood and to to address whether patterns of endophyte diversity are correlated with host phylogeny, environmental variables and distance we designed a geographic survey of related host tree of Myrtae tribe. The study was performed at the following spatial scales: regional, 101-5,000 km; local: 0-100 km and microscale: 0-1 km. A total of 960 isolates of endophytic fungi were obtained from 3,000 leaf fragment samples. These isolates were grouped and identified into 52 species. We found that the similarity of endophytic fungal communities decreases with increasing geographical distance, showed by the distance decay analysis. The nonmetric multidimensional scaling (NMDS) analysis showed that the fungal endophytes communities were grouped at a local scale. The multiple regression on matrices (MRM) suggested that the fungal endophytic community similarity depends on the scale sampled used. Other study showed that endophytic fungi can interact with arbuscular micorryzal, increasing the growth plant. A total of 150 isolates of endophytic fungi were obtained from roots of *Myrtus communis* and seven groups were selected to interactions study with *Sorghum vulgare.* None of the root endophytic fungi tested colonized the root of sorghum, but significantly increased the growth and arbuscular mycorrhizal colonization of sorghum. The fourth part of this work evaluated the endophytic community diversity of *Myrtus communis* by classical isolation from leaves fragments and denaturing gel gradient electrophoresis (DGGE). A total of 113 fungal isolates were obtained and identified by classical isolation in potato dextrose agar. It was found that the similarity of endophytic fungal communities did not decrease with increasing geographical distance. The results obtained in this work suggest a high endophytic fungal diversity associated with Myrtaceae host tree presents indifferent ecossistems. Moreover, these micro-organismos represents a potencial source of substances that can be used to development of new antimicrobial drugs and as a growth promoting fungi.

Relevância e justificativa

Micro-organismos endofíticos são aqueles que, pelo menos durante parte do seu ciclo de vida, habitam tecidos vegetais vivos sem causar sintomas aparentes de doença ou efeitos negativos ao hospedeiro. Estima-se que existam cerca de 300 mil espécies de plantas nos diferentes biomas do planeta e cada planta pode ser hospedeira de um ou mais micro-organismo endofítico, sendo que pesquisas sugerem que a maioria das plantas, senão todas, em ecossistemas naturais formam associações simbióticas com fungos endofíticos. Estes micro-organismos apresentam uma grande diversidade genética e desempenham importantes funções na manutenção dos ecossistemas. Vários trabalhos sugerem que este grupo de micro-organismos é uma fonte promissora de metabólitos secundários de interesse econômico. Além disso, os fungos endofíticos podem interagir com outros micro-organismos promovendo o crescimento vegetal de plantas de interesse agronômico. Os padrões sobre a distribuição dos endofíticos também vem sendo realizados a fim de determinar a influência das características ambientais na distribuição da comunidade de fungos.

Na interação entre fungos endofíticos e planta os fungos endofíticos são beneficiados pelos fotossintatos produzidos e proteção conferida pelo hospedeiro; em troca, garantem maior adaptabilidade ecológica ao hospedeiro, como tolerância ao estresse ambiental, resistência à fitopatógenos e herbívoros, entre outros. Neste tipo de interação os fungos endofíticos podem produzir metabólitos secundários, os quais podem apresentar diferentes atividades biológicas. Os metabólitos secundários, ou produtos naturais, podem ser definidos como substâncias de baixo peso molecular, normalmente produzidos em resposta a condições ambientais específicas. Entre as atividades biológicas envolvendo metabólitos produzidos por micro-organismos endofíticos pode-se mencionar a ação antibacteriana, antifúngica, antioxidante, antiviral, antiparasitária e inseticida. Plantas com histórico etnobotânico são um dos alvos para estudos de bioprospecção, pois acredita-se que algumas propriedades medicinais atribuídas ao vegetal também possam estar relacionadas à produção de metabólitos secundários por seus fungos associados. Neste contexto, os fungos endofíticos associados às medicinais *Eugenia* aff. *neomyrtifolia*, *Myrciaria floribunda* e *Alchornea castaneifolia* presentes em ecossistemas brasileiros foram avaliadas com respeito a atividade antimicrobiana contra micro-organismos de interesse clínico.

Relatos fósseis indicam que a interação fungo endofítico-planta iniciou-se há milhões de anos, quando vegetais superiores surgiram no planeta pela primeira vez. Sabe-se que muitos micro-organismos exibem padrões de diferenciação genética e molecular que podem estar relacionados com a distribuição dos seus hospedeiros. Nesse sentido, no presente trabalho foi avaliado se a comunidade de fungos endofíticos varia ao longo de um gradiente geográfico ou se

os fungos endofíticos apresentam correlação filogenética com seus hospedeiros. A tribo Myrtae (família Myrtaceae) foi escolhida para este objetivo por formar um grupo monofilético e por existirem diversos estudos sobre a sua filogenia.

Outro aspecto importante da biologia de fungos endofiticos é que estes micro-organismos também podem interagir com fungos micorrízicos arbusculares melhorando a adapatabilidade das plantas por meio da promoção do crescimento vegetal, proteção contra doenças ou aumentando a absorção de fósforo. Desta forma, os fungos endofíticos associados a raízes de *Myrtus communis* foram isolados e os mais frequentes foram inoculados na rizosfera de raízes de *Sorghum vulgare*. O sorgo foi escolhido como um modelo de planta de interesse agronômico mundial e constitui o quinto cereal mais consumido no mundo.

O estudo da micota endofítica associada às diversas plantas avaliadas neste trabalho será de grande importância para o conhecimento micológico. Os estudos sobre o potencial biotecnológico dos fungos pode levar ao descobrimento futuro de processos metabólicos utilizados por estes micro-organismos úteis para utilização biotecnológica. Além disso, os estudos biogeográficos são fundamentais para melhor entender os padrões de distribuição da comunidade endofítica a fim de determinar a influência das variáveis ambientais ou históricas na distribuição atual da micota endofítica.

Formatação da tese

Esta tese é apresentada em sete capítulos. O capítulo um refere-se à revisão bibliográfica sobre o tema proposto. O capítulo de 2 trata do estudo da diversidade e da atividade antimicrobiana de fungos do cerrado do Tocantins, Norte do Brasil. Este trabalho foi aceito para publicação na revista African Journal of Microbiology Research. O capítulo 3 fala sobre a diversidade e biogeografia de fungos endofíticos associados espécies vegetais pertencentes à família Myrtaceae presentes em ecossistemas brasileiros e argentinos. O capítulo 4 é sobre as interações de fungos endofiticos com micorrizas arbusculares, e este trabalho foi aceito para publicação na revista Soil Applied Ecology. O capítulo 5 é sobre a diversidade de fungos endofíticos associados a folhas de *Myrtus communis* estudada por métodos tradicionais de isolamento de fungos e por eletroforese em gel de gradiente desnaturante (DGGE). O capítulo seis aborda uma discussão integrada dos resultados obtidos e o capítulo sete as conclusões do trabalho.

Capítulo 1

Revisão bibliográfica

1. Micro-organismos endofíticos

Os micro-organismos endofíticos foram mencionados pela primeira vez no início do século XIX, mas foi De Bary (1866) quem primeiro delineou a diferença entre eles e os patógenos de plantas. Os primeiros estudos com endófitos foram realizados com plantas da família das gramíneas. No final do século XIX, vários pesquisadores descreveram a presença de micélio fúngico nos carpelos e sementes de plantas sadias de *Lolium arvense*, *L. linicolum*, *L. remotum* e *L. temulentum* (Guerin, 1898; Hanausek, 1898; Vogl, 1898; Rodriguez *et al.* 2009). Na década de 1930 pesquisas demonstraram a relação entre fungos endofíticos e a toxicidade de *L. temulentum* para o gado (Kingsbury, 1964). Entretanto os estudos sobre os fungos endofíticos presentes em outras famílias de plantas, que não as gramíneas, intensificaram-se somente a partir de 1970, quando foi verificado que estes micro-organismos apresentam interações simbióticas com os hospedeiros, protegendo as plantas dos ataques de insetos, de doenças e de mamíferos herbívoros (Azevedo, 1999). Desde então vários trabalhos mostraram a presença de espécies fúngicas em plantas pertencentes a grupos como briófitas (U'Ren *et al.*, 2010), pteridofitas (Petrini *et al.*, 1992), gimnospermas (Soca-Chafre *et al.*, 2011), angiospermas monocotiledôneas (Pinruan *et al.*, 2010) e dicotiledôneas (Vaz *et al.*, 2009; Vaz *et al.* 2012 a,b; Vieira *et al.*, 2012). Webber (1981) foi provavelmente o primeiro pesquisador a relatar um exemplo de proteção à planta conferida pelo fungo endofítico *Phomopsis oblonga*, que reduzia a dispersão de *Ceratocystis ulmi*, fungo causador da doença do olmo, pelo controle de seu vetor, o besouro *Physocnemum brevilineum*. O autor associou o efeito repelente contra os insetos à compostos tóxicos produzidos pelos fungos. Isto foi confirmado posteriormente por Claydon *et al.* (1985), os quais mostraram que fungos endofíticos pertencentes à família *Xylariaceae*, associados à espécies de plantas do gênero *Fagus*, sintetizavam metabólitos secundários que afetavam larvas de besouros. Desde então vários pesquisadores apresentaram definições para o termo endofítico. Uma das definições mais aceita infere que os micro-organismos endofíticos são aqueles que, pelo menos durante parte do seu ciclo de vida, habitam tecidos vegetais vivos sem causar sintomas aparentes de doença ou efeitos negativos (Bacon & White, 2000). Coletivamente, mais de cem anos de pesquisa sugere que a maioria das plantas, senão todas, em ecossistemas naturais formam associações simbióticas com fungos endofíticos (Petrini *et al.* 1986; Rodriguez *et al.*, 2009).

Rodriguez *et al.* (2009) propõe um divisão dos fungos endofíticos em quatro classes. A primeira é formada pelos fungos clavicipitaceos (endofíticos-C), os quais represetam um pequeno número de espécies de fungos que formam infecções sistêmicas em gramíneas. A maioria destes fungos pertence aos gêneros *Epiclhoë* e *Balansia*, e seus anamorfos

Neotyphodium e *Ephelis*, respectivamente, ambos da família Clavicipitaceae (Hypocreales, Ascomycota). Os benefícios conhecidos desta interação para a gramínea hospedeira incluem o aumento da tolerância a metais pesados; aumento da resistência à dessecação; redução no herbivorismo e resistência contra patógenos. A proteção contra herbivoria ocorre por meio de metabólitos secundários bioativos produzidos pelos fungos, como a peramina e lolina (atividade inseticida), e lolitrem B e ergovalina (atividade citotóxica) (Alexopoulos *et al.*, 1996; Schulz & Boyle, 2005). O fungo endofítico beneficia-se dos nutrientes presentes no espaço apoplástico do tecido vegetal, da proteção contra estresses abióticos e da competição com micro-organismos epifíticos (Saikkonen *et al.*, 1998).

Os fungos não-clavicipitaceos (endofíticos-NC) apresentam uma alta diversidade de fungos e são representados por três grupos funcionais distintos baseados em características de história de vida e sua significância ecológica (Rodriguez *et al.*, 2009; Saikkonen *et al.*, 1998). Nesta interação os fungos endofíticos são beneficiados pelos fotossintatos produzidos e proteção conferida pelo hospedeiro; em troca, garantem maior adaptabilidade ecológica ao hospedeiro, como tolerância ao estresse ambiental, resistência à fitopatógenos e herbívoros, entre outros (Tan & Zou, 2001; Petrini *et al.*, 1992; Saikkonen *et al.*, 1998; Strobel & Dayse 2003; Linnakoski *et al.*, 2011). A classe dois é formada principalmente por fungos pertencentes ao filo Ascomycota, subfilo pezizomicotina e em minoria também são encontrados fungos do filo Basidiomycota, subfilo Agaricomycotina e Pucciniomycotina. Estes fungos colonizam raízes, caules e folhas; são capazes de formar infecções extensas nas plantas; sua trasmissão pode ocorrer tanto horizontalmente quanto verticalmente; e, apresentam uma alta taxa de infecção (90-100%) em plantas presentes em habitats sob condições de estresse. Um exemplo seria a alga marrom *Ascophyllum nodosum* que necessita do fungo endofítico *Mycophycia ascophylli* para crescer e desenvolver (Garbary & Mcdonald, 1995). Quando o fungo *Curvularia protuberata* e a planta *Dichanthelium lanuginosum* encontram-se associados ambos são capazes de tolerar temperaturas de ate 65ºC, enquanto em condições não simbióticas esta tolerância não passaria de 40ºC (Márquez *et al.*, 2007). Outra característica seria a tolerância a estresse salino. A associação do fungo *Fusarium culmorum* com tecidos de *Leymus mollis* (Poaceae) eleva a tolerância de ambos a níveis de salinidade entre 300 e 500 mM de NaCl (Rodriguez *et al.*, 2008).

A terceira classe é composta de fungos que infectam principalmente tecidos aéreos, que se transmitem horizontalmente e a sua colonização nos tecidos vegetais é normalmente localizada. Neste grupo incluem-se a maioria dos fungos endofíticos obtidos de hospedeiros tropicais (Arnold *et al.*, 2001; Gamboa & Bayman, 2001; U'Ren *et al.*, 2009; Vaz *et al.*, 2009; Vaz *et al.*,

2012a; Vieira *et al.*, 2012) e temperados (Higgins *et al.*, 2007; Murali *et al.*, 2007; Davis & Shaw 2008). A maioria dos fungos deste grupo pertencem principalmente ao filo Ascomycota e em menor frequência ao Basidiomycota (Rodriguez *et al.*, 2009) A frequência de isolamento, riqueza e diversidade destes fungos geralmente seguem forte gradiente latitudinal com os maiores valores encontrados em ambientes tropicais quando comparados com ambientes temperados (Arnold & Lutzoni 2007). A ocorrência de fungos endofíticos varia com o habitat e alguns gêneros são comuns tanto em vegetais de ecossistemas tropicais quanto temperados, tais como *Fusarium, Phomopsis* e *Phoma*. Já em ecossistemas tropicais, os gêneros comumente encontrados são *Cladosporium, Corynespora, Colleotrichum, Guinardia, Lasiplodia, Phyllostica, Pestalotiopsis, Sporormiella* e *Xylaria* (Rodrigues & Samuels, 1999; Suryanarayanan *et al.*, 2002; Schulz & Boyle, 2005; Joshee *et al.*, 2009; Gazis *et al.*, 2010; Veja *et al.*, 2010).

A classe quatro é formada por fungos septados negros ("Dark spetate endophyte", DSE), os quais normalmente colonizam tecidos da raiz, sua transmissão ocorre horizontalmente e colonizam os tecidos vegetais extensivamente (Rodriguez *et al.*, 2009). Estes fungos são pertencentes ao filo Ascomycota e formam estruturas com presença de melanina tais como hifas inter e intracelulares e microesclerocios nas raízes de plantas. Os fungos DSE provavelmente constituem os fungos mais abundantemente encontrados em associação com raízes em paralelo com os fungos micorrízicos arbusculares (Mandyam & Jumpponen, 2005; Aveskamp *et al.*, 2009). Além disso, a maioria destes fungos não apresenta especificidade de hospedeiros. Estudos demonstraram que os fungos septados negros, *Chloridium paucisporum*, *Leptodontidium orchidicola*, *Phialocephala dimorphosphora*, *Phialocephala fortinii* e *Phialocephala Finlândia* são capazes de colonizar uma ampla faixa de hospedeiros, sendo *P. fortinii* capaz de colonizar mais de 20 hospedeiros vegetais.

Rodriguez *et al.* (2009) não inclui nesta classificação os fungos micorrízicos arbusculares (MA). Esta associação constitue o sistema radicular absortivo de aproximadamente 90% das plantas terrestes (Smith & Read 2008) e podem ser classificadas em sete tipos (Harley & Smith 1983, Peterson *et al.*, 2004), sendo as micorrizas arbusculares (MA), o tipo mais abundantemente encontrado na natureza. Esta simbiose propicia o melhor desenvolvimento do vegetal devido ao aumento na absorção de água e nutrientes, principalmente fósforo, pelas hifas do fungo MA (Smith e Read, 2008). Apesar dos fungos micorrízicos também serem endofíticos muitos autores os consideram em um grupo separado por ser uma simbiose altamente especializada e bem caracterizada (Stone *et al.*, 2000).

Estima-se que cerca de 99% dos micro-organismos presentes na natureza não são cultiváveis utilizando-se as metodologias padrões de isolamento (Amann *et al.*, 1995). Os métodos dependentes de cultivo são os mais utilizados para o estudo da comunidade de fungos endofíticos (Arnold *et al.*, 2001; Bussaban *et al.*, 2001; Wilberforce *et al.*, 2003). Estes métodos apresentam alguns incovenientes, como por exemplo, fungos que apresentam crescimento rápido são capazes de crescer mais rapidamente nos meios de cultura tradicionalmente utilizados impedindo o crescimento de fungos de crescimento lento (Hyde & Soytong 2008). Além disso, fungos em estágios latente, quiescente ou que necessitam de alguns requerimentos nutricionais frequentemente não são isolados por estas metodologias (Götz *et al.*, 2006). Sendo assim, métodos independentes de cultivo, como eletroforese em gradiente de gel desnaturante (Denaturing Gradient Gel Electrophoresis, DGGE; Muyzer *et al.*, 1993), podem fornecer dados mais completos sobre a biodiversidade de fungos endofíticos. A técnica de DGGE tem sido utilizada com sucesso em estudos de ecologia de comunidades fúngicas (Götz *et al.*, 2006; Vainio *et al.*, 2005; Duong *et al.*, 2006). Esta técnica permite o isolamento tanto de fungos endofíticos já isolados por meio de cultivo quanto de espécies não-cultiváveis (Duong *et al.*, 2006). Além disso, os perfis moleculares gerados pela técnica de DGGE possibilitam a análise de várias amostras ambientais simultaneamente, sendo bastante útil para o monitoramento e compreensão de variações temporais e espaciais de comunidade microbianas (Zilli *et al.*, 2003). Isto mostra a importância do uso destas técnicas para melhor caracterizar os padrões de distribuição da comunidade de fungos endofíticos.

2. Interação fungo endofítico – planta hospedeira

Apesar da natureza assintomática da ocupação fúngica no tecido vegetal induzir à classificação de um relacionamento simbiótico, a diversidade de fungos endofíticos sugere que estes possam ser sapróbios ou patógenos oportunistas (Strobel & Daisy, 2003). De fato, alguns fitopatógenos têm origem endofítica, pois são micro-organismos oportunistas que podem causar infecções sintomáticas na planta quando esta se encontra em condições de estresse ou senescência. Alguns fungos podem ser oportunistas acidentais, os quais são normalmente encontrados em outros substratos e não são especificamente adaptados aos seus hospedeiros, tais como algumas espécies de fungos coprófilos detectadas nos tecidos vegetais (Schulz & Boyle, 2005). Comensalismo e mutualismo requerem um sofisticado balanço entre as respostas de defesa da planta e a demanda de nutrientes pelo endofítico. Desse modo, a interação mutualística não significa ausência de defesa da planta (Kogel, 2006). Promputtha *et al.* (2007) avaliaram que fungos colonizadores primários e secundários de folhas deterioradas têm origem endofítica.

Evidências filogenéticas obtidas por estes autores indicam que alguns fungos, por exemplo, espécies de *Colletotrichum*, *Fusarium* e *Phomopsis*, ocorrem como endofíticos e sapróbios. Portanto, algumas espécies de endofíticos podem mudar sua estratégia ecológica e adotar um estilo de vida saprofítico. Apesar das especulações a respeito da definição do tipo de interação, diferentes autores citam que a distinção entre endofíticos, epifíticos (aqueles que vivem na superfície de plantas) e fitopatógenos (que causam doenças em plantas) é meramente didática. Não existe um claro limite entre os grupos e sim um gradiente entre eles (Azevedo, 1999; Saikkonen *et al.*, 1998). Saikkonen *et al.* (2004) propuseram que a relação dos fungos endofíticos com a planta hospedeira é bem mais complexa, envolvendo interações entre as espécies e também a influência de fatores bióticos e abióticos. Um fungo endofítico, por exemplo, pode tornar-se um patógeno conforme as condições de ambiente ou equilíbrio com outros endofíticos; um micro-organismo epifítico pode, eventualmente, entrar em uma planta, permanecendo por um determinado período, causando ou não danos à mesma.

Pesquisas mostram que a mudança da interação de mutualismo para parasitismo pode ocorrer por mutação em um único gene do genoma microbiano (Kogel, 2006). Tanaka *et al.* (2006) sugerem um mecanismo de simbiose no qual a produção de espécies reativas de oxigênio (ROS) por *Epiclhoë festucae* NoxA *in planta* regula negativamente o desenvolvimento do fungo e o crescimento da hifa, prevenindo assim a colonização excessiva do tecido da planta. O gene NoxA codifica uma NADPH oxidase que cataliza a produção de superóxido pela transferência de elétrons de NADPH para o oxigênio molecular, com geração secundária de H_2O_2. A inativação do gene *Nox*A em *E. festucae* muda a interação do endofítico com o hospedeiro de mutualismo para parasitismo. Sendo assim, interações mutualísticas entre fungos invasores e planta hospedeira são decifradas como um balanço, sobre controle ambiental, fisiológico e genético, que resulta nos benefícios de adaptabilidade para ambos os parceiros (Saikkonen *et al.*, 1998; Azevedo, 1999).

3. Biogeografia de fungos

Estima-se que a interação fungo endofítico-planta iniciou-se há milhões de anos, quando vegetais surgiram no planeta pela primeira vez. Foram encontradas evidências da associação planta-endofítico em tecidos vegetais fossilizados presentes em Rhynie Chert, material fossilífero encontrado no interior da Inglaterra. Rhynie Chert possui a primeira evidência fóssil das plantas terrestres, datadas do Devoniano, com cerca de 396 milhões de anos (Krings *et al.*, 2007). A partir dessas evidências, supõe-se que diversos tipos de interações entre vegetais e endófitos podem ter sido estabelecidas ao longo dos anos, incluindo-se relações de

especificidade entre os micro-organismos e a planta hospedeira (Tan & Zou, 2001; Strobel, 2002) e que podem estar relacionadas com a distribuição dos hospedeiros (Arnold *et al.*, 2010). Estes simbiontes fúngicos apresentam profundos efeitos na ecologia, adaptação e evolução das plantas (Brundrett, 2006; Arnold *et al.*, 2010), porém existem poucos trabalhos que abordam a biogeografia de micro-organismos (Fierer & Jackson, 2006; Martiny *et al.*, 2006).

Biogeografia é o estudo da distribuição da biodiversidade ao longo do espaço e tempo (Martiny *et al.*, 2006). Desde o século dezoito os biólogos têm investigado a biogeografia e diversidade de plantas e animais e mais recentemente os micro-organismos têm sido inseridos nestes estudos (Martiny *et al.*, 2006). Micro-organismos foram considerados como cosmopolitas por muitos anos por possuírem tempos de geração curtos, grandes populações e terem a capacidade de serem dispersos por longas distâncias (Fenchel & Finlay, 2004; Queloz *et al.*, 2011), o que levou a elaboração da teoria de Baas Beckin: "tudo esta em toda parte, mas o ambiente seleciona" ("everything is everywhere, but the environment selects") também conhecida como hipótese EisE (O´Malley, 2007). Entretanto esta hipótese tem sido muito discutida após o advendo das metodologias moleculares para a taxonomia de micro-organismos, o que permitiu a identificação das espécies de forma mais precisa.

Muitos estudos encontraram correlações entre a composição de micro-organismos e características ambientais e geográficas, tais como salinidade, profundidade e latitude (Martiny *et al.*, 2006). Fungos endofíticos já foram investigados em várias escalas espaciais, que vão desde pequenos estudos sobre os padrões de distribuição em uma única folha à aqueles em níveis geográficos (Carrol *et al.*, 1995). Estudos em nível geográfico comparam fungos endofíticos de hospedeiros semelhantes ou relacionados em diferentes áreas geográficas. Estes trabalhos podem considerar hospedeiros em uma distribuição contínua ou disjunta. Hospedeiros em distribuição disjunta são aqueles que são relacionados, mas separados geograficamente (Carrol *et al.*, 1995). Sabe-se que muitos micro-organismos endofíticos exibem padrões de diferenciação genética, morfológica e funcional que estão relacionados à distribuição dos seus hospedeiros (Martiny *et. al.*, 2006). Porém poucos estudos têm comparado os endófitos associados a hospedeiros em ambientes tropicais e temperados para determinar os possíveis efeitos climáticos que poderiam causar diferenças na população de fungos endofíticos (Taylor *et al.*, 1999). Taylor *et al.* (1999) compararam a população de fungos endofíticos associados à palmeiras presentes em regiões temperadas e tropicais. Foram coletadas folhas da palmeira *Trachycarpus fortunei* na China (dois locais), Austrália e Suíça. Houve uma grande sobreposição entre as comunidades de fungos endofíticos encontradas. A maior foi entre os fungos isolados das palmeiras na China, com 14 gêneros sendo encontrados nos dois locais de coleta. A sobreposição entre os gêneros

encontrados na China, Austrália e Suíça foi menor que 10 gêneros para cada local. Oito gêneros encontrados na Austrália também ocorreram na Suíça. Esta diferença na composição de fungos endofíticos parece ser influenciada por fatores climáticos. Fisher *et al.* (1995), por exemplo, isolaram fungos endofíticos de *Gynoxis oleifolia* no Equador. Quando coletadas amostras dentro de latitudes tropicais, mas em habitat temperado (por exemplo, altas altitudes), a composição de endofíticos obtidos foi análoga a aqueles de hospedeiros em habitats temperados. Vujanovic *et al.* (2006) avaliaram a distribuição de espécies de *Fusarium* associadas à *Asparagus officinalis* L. (Aspargos) presentes em 52 plantações no Canadá. Estas plantações estavam incluídas em cinco áreas climáticas. Amostras de plantas saudáveis, doentes e do solo foram coletadas. Observou-se que a abundância de fungos filamentosos tendia a diminuir ao longo de um gradiente de temperatura, das regiões mais quentes do sul para as áreas mais frias a nordeste. Além disso, foi observado que espécies pertencentes ao complexo de *Fusarium* "vermelhos" eram mais adaptadas ao clima da região Nordeste. Estes autores concluíram que espécies de *Fusarium* podem flutuar dentro e entre cultivares depedendo da estação climática. Tais flutuações podem variar de acordo com a localização geográfica, como observado quando comparado com plantações de Aspargos norte-americanos e de outros continentes.

A fim de estudar os padrões de distribuição biogeográfica de fungos endofíticos foram selecionados hospedeiros relacionados filogeneticamente pertencentes à tribo Myrtae (Myrtaceae). Este grupo foi escolhido por formar um grupo filogeneticamente coeso (Wilson *et al.*, 2001), desta forma seria possível determinar se a distribuição fungos endofíticos era devido a condições ambientais contemporâneas ou relacionadas a efeitos de contingência histórica (Martiny *et al.*, 2006). Análises filogenéticas, moleculares e morfológicas sugerem a origem da família Myrtaceae na Gondwana, sendo que a tribo Myrtae provavelmente se originou e diversificou na Australasia, entre 77 e 56 milhões de anos atrás, quando a Australia estava conectada à América do sul por uma ponte de terra formada pela Antartica (Lucas *et al.*, 2007). A tribo Myrtae representa um grupo monofilético e acredita-se que os eventos de especiação são rápidos e recentes, sendo os movimentos de longa distância a mais provável explicação para explicar movimentos intercontinentais ao invés de eventos de vicariância (Lucas *et al.*, 2007).

4. Substâncias bioativas produzidas por fungos endofíticos

A natureza tem sido fonte de agentes medicinais por milhares de anos e continua sendo uma abundante fonte de novos modelos estruturais para o desenvolvimento de diferentes substâncias de interesse medicinal. Produtos naturais ou metabólitos secundários são definidos como substâncias de baixo peso molecular que, aparentemente, não são necessários para o

crescimento do micro-organismo e são produzidos em resposta a condições ambientais específicas (Demain, 1981). Estes produtos exercem um enorme impacto na medicina moderna, uma vez que 40% dos fármacos prescritos são baseados neles. Além disso, de acordo com o "Food and Drug Administration" (USA FDA), órgão americano de controle e registro de fármacos, 49% das novas substâncias químicas bioativas registradas são produtos naturais ou seus derivados (Brewer, 2000).

Antibióticos são produtos naturais orgânicos de baixo peso molecular produzidos por micro-organismos e que são ativos em baixas concentrações contra outros micro-organismos (Demain, 1981). A introdução dos antibióticos sulfonamida (1930) e da penicilina (1940) revolucionaram a prática médica pela diminuição das taxas de mortalidade relacionadas às doenças infecciosas (Butler & Buss, 2006). Isto despertou o interesse da utilização de micro-organismos como fonte para a descoberta de metabólitos secundários com atividade contra patógenos humanos e de plantas (Strobel & Daisy, 2003).

A história científica mostra que as substâncias isoladas dos micro-organismos renderam algumas das drogas mais importantes utilizadas na clínica. Entre estas se destacam as penicilinas, cefalosporinas, aminoglicosídeos, tetraciclinas; ciclosporina, FK506 e rapamicina; agentes que diminuem o colesterol, como mevastatina e lovastatina; drogas antiparasitárias e antihelmínticas, tais como a ivermectina (Cragg *et al.*, 2005). Além disso, vários metabólitos com atividade quimioterápica também foram isolados a partir de micro-organismos, como antraciclina e bleomicina. A partir destes fatos e da descoberta que o fungo endofítico *Taxomyces andreanae*, isolado do vegetal *Taxus brevifolia*, produz o diterpeno paclitaxel, um dos agentes anticâncer mais utilizado na clínica atualmente, aumentaram substancialmente os estudos dos fungos endofíticos como fonte de substâncias bioativas (Stierle *et al.*, 1995).

Os produtos naturais são metabólitos de grande importância nas interações entre o endofítico e a planta hospedeira e podem atuar em processos de sinalização, defesa e regulação da simbiose (Schulz & Boyle, 2005). Fungos endofíticos estão em constante interação com a planta hospedeira e são tidos como excelentes fontes de produtos naturais bioativos, uma vez que podem ocupar nichos biológicos únicos e crescem em diversos ecossistemas (Schulz *et al.*, 2002). De fato, vários trabalhos demonstraram o potencial dos fungos endofíticos como produtores de substâncias bioativas (Strobel *et al.*, 2002; Bills *et al.*, 2002) ou como promissores para futuros estudos de novas substancias bioativas, avaliados por trabalhos de triagem antimicrobiana contra micro-organismos de interesse clínico (Huang *et al.*, 2001; Peláez *et al.*, 1998; Souza *et al.*, 2004; Phongpaichit *et al.*, 2006; Sette *et al.*, 2006; Vaz *et al.*, 2009, Vieira *et al.*, 2012).

A pesquisa por novos antibióticos declinou significativamente nos anos de 1990. Este declínio foi consequência de diversos fatores, dentre os quais incluem principalmente a dificuldade de isolamento e elucidação estrutural de alguns produtos naturais, a falta de interesse das grandes indústrias farmacêuticas e a forte competição com as tecnologias de síntese química combinatorial associada à triagem de alto fluxo e tecnologias mais rápidas para programas de bioprospecção (Peláez, 2006). Como consequência, nos últimos 20 anos, houve um declínio em 56% no número de antibióticos aprovados pelo "Food and Drug administration" (FDA) (Butler & Buss, 2006), sendo que a maioria dos novos fármacos lançados no mercado durante esse período pertencia a antigas classes de antibióticos, como os β-lactâmicos e macrolídeos (Peláez, 2006).

Diferentes estratégias são adotadas para a seleção do grupo vegetal para o isolamento de fungos endofíticos produtores de substâncias bioativas. Dentre elas, o estudo de plantas com caráter etnobotânico, endêmicas, presentes em ambientes com elevada diversidade e inexplorados são bastante promissores em estudos de bioprospecção (Strobel & Daisy, 2003). O conhecimento da existência de micro-organismos endofíticos levou ao desenvolvimento de novos programas de triagem para descoberta de novas drogas. Identificar micro-organismos endofíticos produtores da mesma substância bioativa produzida pelos vegetais eliminaria as etapas de plantio, colheita e extração de plantas raras ou de crescimento lento. Além disso, o preço do produto seria reduzido, uma vez que poderia ser produzido por meio de processos fermentativos (Strobel, 2002). No presente trabalho foram selecionados hospedeiros vegetais presentes no ecossistema Cerrado, o qual abriga uma diversa população de fungos endofíticos (Furlanetto & Dianese, 1997). Os hospedeiros selecionados apresentavam histórico etnobotânico, ou seja, plantas que são utilizadas tradicionalmente como medicamento por tribos, grupos étnicos e pela população de um modo geral. Neste contexto, é razoável supor que a atividade farmacológica atribuída a algumas espécies vegetais possa estar relacionada, de alguma forma, às substâncias produzidas por micro-organismos endofíticos ou pela planta (Arnold *et al.*, 2001; Ferrara, 2006).

Cabe ressaltar ainda que grande parte das substâncias bioativas obtidas a partir de extratos fúngicos podem ser empregadas diretamente como fármacos, ou mesmo serem modificadas de modo a se obter uma nova molécula com diferentes propriedades, tais como o aumento da atividade, toxicidade seletiva e a diminuição de efeitos colaterais indesejáveis. Mesmo quando estas substâncias não apresentam a atividade esperada, elas podem servir como protótipos para o planejamento e desenvolvimento de novas moléculas, o que evidencia ainda mais a importânciado estudo de fungos endofíticos como fontes de novas substâncias.

1 Objetivos

1.1 Geral

Caracterizar a diversidade de fungos endofíticos associados à Myrtaceae presentes em ecossistemas do Brasil, Argentina e Espanha. Avaliar o potencial destes fungos como produtores de substâncias antimicrobianas de interesse biotecnológico e estudar o seu papel como promotores do crescimento vegetal.

1.2 Específicos

- Isolar e caracterizar as comunidades de fungos endofíticos provenientes de ecossistemas de Mata Atlântica, Mata Amazônica, Cerrado, Andino patagônico e Mediterrâneo;
- Identificar os fungos filamentosos endofíticos por meio do sequenciamento da região ITS do gene do rRNA.
- Verificar atividade antimicrobiana dos extratos metanólicos dos fungos isolados de plantas de ecossistemas de Mata Atlântica, Mata Amazônica, Cerrado brasileiros sobre bactérias e leveduras de interesse clínico;
- Avaliar o padrão de distribuição de fungos endofíticos em plantas filogeneticamente relacionadas da família Myrtaceae presentes em ecossitemas de Mata Atlântica (Brasil) e Andino patagônico (Argentina).
- Estudar o papel de fungos endofíticos associados à raízes de *Myrtus communis* na colonização e peso seco de raízes de plantas de sorgo (*Sorghum vulgare*) e avaliar seu efeito na simbiose com micorrizas arbusculares.

Capítulo 2

Diversity and antimicrobial activity of fungal endophyte communities associated with plants of Brazilian savanna ecosystems

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Full Length Research Paper

Diversity and antimicrobial activity of fungal endophyte communities associated with plants of Brazilian savanna ecosystems

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Fungal endophyte communities associated with leaves of *Myrciaria floribunda*, *Alchornea castaneifolia*, and *Eugenia* aff. *bimarginata* were examined, collected from Brazilian Cerrado ecosystems, and studied for their ability to produce antimicrobial activity. A total of 93 isolates of endophytic fungi were obtained and identified by sequencing of internal transcribed spacer (ITS) regions of the rRNA gene, which revealed the presence of 20 *Ascomycota* and three *Basidiomycota* taxa. The genus *Colletotrichum* is the most frequent endophyte associated with *M. floribunda* and *A. castaneifolia*. *Mycosphaerella* is the most frequent genus associated with *E.* aff. *bimarginata*. All fungal endophytic isolates were cultured and the crude extracts were screened to examine the antimicrobial activities against pathogenic microorganisms. Thirty-eight fungal extracts presented antimicrobial activity against at least one of the different target microorganisms tested. *Emericellopsis donezkii* and *Colletotrichum gloesporioides* showed the best minimum inhibitory concentration (MIC) values, which were lower or similar to MICs of known antibacterial and antifungal drugs. Our results suggest that the plants of Brazilian Cerrado shelter a diverse endophytic fungal community, which includes bioactive taxa capable of producing promising antimicrobial metabolites.

Key words: Antifungal and antibacterial activities, Cerrado ecosystem, endophytic fungi, fungal ecology.

INTRODUCTION

Endophytes are microorganisms that inhabit healthy plant tissues during at least one stage of their life cycle without causing any apparent symptom of disease or negative effects on the hosts (Petrini, 1992). Endophytes have been isolated from all plants previously studied, including bryophytes (U'Ren et al., 2010), pteridophytes (Petrini et al., 1992), gymnosperms (Soca-Chafre et al., 2011), and both monocotyledonous (Pinruan et al., 2010) and dicotyledonous angiosperms (Vaz et al., 2009, Rosa et al., 2010). These fungi colonise on all available tissues, such as roots, stems, leaves, bark, fruits, seeds, and floral organs (Petrini et al., 1992; Rodriguez et al., 2009; Vaz et al., 2009). Plants from temperate environments may harbour dozens of endophytic fungi. Many studies on tropical plants have also documented remarkable

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endophyte richness (Arnold and Lutzoni, 2007). Most endophytic fungi isolated are ascomycetous, and *Sordariomycetes* and *Dothideomycetes* classes contain the majority of foliar fungal endophyte species (Arnold and Lutzoni, 2007). Different plant hosts have been studied as targets of fungal endophytes, which can represent a rich source of bioactive metabolites. The secondary metabolites produced by fungal endophytes play an important role in metabolic interactions between microorganisms and their plant hosts, such as signalling, natural defence, and regulation of the symbiosis (Schulz and Boyle, 2005).

According to Strobel (2003), plants living in a unique ecosystem may shelter a specific fungal community, which can be a source for a variety of bioactive metabolites with potential for pharmacological and agrochemical applications. The Brazilian Cerrado is a biome composed of savanna and forest ecosystems that cover approximately 2 million km^2, representing *ca*. 22% of the land surface of Brazil, plus small areas in eastern Bolivia and north western Paraguay. Some research has shown that the Cerrado ecosystems can be harbouring a large and diverse population of plant-associated fungi with many new fungal taxa (Furlanetto and Dianese, 1997). Although interest has increased in the microbial biodiversity in Brazilian Cerrado, the diversity, taxonomic composition, host affinity, and the biotechnological potential of fungi from this ecosystem remain unclear. In this context, the aims of this work were to characterise the endophytic fungal community associated with three plants from Cerrado ecosystems and to determine the ability of these fungi to produce antimicrobial metabolites.

MATERIALS AND METHODS

Strategy of host species selection

In South America, the *Eugenia* genus occurs in Argentina, Uruguay, Paraguay, and Brazil, and some species have been used as food or medicine. The antioxidant, anti-inflamatory and cytotoxic and antimicrobial activities of *Eugenia* species have been reported (Lago et al., 2011; Stefanello et al., 2011). *Eugenia* aff. *bimarginata* Berg. (*Myrtaceae*) was selected to recover fungal endophytes due to its high abundance in the collection area. *Myrciaria floribunda* O. Berg (H. West ex Willd) (*Myrtaceae*) is native of Central and South America. Also known as rum berry or guava berry, *M. floribunda* has been grown for its edible fruits; moreover, Apel et al. (2006) showed that its essential oils have antimicrobial, anti-inflammatory, and antitumor activities. *Alchornea castaneifolia* (Willd) Juss. (*Euphorbiaceae*) is a native medicinal plant of the Amazon and Brazilian Cerrado forests that has been used for centuries by indigenous peoples of the Amazon against skin diseases, ulcers, malaria, inflammatory reactions, and pathogenic microorganisms (Hiruma-Lima et al., 2006; Costa et al., 2008).

Plant collection and identification

The plant specimens were collected in two ecological reserves of Tocantins state, North Brazil. The first area was Jalapão State Park (10°36'043"S, 46°35'836"W, elevation of approximately 740 m), which is the most extensive protected area in the Tocantins state, consisting of a mosaic of Cerrado physiognomies in transition to xerophilic "Caatinga" ecosystems. The second area was Cantão State Park (09°20'526"S, 49°58'347"W, elevation of approximately 170 m), a protected area located in the West of the Tocantins state, which represents an ecotone area among Cerrado, Amazon forest, and Pantanal ecosystems (Santos and Lolis, 2007). *E.* aff. *bimarginata* was collected at Jalapão State Park and *M. floribunda* and *A. castaneifolia* were collected at Cantão State Park in July 2007. All plant vouchers were deposited at the herbarium of the Institute of the Ciências Biológicas (BHCB) of the Universidade Federal of Minas Gerais, Brazil (http://sciweb.nybg.org/science2/IndexHerbariorum.asp).

Endophytic fungal isolation

Leaves from 30 individuals of each plant species were sampled. Three leaves of each individual were collected, placed in sterile plastic bags and stored less than 24 h at 10°C before the isolation of the endophytic fungi. From the leaves, three fragments (approximately 0.5 cm in length and 0.5 cm wide) of each leaf were plated. The fragments were surface sterilised by immersion in ethanol 70% (1 min) and 2% sodium hypochlorite (3 min), followed by washing with sterile distilled water (2 min) (Collado et al., 1996). The fragments were plated on Petri dishes containing potato dextrose agar (PDA, Difco, USA) supplemented with Chloramphenicol at 100 µg/ml (Collado et al., 1996) and were incubated at 25°C. In order to test the effectiveness of surface sterilisation, 100 µl of the final rinsing water was plated on PDA and incubated at 28°C. Hyphal growth was monitored over an eight-week period. Using aseptic technique, endophytes were transferred to axenic culture on PDA in 60 mm Petri plates and photographed after complete growth. Long-term preservation of the filamentous fungal colonies was carried out in cryotubes with 30% sterile glycerol at -80°C and in sterile distilled water at room temperature. All pure cultures of the endophytic fungal isolates were deposited into the Culture Collection of Microorganisms and Cells of Universidade Federal of Minas Gerais. For calculation purposes and statistical analysis, each individual tree was considered a sample unit. When two or more fungal isolates from the same sample were identified as belonging to the same species, they were considered to be a single isolate. The total occurrence of filamentous fungi and yeasts corresponded to the total number of isolates in each sample.

Fungal identification

Pure cultures of the fungal isolates were grouped based on morphological characters including aerial mycelium form, colony colours, surface texture and margin characters. Because most endophytes did not produce sexual or asexual reproduction structures in regular culture media, at least fifty percent of each morphospecies was selected for identification by sequencing of the ITS region of the rRNA gene. The protocol for DNA extraction of the endophytic fungi was performed according to Rosa et al. (2009). The ITS domains of the large subunit of the rRNA gene were amplified using the universal primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´), as described by White et al. (1990). Amplification of ITS and sequencing protocols were

performed as described to Vaz et al. (2009). Sequencing was carried out using an ET dynamic terminator kit in a MegaBACE 1000/automated 96 capillary DNA sequencer (GE Healthcare, Buckinghamshire, UK). Consensus sequence data of the endophytic fungi were deposited in GenBank under the accession numbers FJ466701 to FJ466730 Phylogenetic relationships were estimated using MEGA program Version 4.0 (Tamura et al., 2007). The Maximum Composite Likelihood model was used to estimate evolutionary distance with bootstrap values calculated from 1,000 replicate runs. In the presented study, a 3% cut-off value was used to define the species level based on the sequencing of the ITS domains (Nilsson et al., 2008).

Endophyte richness, diversity, and similarity among plant hosts

Species accumulation curves were generated for each plant species using EstimateS, version 8.0 (Colwell, 2005). Normality testing was performed using the Shapiro-Wilk test using a software package R (R Development Core Team, 2005). To examine similarity of the fungal communities among tree hosts we used similarity Sorenson's and Jaccard indexes based on presence/absence data only. In this study the individual plant was considered the sample unit, being each of the three sampled leaves considered a replicate for calculation purposes. The Sorenson's similarity coefficient (C_S) was calculated according to the following formula:

$$Cs=2c/(a+b),$$

And the Jaccard similarity coefficient (Cj) is defined by the formula:

$$Cj=c/a+b+c,$$

Where c; the number of fungal species coexisting in both plants, a; the total number of fungal species in one plant, and b is the total number of fungal species on the other plant.

All results were obtained with 95% confidence, and the boostrap values were calculated from 1.000 iterations. The antimicrobial results were submitted to a preliminary multiple correspondence analysis (MCA) as described in Vaz et al. (2009). Twelve variables were analyzed: plant (*M. floribunda*, *E.*aff. *bimarginata* and *A. castaneifolia*), antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Salmonella typhimurium*, *Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis* and *Cryptococcus neoformans* (yes or no for each antimicrobial activity). All samples are illustrated in the graphic, but only the representative samples are considered for the results analysis. The MCA and classification analysis were conducted using SPAD 5.5 software (SPAD, Paris, France).

Antimicrobial assays

In vitro antimicrobial susceptibility tests were performed using the protocols described by Vaz et al. (2009) with a panel of the following eleven microorganisms: *E. coli* ATCC 25922, *S. aureus* ATCC 12600, *P. aeruginosa* ATCC 27853, *B. cereus* ATCC 11778, *S. typhimurium* ATCC 14028, *C. albicans* ATCC 18804, *C. krusei* ATCC 2159, *C. parapsilosis* ATCC 22019, *C. glabrata* ATCC 2001, *C. tropicalis* ATCC 750 and *C. neoformans* ATCC 32608. Inoculum of the target microorganisms were adjusted to a McFarland no. 1 standard in optical density for yeasts, corresponding to 3×10^8 cfu/ml. A McFarland 0.5 standard in optical density for bacteria corresponded to 1 to 2×10^8 cfu/ml. The concentrations were confirmed via spectrophotometer readings at 580 and 626 nm for yeasts and bacteria, respectively. Yeast samples were inoculated using a swab on Sabouraud agar (1% peptone, 4% dextrose and 2% agar), and the bacteria were plated on Brain Heart Infusion agar (Difco, USA) also using a swab.

Crude extract stock solutions at 10 mg/ml were prepared in dimethyl sulphoxide (DMSO, Merck, USA) and stored at -40°C. These solutions were diluted with water and assayed at a final concentration of 100 µg/ml (DMSO concentrations remained below 1%). The extract solutions at 10 mg/ml in DMSO were applied (10 µl) on disk blanks on the Petri dishes containing the target microorganisms and incubated for 24 to 48 h at 37°C. After incubation, the inhibition zones around the application points were measured. Amphotericin B (Sigma, USA) and chloramphenicol (Sigma, USA), both at 100 µg/ml, were used as positive controls for yeasts and bacteria, respectively. Solvent (DMSO) and extracts of culture media were used as the negative controls.

Minimum inhibitory concentration

All methanolic extracts that presented antimicrobial activity in the screening above were evaluated for the minimum inhibitory concentration (MIC) against the same target microorganisms used in the screening test. The MIC values of the methanolic extracts were determined using the agar microdilution method according to the National Committee for Clinical Laboratory Standards protocols against bacteria and yeasts (NCCLS, 2004). The MICs values were defined as the lowest concentration of test samples that inhibited the visible growth of microorganisms.

RESULTS

Fungal endophyte communities

A total of 93 isolates of endophytic fungi were obtained (21 recovered from *M. floribunda*, 37 from *A. castaneifolia*, and 35 from *E.* aff. *bimarginata*), which were grouped, and 41 representative fungal isolates were identified by sequencing the ITS region of the large subunit of the rRNA gene. Molecular identification revealed 23 different endophytic taxa, from which 20 represented *Ascomycota* and three *Basidiomycota* species (Table 1). The most frequent endophytic fungus isolated from *M. floribunda* and *A. castaneifolia* was *Colletotrichum gloesporioides* (Penz.) Penz. and Sacc. (anamorph of *Glomerella cingulata*).

Ten fungal endophytes showed high divergence in the ITS region when compared with fungal sequences of other fungi deposited in the GenBank database and were identified at the genus level. These endophytes were identified as belonging to two genera of Sordariomycetes, four genera of Dothideomycetes and one genus of Leotiomycetes. The phylogenetic position of the Sordariomycetes endophytic fungi isolated in the present

Table 1. Frequency of isolation for the endophytic fungi obtained from *Eugenia* aff. *bimarginata*, *Myrciaria floribunda*, and *Alchornea castaneifolia* and closest related species according to % similarity of the ITS region of the rRNA gene by alignment with sequences of related species retrieved from the GenBank database.

UFMGCB code	Closest related species	Similarity	No. of bp analyzed	Identification and GenBank accession number	Number of isolates/abundance (%)		
					EB	MF	AC
2013	*Aspergillus sydowii* [AY373868]	99	528	*Aspergillus sydowii*	1/2.86	-	-
1989, 1990	*Botryosphaeria dothidea* [AY259092]	96	513	*Botryosphaeria* sp. [HQ184171]	-	3/14.29	-
2029	*Cladosporium cladosporioides* [GQ221853]	98	510	*Cladosporium cladosporioides*	3/8.57	-	-
1937, 1997, 2003, 2004	*Colletotrichum gloeosporioides* [EU552111]	98	534	*Colletotrichum gloeosporioides*	3/8.57	8/38.10	14/37.84
1952	*Diaporthe phaseolorum* [AY577815]	99	531	*Diaporthe phaseolorum*	-	-	1/2.70
1941, 1967, 1971	*Didymella bryoniae* [EU167573]	97	526	*Didymella bryoniae*	-	-	7/18.92
1984	*Trametes* sp. [AF519892]	95	604	*Trametes* sp. [HQ184178]	-	1/4.76	-
1966, 2001	*Emericellopsis donezkii* [AY632658]	99	502	*Emericellopsis donezkii*	-	-	2/5.41
1967	*Eutypella scoparia* [EU436688]	96	526	*Eutypella* sp. [HQ184171]	-	-	1/2.70
2014	*Filobasidium capsuligenum* [AF444382]	84	570	*Filobasidium* sp.[HQ184180]	2/5.71	-	-
1974, 1978	*Fusarium decemcellulare* [FJ545369]	97	521	*Fusarium decemcellulare*	-	4/19.05	-
1936, 1953, 1955, 2043	*Gibberella moniliformis* [GQ168842]	99	501	*Gibberella moniliformis*	3/8.57	-	9/24.32
2045	*Glomerella acutata* [DQ286133]	99	8536	*Glomerella acutata*	-	4/19.05	-
2024, 2026, 2038	*Mycosphaerella pseudoellipsoidea* [EU167585]	97	442	*Mycosphaerella* sp. [HQ184178]	11/31.43	-	-
1976	*Neofusicoccum batangarum* [FJ900608]	99	512	*Neofusicoccum batangarum*	-	1/4.76	-
1932	*Nigrospora oryzae* [DQ219433]	98	483	*Nigrospora oryzae*	-	-	1/2.70
2058	*Letendraea helminthicola* [EU715680]	94	612	*Letendraea* sp. [HQ184176]	3/8.57	-	-
1960	*Penicillium sclerotiorum* [AF033404]	98	467	*Penicillium sclerotium*	2/5.71	-	2/5.41
2037	*Phaeosphaeriopsis musae* [DQ885894]	92	516	*Phaeosphaeriopsis* sp. [HQ184175]	1/2.86	-	-
2033	*Pseudeurotium desertorum* [AY129288]	94	519	*Pseudeurotion* sp. [HQ184177]	2/2.86	-	-
2012, 2060	*Pseudozyma* sp. [AJ876488]	91	545	*Pseudozyma* sp. [HQ184179]	2/5.71	-	-
2036	*Rhytidhysteron rufulum* [AM711974]	98	415	*Rhytidhysteron rufulum*	3/5.71	-	-
2020	*Xylaria* sp. [FJ175168]	99	552	*Xylaria* sp. [HQ184172]	1/2.86	-	-

UFMGCB: Culture collection of the Universidade Federal of Minas Gerais. EB: *Eugenia* aff. *bimarginata*, MF: *Myrciaria floribunda*, AC: *Alchornea castaneifolia.*

work is shown in Figure 1. The isolate UFMGCB 2020 presented 99% similarity with Xylaria sp. (GenBank access number FJ175168) and 94% sequence similarity with isolates of endophytic fungi associated with the bryophyte Herbertus alpines and the plant Plagiochila ramosissima from New Zealand (Table 1). In addition, the UFMGCB 2020 isolate showed 93% similarity with 18 nucleotide differences and 12 gaps with Xylaria castorea (GenBank access number AF163030). These fungi formed a branch in the phylogenetic tree that was strongly supported (Figure 1).

The UFMGCB 1967 isolate showed 15 nucleotide differences with sequence of *Eutypella scoparia* (GenBank access number EU436688), which was obtained as an endophytic fungus associated with the tropical fruit tree *Garcinia dulcis* in southern Thailand. Figure 2 shows the phylogenetic position of the fungal species of *Dothideomycetes* isolated in our study. The UFMGCB 1990 isolate showed 99% similarity with *Botryosphaeria* sp. (GenBank access number DQ480360), an endophytic fungus associated with *Garcinia* sp. plant hosts in southern Thailand, and 96% similarity with *Botryosphaeria dothidea* isolated from *Vitis vinifera* (Vitaceae) in Spain (GenBank access number EU650670); our isolate was identified as *Botryosphaeria* sp. The UFMGCB 2026 isolate showed 97% similarity with *Mycosphaerella pseudoellipsoidea* (EU167585) and *M. gregaria* (EU167580), which were obtained from leaves of *Eucalyptus nitens* and *Eucalyptus globule*s in South Africa and Western Australia, respectively. In our work, this isolate was identified as *Mycosphaerella* sp.

The UFMGCB 2058 isolate was identified as *Letendraea* sp. because it showed 94% query coverage and 94% similarity with an endophytic fungus *Letendraea helminthicola* strain SGLMf23 (EU715680) isolated from *Taxus globosa* in Mexico. The UFMGCB 2037 isolate showed a 6% nucleotide difference when compared with *Ascochyta* sp. CBS 117477 (GU230751) and an 8% nucleotide difference in relation to the ITS sequence of *Phaeosphaeriopsis musae* (DQ885894). This isolate was putatively identified as *Ascochyta* sp. (Figure 2).

Figure 3 shows the phylogenetic position of the fungal species of *Leotiomycetes* isolated in our study. UFMGCB 2033 isolate showed a 6% difference with an uncultured fungus (FN397281) obtained from *Tuber melanosporum* truffle-ground soil and a 6% difference in relation to *Pseudeurotium desertorum* CBS 986.72 (AY129288). Based on these data, this isolate was identified as *Pseudeurotiom* sp. The yeast isolate UFMGCB 2012 presented 91% similarity with *Pseudozyma* sp. (GenBank access number AJ876488). The taxon UFMGCB 1984 showed 95% similarity with *Trametes* sp. (GenBank access number AF519892), *Coriolopsis gallica* (GenBank access number AY684172) and *Trametes troggi* strain 4222 (GenBank access number GU199348), and it was identified as *Trametes* sp. The isolate UFMGCB 2014 showed 84% similarity with the type strain of the yeast *Filobasidium capsuligenum* (GenBank access number AF444382), and it was identified putatively as *Filobasidium* sp.

The samples do not fit the assumptions of a normal distribution, which could be confirmed by the low *P* value in the Shapiro-Wilk test (*M. floribunda*, P=2.978 10^{-5}; *A. castaneifolia*, P=4.261 10^{-5}; and *E. bimarginata*, P=9.463 10^{-4}). The accumulation curves of *E.* aff. *bimarginata* and *A. castaneifolia* did not reach an asymptote, and the accumulation curve of *M. floribunda* approximated an asymptote (Figure 4). The similarity index values showed that the taxa composition was variable among the different hosts and the highest similarity was between *A. castaneifolia* and *E.* aff. *bimarginata* (Table 3). This result is likely related to the occurrence of 10 endophytic taxa only in *E.* aff. *bimarginata* samples, whereas six taxa occurred only in *A. castaneifolia* samples and five only in *M. floribunda*.

Antimicrobial activity

Thirty isolates (33.3%) displayed antimicrobial activity against at least one target microorganism with inhibition zones ranging from 7 to 35 mm in diameter. Table 2 shows that the MIC values of methanolic extracts from endophytic fungi ranged from 7.8 to 500 µg/ml against *C. krusei*, 7.8 to >1.000 µg/ml against *C. parapsilosis*, 62.5 to 500 µg/ml against *Cr. neoformans*, 250 µg/ml against *C. albicans* and >1.000 µg/ml against *C. glabrata*. In addition, the MIC values ranged from 62.5 to >1.000 µg/ml against *S. aureus* and 7.8 to >1.000 µg/ml against *B. cereus*. Differing from these, the MIC values for *S. thyphymurium* were higher than 1.000 µg/ml. The extracts of *C. gloesporioides* UFMGCB 2002 and *Emericellopsis donezkii* UFMGCB 2001 showed the strongest antifungal activities, and the best MIC values. Two isolates of *E. donezkii* (UFMGCB 1966 and UFMGCB 2001) showed the strongest antibacterial activity. The data were clustered into five classes using multivariate statistical analyses (Figure 5). The first group was characterized by extracts of endophytic fungi from the *M. floribunda* presenting activity against *S. typhimurium*, The second group was comprised of endophytic fungi from *A. castaneifolia*, and presented activity against *C. glabrata,* with no activity against *C. parapsilosis*. The fifth group was characterized by endophytic fungi collected from *E.* aff. *bimarginata* with activity against *C. parapsilosis* and *C. albicans.* No conclusion about the antimicrobial activity and the species host from which the fungi were collected from could be concluded for the third and fourth groups. The relative proportion of each group is showed in the Figure 5.

DISCUSSION

The most frequent endophytic fungus isolated from the two plant species, *M. floribunda* and *A. castaneifolia,* was *C. gloesporioides*. *C. gloesporioides* has been commonly isolated as an endophyte from several plant species (Joshee et al., 2009; Higgins et al., 2011; Vega et al., 2010), and it is considered a generalist species.

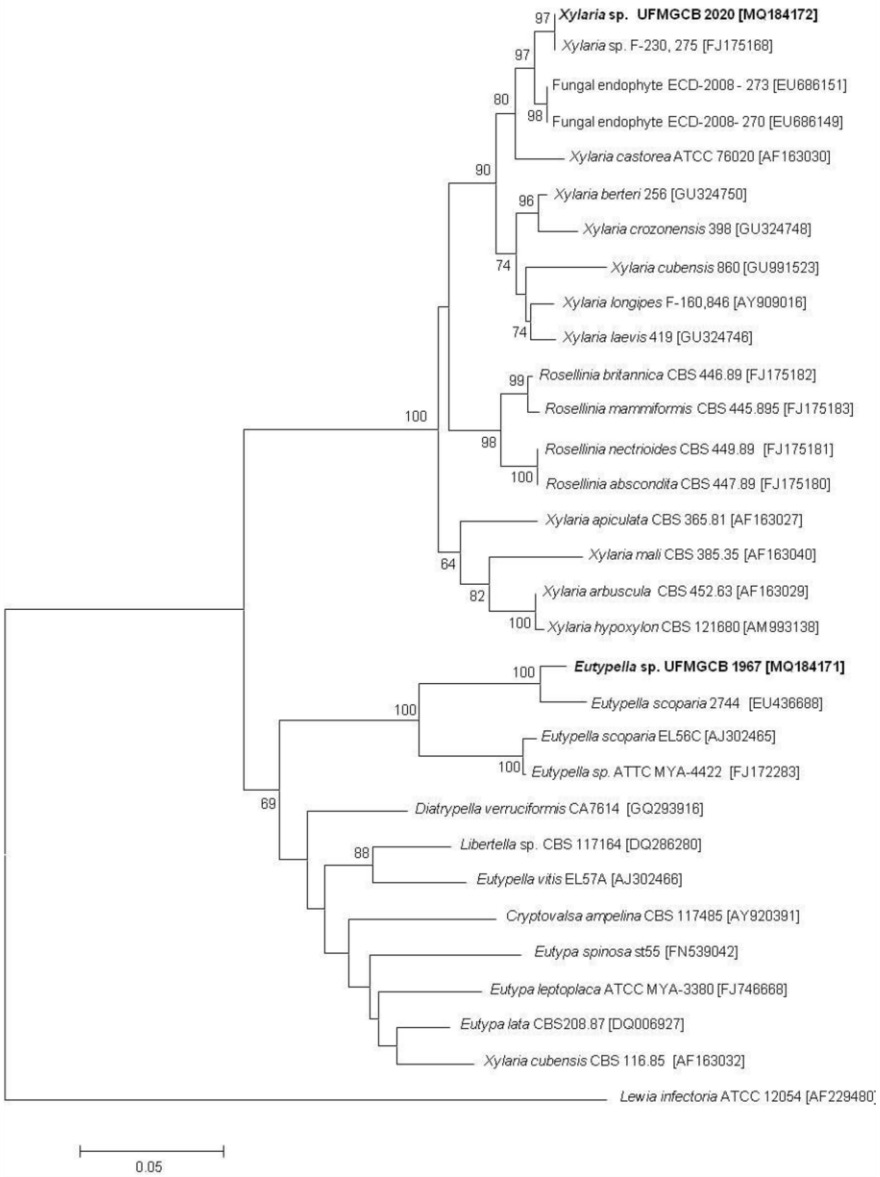

Figure 1. Phylogenetic tree based on the rRNA gene sequence (ITS1-5.8S-ITS2) showing the closest relatives of *Sordariomycetes* endophytic filamentous fungi isolated from *Alchornea castaneifolia*, *Eugenia* aff. *bimarginata* and *Myrciaria floribunda*. The tree was constructed by neighbour-joining analysis (maximum composite likelihood). Bootstrap percentages from 1,000 replicates are shown. The tree was rooted with *Lewia infectoria* ATCC12054 [AF229480] as the out-group.

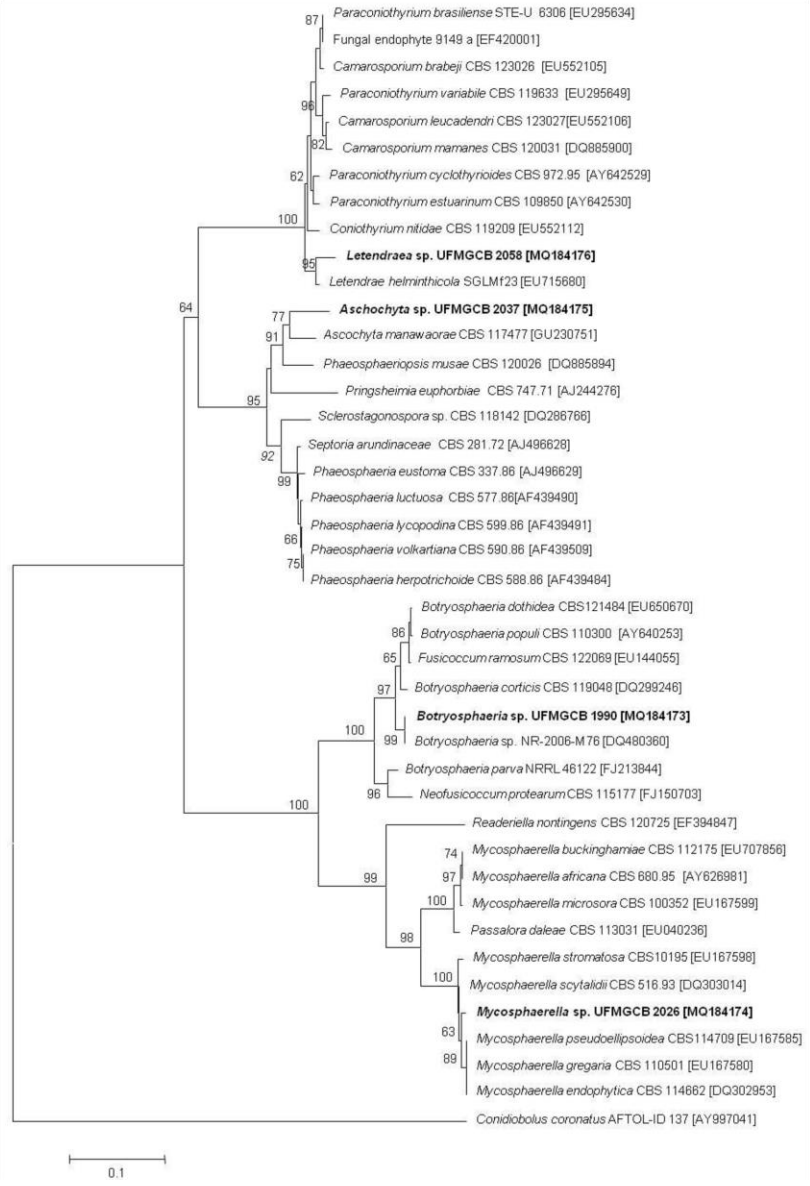

Figure 2. Phylogenetic tree based on the 5 rRNA gene sequence (ITS1-5.8S-ITS2) showing closest relatives of *Dothydeomycetes* endophytic filamentous fungi isolated from *Alchornea castaneifolia*, *Eugenia* aff. *bimarginata* and *Myrciaria floribunda*. The tree was constructed by neighbour-joining analysis (maximum composite likelihood). Bootstrap percentages from 1,000 replicates are shown. The tree was rooted with *Conidiobolus coronatus* AFTOL-ID137 [AY997041] as the out-group.

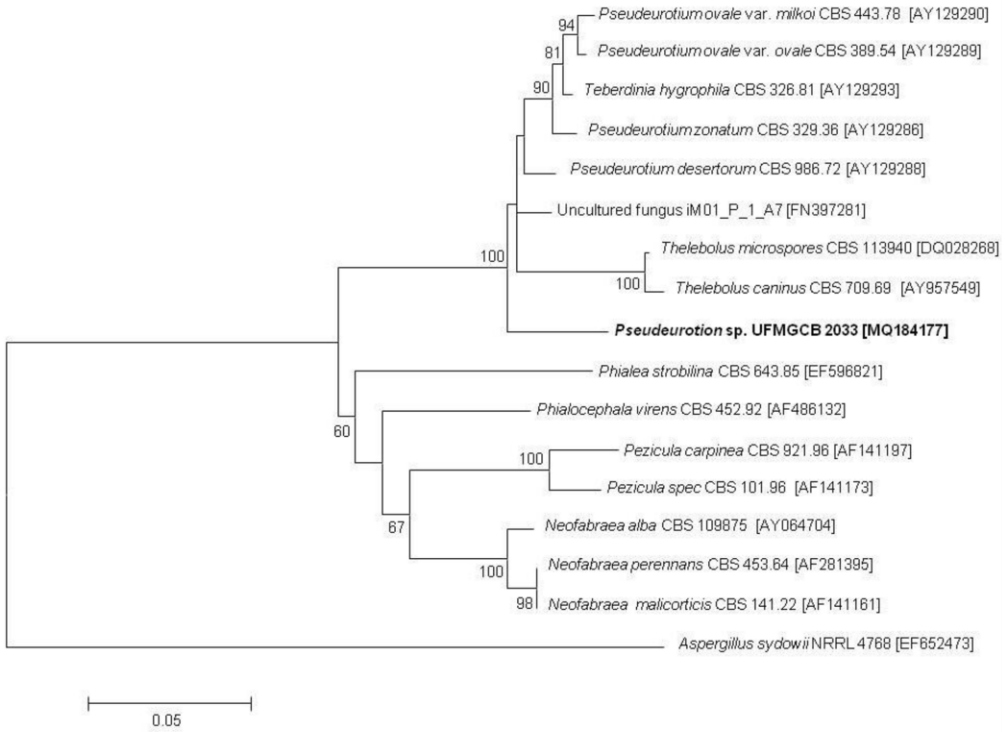

Figure 3. Phylogenetic tree based on the 5 rRNA gene sequence (ITS1-5.8S-ITS2) showing closest relatives of *Leotiomycetes* endophytic filamentous fungi isolated from *Alchornea castaneifolia*, *Eugenia* aff. *bimarginata* and *Myrciaria floribunda*. The tree was constructed by neighbour-joining analysis (maximum composite likelihood). Bootstrap percentages from 1,000 replicates are shown. The tree was rooted with *Aspergillus sydowii* [EF652473] as the out-group.

Mycosphaerella sp. was the most frequent fungus among the 13 species associated with *E.* aff. *bimarginata* in Jalapão State Park and may be considered prevalent among the fungal species associated with this host plant. *Mycosphaerella* species have been recovered as endophytes (Johsee et al., 2009; Márquez et al., 2010), although some species of *Mycosphaerella* are phytopathogenic fungi able to cause serious diseases in different plant species (Crous et al., 2009). *Giberella moniliformis* and *Didymella bryoniae* were also frequent in *A. castaneifolia* samples. *G. moniliformis* and *Penicillium sclerotiorum* were common to *A. castaneifolia* and *E.* aff. *bimarginata,* which were collected in different geographic areas presenting different phytophysiognomies. This result indicated that these two fungal species may be ubiquitous in Cerrado ecosystems. The species of *Colletotrichum*, *Gibberella*, and *Penicillium* are frequently found as endophytes in many tropical, temperate, and polar plants (Rosa et al., 2009; U'Ren et al., 2009; González et al., 2011) and have adapted to a wide range of geographical sites, climatic conditions, ecological habitats, and host plants. Ten isolates of endophytic fungi isolated in our work could represent new species, since their ITS sequences showed high divergence when compared with fungal sequences of other species deposited in GenBank. This result shows that the three plant species can be a reservoir of new fungal species.

The samples do not fit the assumptions of a normal distribution, and the accumulation curves of *Eugenia* aff. *bimarginata* and *A. castaneifolia* did not reach an asymptote, and this is a pattern frequently found

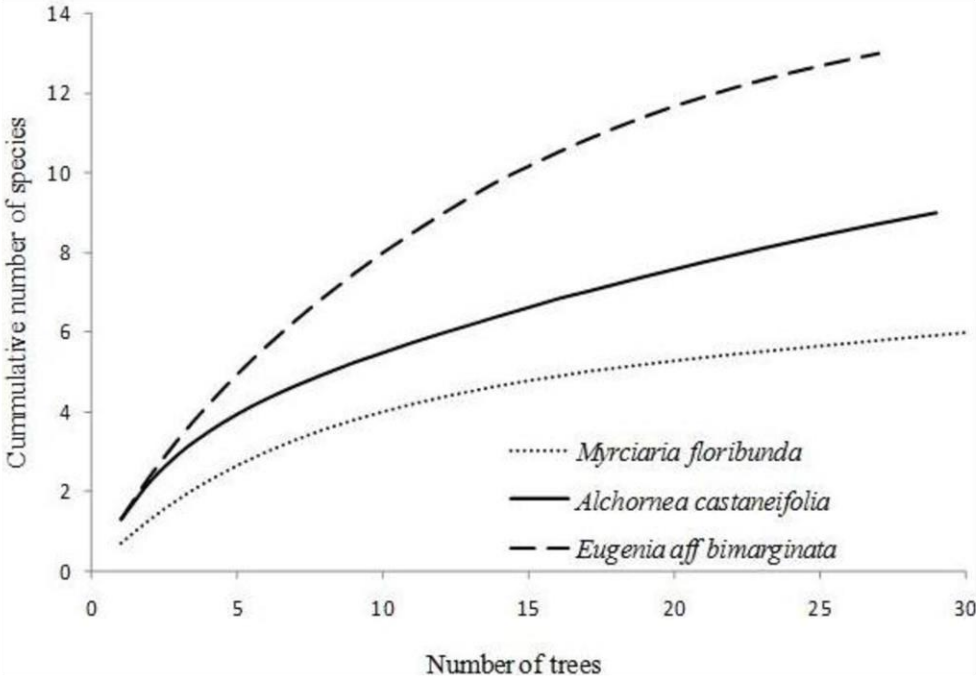

Figure 4. Species accumulation curves for fungal endophytes from healthy leaves of three host species (*Eugenia* aff. *bimarginata*, *Alchornea castaneifolia* and *Myrciaria floribunda*) based on the Mao Tao estimator calculated using EstimateS v.8.0 (6).

in samples from tropical studies (Gazis and Chaverri, 2010, Joshee et al., 2009). This result suggests that further sampling could recover other endophytic fungi associated with these plants. However, the accumulation curve of *M. floribunda* approximated an asymptote, which indicated that increasing sampling will not significantly increase the number of species found, although species richness in *M. floribunda* is lower than in the other host trees sampled.

The similarity index indicated a low similarity among the fungal communities of the host plants, and this result is likely related to the occurrence of 10 endophytic taxa only in *E.* aff. *bimarginata* samples, whereas six taxa occurred only in *A. castaneifolia* samples and five only in *M. floribunda*. It is also related to the common occurrence of accidental species, defined here as those with one or two isolates per host.

Brazil has a long tradition in medicinal plant use and many of them are used in the form of crude extracts, infusions or plasters to treat common infections. Several works have showed the capacity of endophytic fungi to produce bioactive natural products with potential therapeutic interest (Strobel et al., 2003; Rodriguez et al., 2009; Liu et al., 2010; Vaz et al., 2009). Therefore, considering that the plant species studied has a popular use to treat infectious disease we decided test the endophytic fungi associated with these plants against pathogenic microorganisms. Thirty fungal isolates displayed antimicrobial activity against at least one target microorganism. Isolates of *E. donezkii* and *C. gloesporioides* showed the best minimum inhibitory concentration (MIC) values, which were lower or similar to MICs of known antibacterial and antifungal drugs. One isolate each of *C. gloesporioides* (UFMGCB 2002) and *E. donezkii* (UFMGCB 2001) showed the strongest antifungal activities. The *Colletotrichum* genus includes phytopathogenic, saprophytic, and endophytic species (Joshee et al., 2009; Gazis and Chaverri, 2010). Several works have reported that *Colletotrichum* species are a potential source of bioactive metabolites, such as

Table 2. Minimal inhibitory concentration (MIC) in µg/ml against yeasts and bacteria of crude extracts obtained from endophytic fungi isolated from *Eugenia* aff. *bimarginata*, *Myrciaria floribunda*, and *Alchornea castaneifolia*.

Host plant	UFMGCB Code	Endophytic species	Individual tree	Yeasts					Bacteria		
				CA	CK	CP	CG	CN	SA	ST	BC
E. bimarginata	2027	*Gibberella moniliformis*	30	>1.000	-	-	-	250	-	-	1.000
	2015	*G. moniliformis*	11	-	-	1.000	-	-	-	-	-
	2025	*Mycosphaerella pseudoellipsoidea*	28	-	-	>1.000	-	-	-	-	-
	2031	*Mycosphaerella* sp.	5	-	500	-	-	-	-	-	250
	2032	*Mycosphaerella* sp.	6	-	-	-	-	-	1.000	-	-
	2038	*Mycosphaerella* sp.	23	250	-	-	-	-	-	-	-
	2040	*Mycosphaerella* sp.	29	-	-	>1.000	-	250	-	-	-
	2059	*Mycosphaerella* sp.	11	-	-	-	-	-	250	-	-
M. floribunda	1990	*Botryosphaeria* sp.	11	-	-	-	-	-	-	>1.000	250
	1991	*Botryosphaeria* sp.	11	-	-	-	>1.000	-	-	-	-
	1987	*Coletotrichum gloeosporioides*	1	250	-	>1.000	-	-	-	-	-
	1974	*Fusarium decemcellulare*	13	-	-	-	-	500	-	-	-
	1978	*F. decemcellulare*	13	-	-	>1.000	-	-	-	-	-
	1985	*F. decemcellulare*	13	-	-	-	-	500	-	>1.000	>1.000
	1988	*F. decemcellulare*	13	-	250	-	-	500	-	>1.000	-
	2044	*Glomerella acutata*	14	-	500	-	-	-	-	-	-
	1986	*Glomerella acutata*	14	-	-	500	-	-	-	>1.000	500
A. castaneifolia	2002	*C. gloeosporioides*	1	-	**7.8**	**7.8**	-	500	>1.000	-	250
	1935	*C. gloeosporioides*	1	-	-	-	1.000	500	-	-	-
	1938	*C. gloeosporioides*	1	-	-	-	1.000	-	-	-	-
	1959	*C. gloeosporioides*	1	-	-	-	-	500	-	-	-
	1961	*C. gloesporioides*	2	-	-	-	>1.000	-	-	-	-
	2007	*C. gloeosporioides*	1	-	-	125	-	-	-	-	-
	1941	*Didymella bryoniae*	4	-	-	-	-	500	-	-	-
	1946	*Didymella bryoniae*	4	-	-	-	1	-	-	-	-
	1966	*Emericellopsis donezkii*	5	-	-	-	-	-	**62.5**	-	**7.8**
	2001	*Emericellopsis donezkii*	5	-	250	-	-	**62.5**	**62.5**	-	**15**
	1994	*G. moniliformis*	3	-	-	-	-	-	-	>1.000	125

Table 2. Contd

1996	*G. moniliformis*	1	-	-	-	-	1.000	-	-	-
1963	*G. moniliformis*	3	-	-	-	-	500	-	-	250

UFMGCB: Culture collection of the Universidade Federal of Minas Gerais. Ca: *Candida albicans*; Ck: *Candida krusei*; Cp: *C. parapsilosis*; Cg: *Candida glabrata*; Cn: *Cryptococcus neoformans*; Sa: *Staphylococcus aureus*; St: *Salmonella thyphimurium*; Bc: *Bacillus cereus*; - no inhibition. Only results for fungal isolates with at least one antimicrobial activity are shown.

Table 3. Sorenson's and Jaccard similarity coefficients of endophytic fungi from the three plants.

	E.* aff. *bimarginata	***M. floribunda***	***A.castaneifolia***
Eugenia aff. *bimarginata*	-	0.133 / 0.063	0.400 / 0.167
Myrciaria floribunda	0.133 / 0.063	-	0.200 / 0.091
Alchornea castaneifolia	0.400 / 0.167	0.200 / 0.091	-

colletotric acid, which has an antimicrobial substance with activity against bacteria and fungi (Suryanarayanan et al., 2009; Liu et at., 2010), and gloesporone, a fungal germination inhibitor (Meyer et al., 1987). Two isolates of *E. donezkii* showed the strongest antibacterial activity. Berg et al. (1996) isolated bergofungin from the extract of *E. donezki*, a peptaibol-type antibiotic that showed antibacterial activity against *B. cereus*. According to Saleem et al. (2010), promising compounds useful to development of antimicrobial drugs should show MICs values ranging from 0.02 to 10 µg/ml. The extracts of endophytic isolates UFMGCB 2002 and UFMGCB 2001 showed the best MIC values, which were lower or near the MICs of known antibacterial and antifungal drugs. For example, penicillin was effective (range: 0.012 to > 32 µg/ml) against *B. cereus* (Turnbull et al., 2004), and fluconazole was effective against both *C. krusei* (16 to 64 µg/ml) and *C. parasilosis* (2 to 8 µg/ml) (NCCLS, 2004). Fungal crude extracts are generally a mixture of active and non-active compounds, and low MICs may be suggestive of good antimicrobial activity. The extracts of these endophytic fungal are potential candidates for further studies to isolate the bioactive compounds, which can serve as models for specific drugs (antibacterial or antifungal), as well as drugs with broad antibiotic effects. The majority of fungal isolates obtained in this study that belonged to the same species and obtained from the same host tree exhibited different antimicrobial activity patterns against different target microorganisms. Some works also reported differences in the biological activities among fungal isolates of the same plant species (Vaz et al., 2009). These results suggest that more than one fungal isolate of each species should be tested when searching for biological activities. The high number of methanolic extracts with antimicrobial activity may be considered as a possible indicator for the capacity of these endophytic fungi to produce active compounds against the target pathogens

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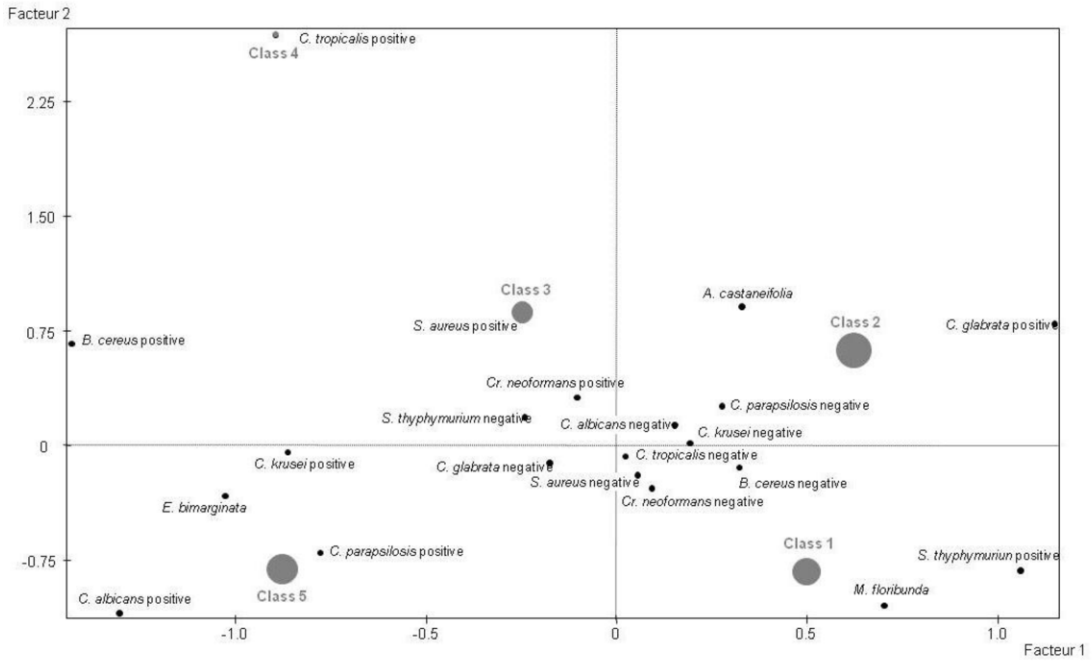

Figure 5. Multiple correspondence analyses about the association among endophytic fungi and species host (*Myrciaria floribunda*, *Alchornea castaneifolia*, and *Eugenia* aff. *bimarginata*) and antimicrobial activity (positive or negative) against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Salmonella typhimurium*, *Candida albicans*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *Cryptococcus neoformans* (yes or no for each antimicrobial activity). Class 1: Endophytic fungi isolated from *M. floribunda* presenting activity against *S. typhimurium*. Class 2: Endophytic fungi from *A. castaneifolia* presenting activity against *C. parapsilosis* and no activity against *C. parapsilosis*. Class 5: Endophytic fungi from *Eugenia* aff. *bimarginata* with antimicrobial activity against *C. parapsilosis* and *Candida albican*. Class 3 and 4: No conclusions about these groups.

Brazilian laws.

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Capítulo 3

Fungal endophyte β-diversity associated with Myrtaceae species in an Andean Patagonian forest (Argentina) and an Atlantic forest (Brazil)

Fungal endophyte β-diversity associated with Myrtaceae species in an Andean Patagonian forest (Argentina) and an Atlantic forest (Brazil)

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Summary

The biogeography of microorganisms is poorly understood, and there is an open debate regarding how microbial diversity is structured. We examined the community similarity of the fungal endophytes of phylogenetically related Myrtaceae trees in a latitudinal gradient. A total of 951 isolates were obtained and identified into 55 distinct OTUs based on the sequencing of ITS regions of the rRNA gene. The study was performed at the following spatial scales: regional, 101-5,000 km; local, 0-100 km; and microscale, 0-1 km. Similarity of fungal endophyte communities decreased with increasing geographical distance. At regional scales, environmental factors were also important for explaining the fungal community similarity. Moreover, dispersal limitation was significant at all of the analysed scales, and there was no relationship between the fungal community similarity and the host tree proximity. We conclude that fungal endophytes display a biogeographic pattern and that considering multiple scales is important for understanding fungal biogeography.

Keywords: Biogeography/ Distance decay/Fungal endophyte/ Myrtaceae/β-diversity

Introduction

Biogeography is the study of the distribution of biodiversity over space and time (Martiny *et al.*, 2006), and its study can illuminate the mechanisms that generate and maintain diversity, such as dispersion, speciation, extinction, and species interactions (Brown *et al.*, 1998). The community similarity between two groups often decreases as the distance between them increases, a pattern observed in communities from all domains of life and known as distance decay or β-diversity (Nekola and White, 1999). Two primary explanations for this pattern have been proposed. Niche theory predicts that community similarity decreases with environmental distance, irrespective of geographic proximity, as a result of species differences along environmental gradients (Tilman, 1982). Neutral theory, in contrast, predicts that the decay of community similarity is caused by spatially limited dispersal, independent of environmental differences between sites (Hubbell, 2001). The past several years have witnessed an increase in debates about the biogeographic patterns of microorganisms and whether these patterns are similar to those observed in macroorganisms.

Microorganisms have long been regarded as cosmopolitan because they exhibit short generation times and large population sizes and because they disperse over long distances (Fenchel and Finlay, 2004), prompted Baas Becking's hypothesis that "*everything is everywhere*, but, *the environment selects*" (the EisE hypothesis) (Baas Becking, 1934; Green & Bohannan, 2006). The distance decay relationship is used to demonstrate how the processes of selection, drift, dispersal, and mutation shape biogeographic patterns (Hanson *et al.*, 2012). When considering the EisE hypothesis, the distance decay curve would be due to a gradient of selective factors, which are thus spatially autocorrelated (Martiny *et al.*, 2011; Hanson *et al.*, 2012). Therefore, organisms with different niche preferences are selected from the available pool of taxa as the environment changes with distance (Martiny *et al.*, 2011). Distance decay patterns, and therefore β-diversity, can also be influenced by dispersal limitation. This effect should reflect the influence of historical processes on the current biogeographic patterns (Martiny *et al.*, 2011).

Microbial biogeography studies were challenged following the advent of the molecular taxonomy of microorganisms, which allowed species to be distinguished more accurately (Fierer, 2008). The internal transcribed spacer (ITS1 and ITS2) and 5.8S regions of the nuclear ribosomal repeat unit are the most widely used molecular markers in fungal endophyte diversity studies (Promputtha *et al.*, 2007; U'ren *et al.*, 2010) and have been used as a primary fungal barcode marker (Schoch *et al.*, 2012). The main advantage of ITS is the ease with which it can be amplified from all lineages of fungi using universal primers (Nilsson *et al.*, 2008) and the

large size of the available ITS sequence database, such as GenBank and EMBL (Vilgalys, 2003). Fungal diversity remains relatively poorly explored, as evidenced by the small proportion of fungi that have been identified to date. Recent estimates have suggested that as many as 5.1 million fungal species exist (Blackwell, 2011), making fungi among the most diverse groups of organisms on our planet. Fungal endophytes inhabit healthy plant tissues during at least one stage of their life cycles without causing any apparent symptoms of disease or negative effects on the host (Petrini *et al.*, 1992, Arnold *et al.*, 2001). Endophytes have been isolated from all studied plant groups, including bryophytes (U'Ren *et al.*, 2010), pteridophytes (Petrini *et al.*, 1992), gymnosperms (Soca-Chafre *et al.*, 2011), and both monocotyledonous (Pinruan *et al.*, 2010) and dicotyledonous angiosperms (Vaz *et al.*, 2009; Vieira *et al.*, 2012). Many studies have documented remarkable endophyte richness in tropical plants (Saikkonen *et al.*, 1998, Arnold & Lutzoni, 2007; Vaz *et al.*, 2009, 2012; Vieira *et al.*, 2012). Similar tendencies have been observed in temperate environments, where a host tree may harbour dozens of fungal endophytes (Saikkonen *et al.*, 1998; Stone *et al.*, 2000).

Little is known about how fungal endophyte communities vary across a geographic range or whether endophytes show phylogenetic correlations with their hosts. Many host-associated microorganisms exhibit patterns of genetic, morphological and functional differentiation that are related to the distribution of their hosts (Papke *et al.*, 2004). Myrtaceae species are found in diverse habitats, and a robust phylogeny exists for this group, making them ideal subjects for comparisons of endophyte community diversity. Phylogenetic analyses of morphological and molecular data have suggested a Gondwanan origin of Myrtaceae, with the Myrtae tribe originating and diversifying in Australasia between 77-56 ma, when Australia was connected to South America via warm-temperate Antarctic land bridges (Lucas *et al.*, 2007). Tribe Myrtae DC. is a monophyletic group of plants that probably has experienced a recent and rapid speciation, with long-distance dispersals more likely than vicariance to explain at least some of the intercontinental movements (Lucas *et al.*, 2007).

We studied a phylogenetically related Myrtae (Myrtaceae) host species to determine the β-diversity, and therefore the distance decay patterns, of its fungal endophyte communities. We developed a phylogenetic hypothesis for the relationships among the fungal endophytes associated with the Myrtaceae host samples. It was considered *Luma apiculata* and *Myrceugenia ovata* var. *nanophylla* which constitute a single clade. We included one Myrtaceae species not present in this clade, *Eugenia neomyrtifolia,* as an "outgroup". The objectives of the present study were the following: (1) to determine whether the fungal endophyte β-diversity, specifically the slope of distance decay curve, varies over regional, local or microscale scales; and (2) to

determine whether environmental variation or dispersal limitation explains the fungal community assemblage diversity along latitudinal gradients.

Experimental procedures

Study areas

Three different sites in Patagonia, Argentina were studied. These sites were located in the Andean Patagonian region, near the city of San Carlos de Bariloche, which is situated within Nahuel Huapi National Park. This region is characterised by native forests, which are dominated by *Nothofagus* species or native conifers, such as *Austrocedrus chilensis, Araucaria araucana, Fitzroya cupressoides* and *Pilgerodendrum uviferum* (Donoso, 2006). In Argentina, *Luma apiculata* was collected in two different sites, the Arrayanes Forest and Puerto Blest; *Myrceugenia ovata* var. *nanophylla* was collected in one site, Espejo Lake. In Brazil, the study was conducted at the Centro de Pesquisas e Conservação da Natureza Pró-Mata of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), in São Francisco de Paula, Rio Grande do Sul state. This area is a confluence of three phytoecological regions, including Araucaria forest, Atlantic rainforest and an herbaceous-bushy formation known regionally as "hill-top fields". *Myrceugenia ovata* var. *nanophylla* and *Eugenia neomyrtifolia* were collected in this Atlantic rainforest site (Figure 1). All the sampled sites were characterised by low antropic impact and minimal atmospheric pollution.

Fungal endophyte isolation

Five apparently healthy leaves were collected from each of 20 individuals of all Myrtaceae species that occur in the studied sites. The trees were spaced approximately 5 m apart. All the leaves were stored in sterile plastic bags, and fungal isolation was performed on the same day of the collection. The leaves were surface-sterilised via successive dipping in 70% ethanol (1 min) and 2% sodium hypochlorite (3 min), followed by washing with sterile distilled water (2 min). After the leaf surface sterilisation, six fragments (approximately 4 mm^2) were cut from each leaf: one from the base, two from the middle vein, one from the left margin, one from the right margin and one from the tip (6 leaf fragments/leaf; 30 leaf fragments/tree; 600 leaf fragments/site; 3,000 leaf fragments overall) . All the fragments were plated onto potato dextrose agar (PDA, Difco, USA) supplemented with 100-µg/ml chloramphenicol (Collado *et al.*, 1996). The plates were incubated at 15°C for up to 60 days. To test the effectiveness of the surface sterilisation, 100 µl

of the water used during the final rinse was plated on PDA to test for epiphytic microbial contaminants. Individual colonies were purified on PDA, and their morphologies were documented and photographed. For filamentous fungi, the long-term preservation of mycelial samples was performed in sterile distilled water at room temperature. All the fungal isolates were deposited in the Culture Collection of Microorganisms and Cells of the Universidade Federal of Minas Gerais.

Fungal identification

Pure cultures of the fungal isolates were grouped based on their morphological characteristics, including aerial mycelium formation, colony colour, surface texture and margin characters. At least fifty percent of the fungal isolates of each morphospecies were identified by directly extracting their total genomic DNA and sequencing the ITS region of the rRNA gene. The extraction of DNA from filamentous fungi was performed according to Rosa *et al.* (2009). The ITS domains of the rRNA gene were amplified using the universal primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´), as described by White *et al.* (1990). The amplification of ITS and the sequencing were performed as described by Vaz *et al.* (2009).

Analysis of ecological data

The diversity was estimated using the Shannon (H`) index ($H = -\Sigma\ ni/n\ ln\ (ni/n)$, where *ni* is the number of individuals in the taxon *i* and *n* is the total number of individuals). Values of the Shannon index usually lie between 1.5 and 3.5, with 1.5 representing the lowest diversity and 3.5 the highest (Gazis & Chaverri, 2010). Species accumulation curves were used to determine whether a sufficient number of samples had been obtained from each study site and were generated for each host tree species in each site using EstimateS, version 8.0 (Colwell, 2005). For calculations and statistical analyses, each individual fragment was considered a sample unit; a total of 3,000 sample units were evaluated. To visualise the trends and groupings of the fungal endophytes, Nonmetric Multidimensional Scaling (NMDS) analyses were conducted. The Jaccard distance was used in these analyses due to its simplicity, widespread use and more conservative measurement of community similarity than distances based on species abundance data, which are more sensitive to disturbances and local environmental differences (Nekola & White, 1999). To reduce the influence of the most abundant species, the data were square-root transformed prior to the NMDS analysis (Clarke & Warwick, 2001; Joshee *et al.*, 2009).

The rate of distance decay of the fungal endophyte communities was calculated according to Nekola and White (1999), with the assumption that community similarities decrease with increasing geographical distance. The distance decay relationship was calculated as the slope of a least-squares linear regression on the relationship between the (ln-transformed) geographic distance and the fungal endophyte community similarity measured by the Jaccard index. The slope of the distance-similarity relationship is one of the most common measures of β-diversity in ecological studies (Nekola & White, 1999). We chose to transform the geographic distance because of our sampling scheme, which purposely sampled over many orders of magnitude; otherwise, the data points would have been highly skewed (Martiny *et al.*, 2011). In addition, we tested whether the slope of the distance decay curve of each collection site was significantly different from zero using a randomisation procedure with 1,000 iterations.

To create geographic distance matrices between all the sampling points, we recorded the location and compass direction of each sampling transect with a handheld GPS unit (Martiny *et al.*, 2011). We used the GPS points, bearing angle, and sample spacing along each transect to calculate the geographic distance between samples within each collection site. To investigate the relationships among the fungal endophyte community similarity, geographic distance and environmental characteristics across all the spatial scales, we first used the ecodist R package (R Development Core Team, 2005; Goslee & Urban, 2007; Martiny *et al.*, 2011) to apply a ranked partial Mantel test (which assumes a monotonic, but not linear, relationship). We performed a principal components analysis (PCA) of several environmental variables (water precipitation, altitude and temperature) and then computed the dissimilarities for the first component. To compare additional variables, we created a distance matrix that considered geographic distances. Correlations were examined with the Spearman correction, and the *p* values were based on 10,000 permutations.

To tease apart the relative importance of the environmental variables on fungal endophyte community similarity, we used multiple regression on matrices (MRM) (Goslee & Urban, 2007). To reduce the effects of spurious relationships between variables, we performed the MRM test, removed the nonsignificant variables, and then repeated the test (Harrel, 2001, Martiny *et al.*, 2011). The model results reported here are from the second performance of the test. To further examine the relative importance of each predictor variable at the three spatial scales (regional: 101-5,000 km, local: 0-100 km and microscale, 0-1 km), we investigated scale-specific MRM models. We tested the significance of each model by performing 10,000 permutations. All the analyses were performed using the R program (R Development Core Team, 2005).

Results

Diversity of fungal endophytes

A total of 951 fungal endophyte isolates were obtained from 3,000 leaf fragments. Fifty-five distinct operational taxonomic units (OTUs) were identified based on the sequencing of the ITS region of rRNA. Twenty-three taxa were identified at the species level. Thirty-two OTUs exhibited a high divergence in the ITS region, with nucleotide differences from the other fungal sequences deposited in GenBank that ranged from 4 to 11 % (Supplementary table 1). All the taxa belong to the Ascomycota phylum, except Trametes, which belongs to the Basidiomycota phylum. The ascomycetous fungi were identified as members of the Sordariomycetes, Dothideomycetes, Leotiomycetes, Euromycetes and Pezizomycetes classes. *Pseudocercospora basintrucata* and *Xylaria* sp.1 were the most frequent taxa isolated from the *L. apiculata* present at the Arrayanes Forest and Puerto Blest collection sites (Andean Patagonian forest), respectively. *Mycosphaerella* sp. and *Xylaria enteroleuca* were most frequently isolated from *M. ovata* var. *nanophylla* from Espejo Lake (Andean Patagonian forest, Argentina) and the Atlantic rainforest (Brazil), respectively. The most frequent fungal species isolated from *E. neomyrtifolia* (Atlantic rainforest, Brazil) was *Colletotrichum* sp. Only species of the genus *Xylaria* were isolated simultaneously from all the host plants studied.

The accumulation curve reached an asymptote when the singletons were removed, which indicates that the sampling effort had sufficient statistical power to capture the total species richness of the cultivable fungal endophytes (Figure 2). The results of the bootstrap analysis of the observed species richness fell within the 95% confidence interval, which indicated that the sampling of fungal endophytes was statistically complete (data not shown). The diversity indexes are shown in Supplementary table 2. The host trees present in Atlantic rainforest ecosystem in Brazil, *M. ovata* and *E. neomyrtifolia*, displayed the highest Shannon values. In contrast, the *L. apiculata* host trees present in the Andean Patagonian forests exhibited low Shannon index values. The *M. ovata* that grew in the Atlantic rainforest (Brazil) presented greater diversity than the same species collected in the Andean Patagonian forest (Argentina). This species did not share any fungal endophyte species between the two sampled sites. The *Luma apiculata* collected in two different Argentinean sampling sites shared six fungal endophyte species. The *M. ovata* present in the Atlantic forest shared three fungal endophyte species with the *E. neomyrtifolia* present at the same site.

The NMDS plots revealed a clear separation between the groups found at the three Andean Patagonian forest sites (Argentina) and those at the Atlantic rainforest site (Brazil) (Figure 3). A plot of the fungal endophyte community similarity versus the distance for each pairwise set of

samples revealed a significant, negative distance decay curve for the fungal endophyte community (slope = - 0.01, $P < 0.0001$) (Figure 4). Furthermore, a microscale analysis of the slope of this curve revealed significant variation between the host tree transects. There were significantly negative distance decay slopes for *M. ovata* (slope = - 0.02, $P = 0.03$) from Puerto Blest in the Andean Patagonian forest and for *L. apiculata* (slope = - 0.01, $P = 0.01$), *M. ovata* (slope = - 0.11, $P < 0.0001$) and *E. neomyrtifolia* (slope = - 0.03, $P = 0.002$) from the Atlantic rainforest in Brazil. However, the distance decay slope for *L. apiculata* in the Arrayanes Forest did not differ significantly from zero ($P = 0.447$).

Over a regional scale, geographic distance appears to influence the fungal endophyte community β-diversity. The ranked partial Mantel tests revealed that the dissimilarity in the fungal community was highly correlated with geographic distance ($\rho = 0.27$, $P < 0.05$), but not with environmental distance ($P = 0.9$) (Table 1). MRM were used to investigate the relative importance of the factors contributing to these correlations. Over a regional scale, the MRM model explained a low proportion of the variability in the fungal endophyte community similarity ($R^2 = 5\%$, $P = 0.05$; Table 2). The geographical distance ($\beta = 0.95$, $P = 0.0001$), altitude ($\beta = - 0.27$, $P = 0.05$) and air temperature ($\beta = - 0.75$, $P = 0.001$) significantly contributed to the partial regression coefficients. Water precipitation did not play a significant role ($P = 0.957$) in describing the model. Geographic distance had a strong effect on community similarity when considered at a local scale; i.e., neither the two different ecosystems (the Andean Patagonian and Atlantic rainforests) nor the environmental variables were significant in explaining the model. Geographic distance did not influence the similarity of the fungal endophyte community to that of *L. apiculata*, which were spaced approximately 24 km apart. However, the geographic distance (approximately 2,300 km) between the *M. ovata* samples from Argentina and Brazil was statistically significant ($R^2 = 0.11$, $P = 0.0001$; $\rho = 0.34$, $P = 0.0001$).

Discussion

We used a culture-based approach and the sequencing of ITS region of the rRNA gene to assess the diversity and taxon composition of fungal endophytes associated with phylogenetically related Myrtaceae species. The ITS region was chosen because this region has the highest probability of allowing the successful identification of a broad range of fungi (Schoch *et al.*, 2012). The diversity patterns of the host trees in an Atlantic forest in Brazil (Supplementary table 2) were similar to those determined by extensive studies of foliar fungal endophytes of *Podocarpaceae* in New Zealand (Joshee *et al.*, 2009); *Euphorbiaceae* in Peru

(Gazis & Chaverri, 2010); *Lycopodiaceae*, *Rosacea* and *Pinaceae* in Canada (Higgins *et al.*, 2007); and *Cupressaceae* in the USA (Hoffman & Arnold, 2008). However, this pattern was not present in the host trees sampled in the Andean Patagonian forest (Argentina) in the present work, which exhibited less diversity than that observed in temperate ecosystems (Higgins *et al.*, 2007). The sampling of 3,000 leaf fragments was sufficient to adequately capture the richness of the cultivable endophytes, which was confirmed by a species accumulation curve analysis. The curves of all the host tree species reached a plateau, indicating that our sampling strategies allowed a robust estimate of the fungal endophyte species number and abundance. Both the number and size of the sampled fragments have important effects on the number of species isolated: when the size of the leaf fragments is reduced while their number is increased, the number of isolated fungal species increases (Gamboa *et al.*, 2002). The number of leaf fragments sampled in this work was greater than those of other studies of fungal endophytes (Higgins *et al.*, 2007; Vega *et al.*, 2010), making the diversity pattern obtained here more likely to be genuine. This reliability is confirmed by the low number of singletons obtained in the present study when compared with studies of tropical and temperate angiosperms (Arnold *et al.*, 2001; Higgins *et al.*, 2007).

There is an ongoing debate as to whether microorganisms exhibit biogeographical patterns similar to those of macroorganisms or whether such patterns are obscured by the population sizes and dispersal capabilities of microorganisms (Martiny *et al.*, 2011). This work compared the spatial variations in fungal endophyte communities across a latitudinal gradient and with phylogenetically related host tree species. The distance decay analysis showed that the fungal endophyte similarity decayed with increasing distance when analysed at regional and local scales. However, the slope of the distance decay curve varied in the microscale analysis. The only non-significant distance decay slope was observed for the *L. apiculata* collected in the Arrayanes Forest, which is dominated by this species. In wood plants, non-systemic endophytes are horizontally transmitted by spores from plant to plant (Saikkonen *et al.*, 1998), and infection by airborne inoculum likely plays an additional role (Carroll *et al.*, 1977; Bertoni & Cabral, 1988). These modes of transmission may explain the absence of a geographic distance effect on the distance decay in this study and suggest that the mycota associated with the forest trees surrounding are probably responsible for the fungal endophytes associated with *L. apiculata.* Therefore, fungal dispersal counteracts the distance decay relationship at the microscale level (Hanson *et al.*, 2012).

The NMDS analysis suggested that the fungal endophyte assemblages were shaped at a local scale (Figure 3). This fact is further indicated by the clear separation of groups between the

three Andean Patagonian forest sites (Argentina) and the Atlantic rainforest site (Brazil). The partial Mantel test showed that dispersal limitation significantly influenced the fungal endophyte community β-diversity at a regional scale ($\rho = 0.27$, $P < 0.05$). The MRM analysis allowed us to evaluate the independent contributions of geographic distance and environmental variables to the fungal endophyte community distribution. The varying importance of geographic distance and environmental parameters at different spatial scales likely reflects differences in their underlying variability at these scales (Martiny *et al.*, 2011). The MRM analysis indicated that at a regional scale, the measured variables could not explain most of the variability in fungal endophyte community similarity, which was reflected in an unexplained variance of 95% (Table 1). We may have missed spatially autocorrelated abiotic or biotic factors with strong influences on the fungal endophyte communities. However, geographic distance and environmental variables (altitude and temperature) were found to influence the fungal endophyte community at different scales (Table 1). This fact likely reflects the large environmental differences between the ecosystems sampled in Argentina and Brazil because these parameters were not significant when separately analysed in each region (at a local scale) (Table 1). The distance (2,324 km) between these two ecosystems encompasses a latitudinal gradient on the east to the southwest of South America, which results in a high amount of variability in environmental distance.

Previous studies have shown that the fungal endophyte community assemblage is affected by weather, with temperature and moisture being the most important variables for explaining fungal diversity (Talley *et al.*, 2002). This pattern was also observed by Martiny *et al.* (2011) in an analysis of the distribution of Nitrosomadales bacteria along a large transect in North America. It has been suggested that in some systems, both deterministic (niche-based) and stochastic (neutral-based) processes are responsible for structuring ecological communities (Chave, 2004). A literature review performed by Hanson *et al.* (2012) indicated that both contemporary selection and historical processes shape microbial biogeographic patterns. Furthermore, that work revealed that most studies have found that at least one environmental factor or the distance effects, including dispersal limitation, influence microbial composition. The results of the present work suggested that the distance and environmental influence will influence depend on the scale sampled used.

When considered at local scales, only geographic distance was significant in explaining the β-diversity (Table 1). The MRM model was exceptionally robust in explaining the local-scale variation in the fungal endophyte community similarity at the Argentinean collection sites (the Andean Patagonia ecosystem) ($R^2 = 0.86$, $P = 0.0001$). Furthermore, our results suggest that the primary control of fungal endophyte diversity is exerted by dispersal limitation, independent of

environmental differences between sites in the same ecosystem. When the *M. ovata* var. *nanophylla* samples present in the Andean Patagonian forest (Argentina) and the Atlantic rainforest (Brazil) were compared, only geographic distance was significant in explaining the fungal endophyte community similarity. Therefore, there was no relationship between fungal endophyte community similarity and the phylogenetic relationship of the host tree species. We conclude that fungal endophytes may show distribution patterns that are not profoundly different from those of higher organisms (Astorga *et al.*, 2012).

Conclusion

It has long been assumed that microorganisms have cosmopolitan distributions however the results of this work show that fungal endophytes exhibit a biogeographic pattern, corroborating some previous studies which show that microorganism are not randomly distributed. We conclude that fungal endophyte communities are influenced by both environmental factors and geographic distance and that these effects depend on the spatial scale analysed. Overall, our study shows that there is no relationship between fungal community similarity and the host tree phylogenetic proximity.

Acknowledgements

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Figure 1. Map of the geographical distribution of the host tree species.

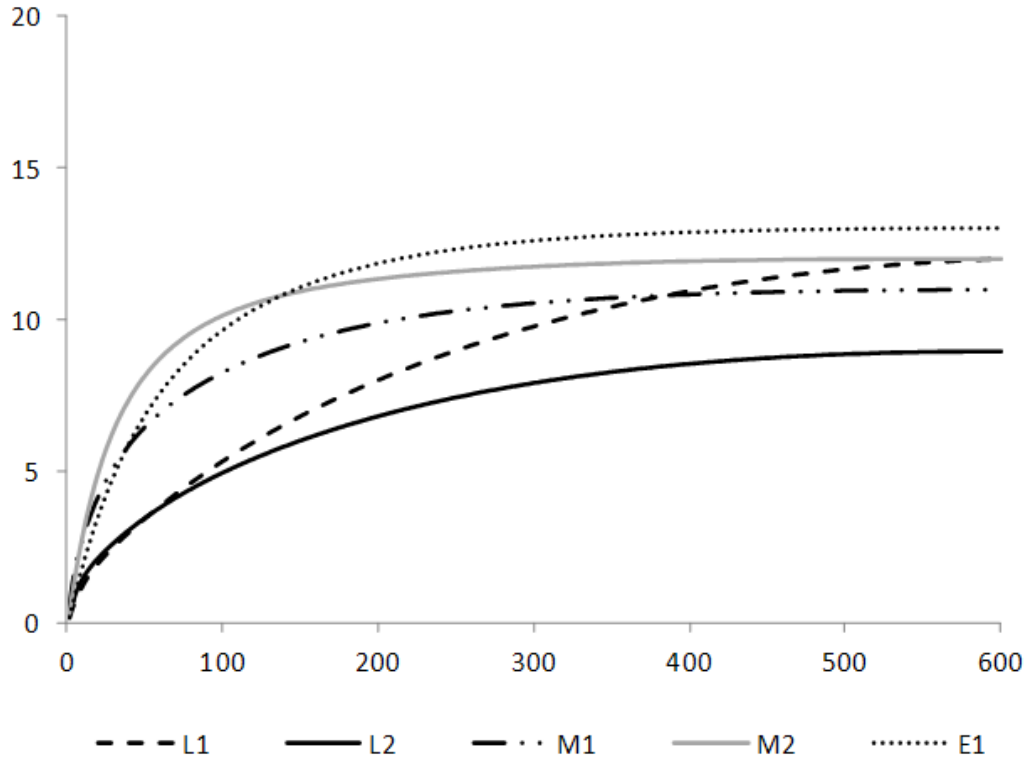

Figure 2. Accumulation curves for the fungal endophytes of *Luma apiculata* (Arrayanes forest) (L1), *Luma apiculata* (Puerto Blest) (L2), *Myrceugenia ovata* var. *nanophylla* (Argentina) (M1), *Myrceugenia ovata* var. *nanophylla* (Brazil) (M2), and *Eugenia neomyrtifolia* (Brazil) (E2). Singleton fungal species were not included in the analysis.

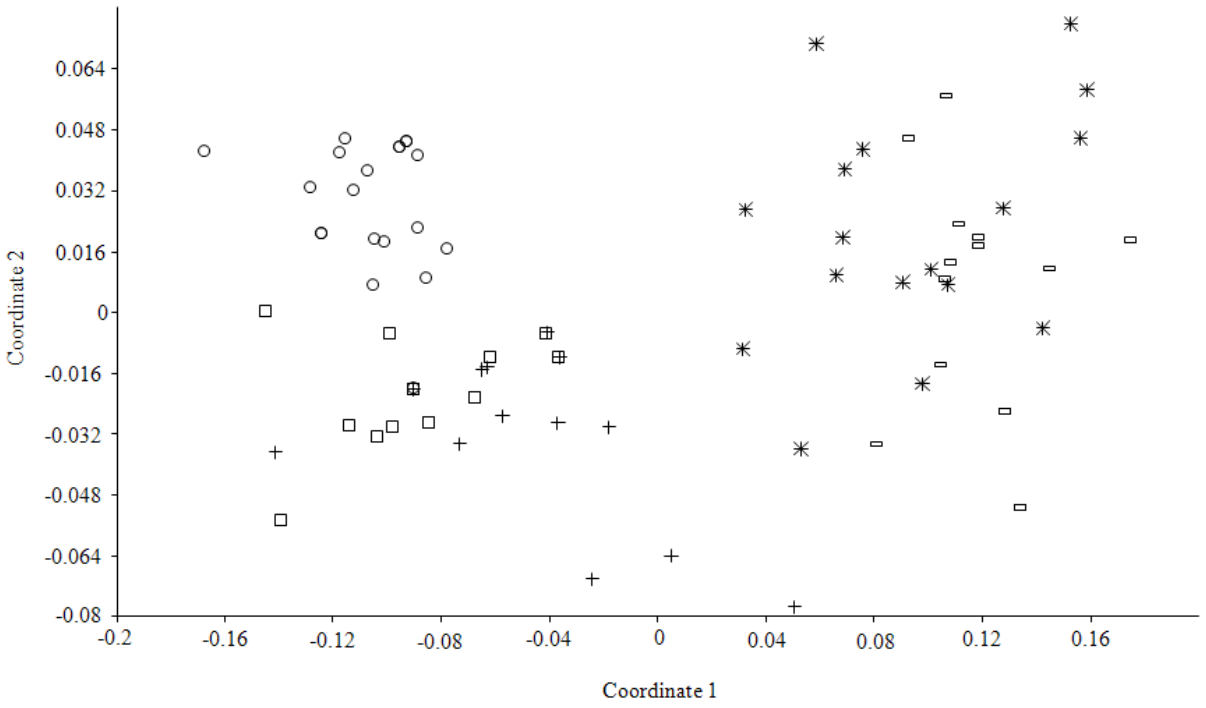

Figure 3. Nonmetric multidimensional scaling (NMDS) plots based on the Jaccard index that show the differences between fungal endophyte community compositions. Each symbol represents a single host tree species. Argentina: the squares represent *Luma apiculata* from Puerto Blest, the crosses represent *Luma apiculata* from the Arrayanes Forest, and the circles represent *Myrceugenia ovata* var. *nanophylla* from Lago Espejo. Brazil: the rectangles represent *Myrceugenia ovata* var. *nanophylla*, and the stars represent *Eugenia neomyrtifolia*. The R^2 values between the ordination distance and the distance in the original space are 0.23 and 0.15 for axis 1 and axis 2, respectively.

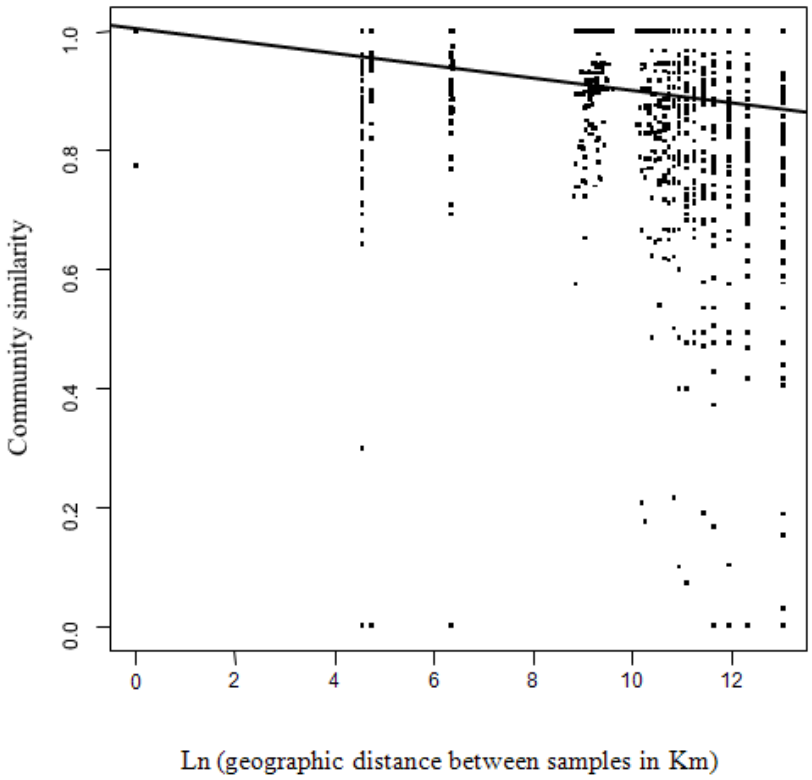

Figure 4. Distance decay relationship for fungal endophyte communities from an Andean Patagonia forest (Argentina): *Luma apiculata* (Arrayanes Forest), *L. apiculata* (Puerto Blest) and *Myrceugenia ovata* var. *nanophylla* (Espejo Lake) and from an Atlantic forest (Brazil): *Myrceugenia ovata* var. *nanophylla* and *Eugenia neomyrtifolia*. Pairwise community similarities were calculated using the Jaccard index and plotted against the natural logarithms of the distances among the study sites. The solid black line denotes the linear regression across all the spatial scales, and the slope is significantly smaller than zero (slope = - 0.01, $P < 0.0001$).

Table 1. Comparison of the fungal endophyte partial Mantel test results, where Spearman ρ is the correlation between the fungal endophyte community dissimilarity and either geographic distance or environmental distance.

Correlation between fungal endophyte community dissimilarity and:	Controlling for:	Continental scales	
		ρ	*P*
Geographic distance	Environmental distance	0.27	< 0.05
Environmental distance	Geographic distance	- 0.21	0.99

The environmental variables (Environment) were first examined using a principal components analysis (PCA) that considered the elevation, pluviomietry and temperature of each collection site. The *P* values are one-tailed and based on 10,000 permutations.

Table 2. Results of the multiple regressions on matrices (MRM) analysis of the fungal endophytes by spatial scale.

	Regional scale $R^2 = 0.05$ *	Local scale	
		Argentina $R^2 = 0.86$ ***	Brazil $R^2 = 0.32$ ***
Ln (geographic distance)	0.95 ***	0.93 ***	0.56 ***
Altitude	- 0.27 *		
Temperature	- 0.75 **		

The variation (R^2) of the community dissimilarity that is explained by the remaining variables and the partial regression coefficients (β) of the final model are shown. Where a partial regression is shown, its significance level (via one-way tests) is < 0.005. * $P \leq 0.01$, ** $P \leq 0.001$, *** $P \leq 0.0001$. The water precipitation, altitude and temperature were measured at each collection site. The water precipitation was not significant at any scale and is not shown in the table.

Table 3. Comparison of the fungal endophyte partial Mantel test results, where Spearman ρ is the correlation between the fungal endophyte community dissimilarity and either geographic distance or environmental distance.

Correlation[1] between fungal endophyte community dissimilarity and:	Controlling for:	Continental scales	
		ρ	*P*
Geographic distance	Environmental distance	0.27	< 0.05
Environmental distance	Geographic distance	- 0.21	0.99

[1]The environmental variables (Environment) were first examined using a principal components analysis (PCA) that considered the elevation, pluviomietry and temperature of each collection site. The *P* values are one-tailed and based on 10,000 permutations.

Table 4. Results of the multiple regressions on matrices (MRM) analysis of the fungal endophytes by spatial scale. The variation (R^2) of the community dissimilarity that is explained by the remaining variables and the partial regression coefficients (β) of the final model are shown. Where a partial regression is shown, its significance level (via one-way tests) is < 0.005.

	Regional scale[1] $R^2 = 0.05$[2]	Local scale	
		Argentina $R^2 = 0.86$[3]	Brazil $R^2 = 0.32$[3]
Ln (geographic distance)	0.95[3]	0.93[3]	0.56[3]
Altitude	- 0.27[2]		
Temperature	- 0.75[4]		

[1]The water precipitation, altitude and temperature were measured at each collection site. The water precipitation was not significant at any scale and is not shown in the table. [2]$P \leq 0.01$, [3]$P \leq 0.0001$. [4]$P \leq 0.001$.

Supplementay table 1. Closest related fungal species according to the percent similarities between the ITS regions of the rRNA gene revealed by alignment with sequences from related species retrieved from the GenBank database and the number of fungal endophyte isolates obtained from *Luma apiculata*, *Myrceugenia ovata* var. *nanophylla* and *Eugenia neomyrtifolia* from an Andean Patagonian forest (Argentina) and an Atlantic forest (Brazil).

Closest related species	UFMGCB	Identification	Pb	Similarity %	L1*	L2*	M1*	M2*	E1*
Sordariomycetes, Coniochaetales									
Coniochaeta velutina STE-U [GQ154545]	3827	*Coniochaeta velutina* [JQ346221]	706	100			5		
Sordariomycetes, Diaphortales									
Amphilogia gyrosa [EF026147]	3826	*Amphilogia* sp. [JQ346197]	616	93			6		
Diaporthe australafricana [AF230760]	3841	*Diaphorte* sp.1 [JQ327869]	577	96	2	2			
Diaporthe helianthi [AJ312356]	5242	*D. helianthi* [JQ346194]	478	99					4
Diaporthe phaseolorum [EU272530]	5311	*Diaphorte* sp.2 [JQ327871]	434	90					2
Diaporthe phaseolorum [EU272530]	5212	*Diaphorte* sp.3 [JQ327870]	498	93				3	7
Diaporthe phaseolorum [EU272530]	5173	*Diaphorte* sp.4 [JQ327872]	484	95				12	
Diaporthe phaseolorum [EU272530]	5211	*Diaphorte* sp.5 [JQ327871]	464	96				10	
Diaporthe phaseolorum [EU272530]	3855	*D. phaseolorum* [JQ327873]	467	97	5				
Diaporthe phaseolorum [EU272530]	5203	*Diaphorte* sp.6 [JQ327874]	485	94				16	8
Diaporthe stewartii [FJ889448]	5264	*Diaporthe stewartii*	329	99					6
Greeneria uvicola [GU907101]	3949	*Greeneria* sp.1 [JQ346195]	599	89			34	1	

Greeneria uvicola [GU907101]	5274	*Greeneria* sp.2	453	90			1	
Sordariomycetes, Glomerellales								
Colletotrichum boninense [AB042313]	5233	*Colletotrichum* sp.1 [JQ346206]	531	98				23
Colletotrichum boninense [AB042313]	5230	*Colletotrichum boninense* [JQ346207]	532	99				5
Colletotrichum boninense [AB042313]	5253	*Colletotrichum* sp. 2 [JQ346208]	507	98				7
Colletotrichum boninense [AB042313]	5201	*Colletotrichum* sp.3 [JQ346209]	520	97			9	
Colletotrichum boninense [AB042313]	5197	*Colletotrichum* sp.4 [JQ346210]	467	98			20	
Colletotrichum boninense [AB042313]	5259	*Colletotrichum* sp.5 [JQ346211]	471	98				5
Colletotrichum boninense [AB042313]	5177	*Colletotrichum* sp.6 [JQ346212]	484	97			22	
Colletotrichum boninense [AB042313]	5171	*Colletotrichum* sp.7 [JQ346213]	526				13	
Colletotrichum boninense [AB042313]	5215	*Colletotrichum* sp.8	402	96			3	
Sordariomycetes, Hypocreales								
Cephalosporium curtipes var. *uredinicola* CBS 650.85 [AJ292405]	3956	*Cephalosporium* sp. [JQ346222]	557	89		6		
Sordariomycetes, Xylariales								
Annulohypoxylon bovei [JN225906]	3885	*Annulohypoxylon* sp.3 [JQ327866]	567	92	2			
Annulohypoxylon squamulosum [EF026139]	3852	*Annulohypoxylon* sp.1[JQ327864]	547	92	3			
Annulohypoxylon squamulosum [EF026139]	5249	*Annulohypoxylon* sp.2 [JQ327865]	485	82				13
Annulohypoxylon squamulosum [EF026139]	3946	*Annulohypoxylon* sp.4 [JQ327867]	560	92		1		
Biscogniauxia cylindrispora [EF026133]	3834	*Biscogniauxia* sp.1 [JQ327868]	706	85	1			
Nemania aenea [AF201704]	3884	*Nemania aenea* [JQ327863]	588	97				

Nemania illita [026122]	3918	*Nemania* sp. [JQ327862]	481	94		4	27		
Xylaria berteri [GU324750]	5268	*Xylaria berteri* [JQ327861]	577	99				34	17
Xylaria catorea [GU324751]	3880	*Xylaria castorea* [JQ327858]	574	99	2	5			
Xylaria catorea [GU324751]	3872	*Xylaria* sp.1 [JQ327859]	581	94	9	112	11		
Xylaria enteroleuca [163033]	5292	*Xylaria enteroleuca* [JQ327860]	494	98				46	
Dothideomycetes, Botryosphaeriales									
Guignardia sp. MUCC 0441 [AB454355]	3916	*Guinardia* sp. 1 [JQ346219]	561	92			1		
Guignardia mangiferae CBS 356.52 [FJ538342]	5169	*Guinardia mangiferae*	513	99				37	21
Dothideomycetes, Dothideales									
Dothiora cannabinae [DQ470984]	3894	*Dothiora cannabinae* [JQ346227]	556	99	4				
Dothideomycetes, Capnodiales									
Cladosporium cf. *subtilissimum* CBS 172.52 [EF679390]	3843	*Cladosporium subtilissimum* [JQ346203]	548	99	2	6			
Cladosporium colombiae [FJ936159]	3901	*Cladosporium colombiae* [JQ346204]	544	99	1	2			
Mycosphaerella brassicicola CBS 174.88 [EU167607]	3928	*Mycosphaerella* sp. [JQ346202]	528	89			191		
Pseudocercospora basitruncata CBS 114664 [DQ303071]	3898	*Pseudocercospora basintrucata* [JQ346205]	534	97	72	18	1		
Dothideomycetes, Pleosporales									
Camarosporium brabeji CBS 123026 [EU552105]	3848	*Camarosporium brabeji* [JQ346215]	538	99	2				
Didymella phacae strain CBS 184.55 [EU167570]	3925	*Didymella* sp. [JQ346225]	415	94	1				
Lewia infectoria ATCC 12054 [AF229480]	3824	*Lewia infectoria* [JQ346214]	566	99			8		
Microsphaeropsis olivacea CBS 432.71 [GU237863]	3886	*Microsphaeropsis olivacea* [JQ346217]	444	98		1			

Paraconiothyrium sporulosum [GU566257]	3837	*Paraconiothyrium* sp. [JQ346216]	510	96		1	2	
Leotiomycetes, Helotiales								
Cryptosporiopsis actinidiae [EU482298]	3887	*Cryptosporiopsis actinidiae* [JQ346199]	554	98		1		1
Mollisia cinerea [DQ491498]	3832	*Mollisia* sp. [JQ346200]	554	94			1	
Mollisia cinerea [DQ491498]	3900	*Mollisia cinerea* [JQ346201]	554	99		1		
Pezicula corylina [AF141176]	3840	*Pezicula corylina* [JQ346198]	554	97		3	34	
Eurotiomycetes; Chaetothyriales;								
Exophiala capensis CBS 128771[JF499861]	LAB 2IIIB4	*Exophiala capensis* [JQ346226]	448	97				
Eurotiomycetes; Eurotiales								
Penicillium restrictum NRRL 25744 [AF033459]	3803	*Penicillium restrictum* [JQ346224]	510	99			1	
Pezizomycetes; Pezizales								
Peziza varia [AF491570]	3892	*Peziza* sp. [JQ346218]	579	94	2			
Fungi incertae sedis; Mortierellales								
Mortierella sclerotiella CBS 529.68 [HQ630310]	3802	*Mortierella sclerotiella* [JQ346223]	582	98			3	
Badisiomycete								
Trametes hirsuta LE 231668 [HQ435841]	5251	*Trametes hirsute* [JQ346220]	486	97				15

L1: *Luma apiculata* (Arrayanes forest); L2*: Luma apiculata* (Puerto Blest); M1: *Myrceugenia ovata* (Espejo lake, Argentina); M2: *Myrceugenia ovata* (Brazil); E1: *Eugenia neomyrtifolia* (Brazil). UFMGCB: Culture collection of the Universidade Federal of Minas Gerais. * Number of segments plated per plant species: 600.

Supplementary table 2. Study site and fungal endophyte community characteristics for each collection site in Argentina and Brazil.

	Luma apiculata		*Myrceugenia ovata* var. *nanophylla*		*Eugenia neomyrtifolia*
Country	Argentina	Argentina	Argentina	Brazil	Brazil
Collection area	Arrayanes Forest	Puerto Blest	Espejo lake	PUCRS	PUCRS
Coordinates	40º 51´ S 71º 36´ W	41º 01´ S 71º 48´ W	40º 47´ S 71º 40´ W	29º 27 ´ S 50º 0.6´ W	29º 27 ´ S 50º 0.6´ W
Alt[a]	850	850	970	912	905
ºC[b]	10	9	10	15	15
mm[c]	1500	3000	1800	2252	2252
Number of isolates obtained	106	159	336	225	134
Number of fungal species	12	15	19	15	14
Singletons	1	6	8	3	1
Shannon diversity index	1.29	1.04	1.48	2.24	2.36

Number of leaves sampled: 100; number of segments plated per plant species: 600. [a]Alt: altitude above sea level. [b]Annual mean temperature in ºC.

[c]Annual precipitation in mm. The calculation of the diversity indexes excluded singleton fungal species.

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Capítulo 4

Arbuscular mycorrhizal colonization of *Sorghum vulgare* in presence of root endophytic fungi of *Myrtus communis*

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Arbuscular mycorrhizal colonization of *Sorghum vulgare* in presence of root endophytic fungi of *Myrtus communis*

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ABSTRACT

A total of 150 isolates of endophytic fungi were obtained from roots of *Myrtus communis* grown in the Mediterranean area. Seven different endophytic fungi taxa, all belonging to the Ascomycota phylum were found. Arbuscular mycorrhizal (AM) fungi, belonging to the *Glomus* genus, were also present in roots of *M. communis*. None of the root endophytic fungi tested, except *Drechslera* sp., colonized the root of sorghum. Among all fungi tested, only *Phoma schachtii* and *Cylindrocarpon destructans* significantly increased the shoot dry weight of sorghum grown in sterilized soil. *Phomopsis columnaris*, *P. schachtii*, *Eucasphaeria* sp. and *Cylindrocarpon pauciseptatum* increased the dry matter of sorghum grown in unsterilized soil. The percentage of AM colonization of sorghum was increased significantly only when *Ph. columnaris*, *P. schachtii*, the two strains of *Bionectria ochroleuca*, *Eucasphaeria* sp. and *C. pauciseptatum* were applied to unsterilized soil. The number of CFUs of endophytic fungi inoculated in sorghum rhizosphere was similar throughout the experiment, except *B. ochroleuca*-1 whose population increased at the end of the experiment either in sterilized or in unsterilized soil. No significant effect of the endophytic fungi isolated from *M. communis* on shoot and root P, K, Ca, Na, Mg, Cu and Zn concentrations of plants grown in sterilized or unsterilized soils, except *Eucasphaeria* sp. which increased the shoot P concentration of sorghum grown in unsterilized soil.

1. Introduction

Soil and rhizosphere fungi can confer plant abiotic and biotic stress tolerance, increasing biomass and decreasing water consumption, or can alter resource allocation (Smith and Read, 2008; Bever et al., 2010). Endophytic fungi are microorganisms that inhabit healthy plant tissues at least one stage of their life cycle, without causing any apparent symptom of disease or negative effects on their hosts (Petrini et al., 1992). Arbuscular mycorrhizal (AM) fungi are the most widespread root-endophyte associate fungi. This symbiosis can benefit plant growth, particularly through enhanced phosphorus, water and mineral uptake (Smith and Read, 2008). Ascomycetous root endophytic fungi can be considered as two groups, the dark septate endophyte (DSE) and fungi with hyaline and pale hyphae (Addy et al., 2005). These two groups are considered at least as ubiquitous as mycorrhizal associations among temperate-zone plants (Arnold et al., 2001). The ascomycetous DSE probably constitute the most abundant and most widespread group of root colonizer and parallel AM fungi in occurrence and colonization of plant species (Mandyam and Jumpponen, 2005). Fungi with hyaline and pale hyphae are also commonly found in plants root but are less studied because they are less conspicuous and easier to overlook than the DSE (Addy et al., 2005).

The interactions of ascomycetous root endophytic fungi with host plants can vary from pathogenic to beneficial mutualism (Smith and Read, 2008). Endophytic fungal colonization is important to improve the ecological adaptability of host enhancing tolerance to biotic and abiotic stresses (Schulz and Boyle, 2005). Moreover, root colonization by endophytic fungi may confer benefits to the host plant by means of growth promotion, protection against disease or assistance in phosphorous uptake (Sieber, 2002).

It is known that AM symbiosis is influenced by the activities of microorganisms in the soil (Lecomte et al., 2011). DSE

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coexist often with different AM fungi. The role of endophytic fungi in ecological situation with AM fungi must be important for plant development and plant communities (Mandyam and Jumpponen, 2005). It is known that some DSE, such as *Drechslera* sp., are able to colonize root of grasses such as sorghum and the exudates produced by the host stimulated the presymbiotic stage development of AM fungi (Navarro, 2008; Scervino et al., 2009). However, studies on the effect of root endophytic fungi on AM fungi are scarce.

Myrtus communis (Myrtaceae) is an evergreen shrub up to 4-m high that is distributed throughout Mediterranean ecosystems. It is well adapted to waterstress conditions and can also be used for revegetation of arid and degraded zones. Many endophytic fungi as well as AM and DSE fungi in Myrtaceae roots under nature conditions have been found (Matosevic et al., 1997; Jumpponen and Trappe, 1998). Some of the endophytic fungi present in *M. communis* roots may be able to colonize and improve the development and growth of agricultural plants.

The aims of this work is to study the role of endophytic fungi, isolated from roots of *M. communis*, on root colonization and dry matter of agricultural plants as well as their effects on the AM symbiosis. We selected sorghum (*Sorghum vulgare*) as a model of agricultural plants. The fungus *Drechslera* sp. has been used as a fungal model of endophyte able to colonize root of sorghum. To our knowledge, this is the first report about the presence of non-mycorrhizal endophytic fungi in root of *M. communis* and their effects on AM fungi.

2. Materials and methods

2.1. Site description

The study site was in Sierra Tejeda, Almijara y Alhama Natural Park (36°51′25″N, 3°41′40″W, elevation of around 360 m). The Park is located in the south of Andalucia, nearby Mediterranean Sea, between Malaga and Granada and consists of several dolomitic mountain ranges.

Soil samples were dry ashed for mineral analysis. Total concentrations of K, P, Fe, Ca, Mn, Na, Mg, Cu and Zn of soil samples were determined by digestion with $HNO_3 + H_2O_2$, followed by ICP-OES 720-ES (*inductively coupled plasma-optical emission spectrometry*) (Agilent, Santa Clara, EEUU). Soil pH was 7.9 and was determined on aqueous suspension of soil (1:10, w/v).

2.2. Fungal isolation

Eight adults shrubs of *M. communis* were randomly sampled in October 2010. From each individual, fine roots and approximately 250 g of adjacent soil were collected by tracing thick roots from the base of the trunk to their ultimate branching. Each of the three replicate sample consisted of five bulked subsamples taken from the top 10 cm of soil of each shrub. All root samples were held in sterile plastic bags and fungal isolation occurred on the same day as plant collection.

The AM spores were isolated by wet-sieving 5 g of soil from each sample through 50–100–250–700 μm sieves and centrifuging in a 50% (w/v) sucrose gradient (Walker et al., 1982). It was counted cytoplasm-filled, viable-looking individual spores in a Doncaster dish (Doncaster, 1962), using a dissecting microscope. We did not count floating spores which looked empty or parasitized.

For surveys of endophytic fungi, the roots were washed in running tap water and then surface-sterilized via successive dipping in ethanol 70% (1 min) and 2% sodium hypochlorite (3 min), followed by washing three times with distilled water (2 min). The roots were cut into ca. 4 mm pieces, mixed and plated onto potato dextrose agar (PDA, Difco, USA) supplemented with chloramphenicol at a concentration of 100 μg/ml which allow to isolate most of the endophytic fungi (Collado et al., 1996; Kumar and Hyde, 2004). Twenty five tissue segments were plated per individual totalizing 200 segments. Plates were sealed and incubated for up to 60 days at 28 °C in the dark and emergent hyphae were transferred and purified on new PDA (Difco, USA) plates. To test the effectiveness of surface sterilization, 100 μl of water used during the last rinsing step was placed on PDA to detect epiphytic microbial contaminants. Long-term preservation of mycelial samples was carried out in sterile distilled water at room temperature.

2.3. Fungal identification

Pure cultures of the fungal isolates were grouped based on morphological characters including aerial mycelium form, colony colours, surface texture and margin characters. At least one representative isolate of each morphospecies was selected for identification by sequencing of ITS region of the rRNA gene. The protocol for DNA extraction of filamentous fungi was done according to Rosa et al. (2009). The internal transcribed space (ITS) domains of rRNA gene were amplified using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described by White et al. (1990).

Amplification of ITS and sequencing protocols were performed as described to Vaz et al. (2009). Sequencing was carried out using an ET dynamic terminator kit in a MegaBACE 1000/automated 96 capillary DNA sequencer (GE Healthcare, Buckinghamshire, UK). The quality of each Electropherogram was evaluated using Phred-Phrap software and consensus sequences were obtained by alignment of both sequence strand using CAP3 software available at the web page Electropherogram quality analysis (http://asparagin.cenargen.embrapa.br/phph). The nucleotide sequences and other related sequences were aligned using the CLUSTALW software package (EMBL-EBI) (http://www.ebi.ac.uk/clustalw/). In this study it was defined a 3% cut-off value to define species level based on the sequencing of the ITS domains (Nilsson et al., 2008).

2.4. Greenhouse experiments

2.4.1. Experimental design

In order to see if there were beneficial effect of endophytic fungi isolated from *M. communis* on the dry matter of sorghum and if there were synergistic, neutral or antagonistic action between these endophytic fungi and the AM indigenous fungi from the Rio Verde soil, we inoculated these endophytic fungi in Rio Verde soils. Sorghum (*S. vulgare* L.) plants were grown in 0.5 l pots either in sterilized on unsterilized soils from Rio Verde site, with the following treatments: (1) non inoculated soil (2) soil inoculated with each of the most frequent endophytic fungi isolated from *M. communis*. Plants were grown in a greenhouse with natural light supplemented by Sylvania incandescent and cool-white lamps, 400 E m^{-2} s^{-1}, 400–700 nm, with a 16/8 h day/night cycle at 25–19 °C and 50% relative humidity After five weeks plants were harvested and the shoot and root dry weight, the percentage of root length colonized by the fungi and the number of colony forming units (CFUs) of endophytic fungi were determined. There were four replicate pots per treatment and the experiments were repeated twice.

The shoot and root dry weight were determined after drying at 70 °C for 24 h. Mineral concentrations of shoot and root of sorghum plants were measured by after digestion with $HNO_3 + H_2O_2$, followed by ICP-OES 720-ES (*inductively coupled plasma-optical emission spectrometry*) (Agilent, Santa Clara, EEUU).

2.5. Fungal inoculation

Endophytic fungi isolated from *M. communis* root and *Drechslera* sp. BAFC 3419 from the University of Buenos Aires (GenBank accession number FJ868975), were grown at 28 °C in 250 ml Erlenmeyer flasks containing 70 ml of a potato dextrose medium on a rotary shaker at 80 rpm. The flasks were inoculated with 2 cm^2 agar plugs covered by fungi mycelia from a 7-day-old culture. After 7 days, the fungal suspension was centrifuged at 1000 rpm to 25 min, the supernatant was discharged and the dry weight was determined. The pellet was resuspended in 50 ml distilled water and 5 ml was dried at 70 °C for 24 h. A suspension at 6 mg/ml was prepared in distilled water and 17 mg of mycelia was used as inoculum per pot. Non inoculated controls were given a filtrate (Whatman no. 1 paper) of the inoculum containing the common soil microflora, but free of fungal propagules.

2.6. Fungal measure

Because endophytic fungi were not detected in sorghum root, we evaluate the population of these fungi in the rhizosphere soil of sorghum. The population density was determined according to Ravnskov et al. (2006). The number of CFUs in suitable dilutions of such samples, taken from the four replicate pots of each treatment, were counted 48 h after inoculation and at the end of the experiment. The fungi were isolated and identified up to genera (Domsch et al., 1980). All the endophytic fungi were identified using microscopic structures (conidiophores, conidia, chlamidospores) and using colony characteristics such as growth rate, aerial mycelium, pigmentation and sclerotial bodies (Domsch et al., 1980). Soil was dried at 105 °C and weighed. The number of CFUs was expressed per g of dry soil.

Roots of *M. communis* and sorghum were rinsed in distilled water to remove any trace of soil, cleared and stained according to the Phillips and Hayman method (1970). Presence of root endophytic fungi and percentage of root length with endophytic fungi colonization was determined under compound microscope (Ocampo et al., 1980). The percentage of AM colonization was measured by the grid line intersect method (Giovannetti and Mosse, 1980).

2.7. Statistical analysis

Two way analysis of variance (ANOVA) was used to examine levels of significance ($P < 0.05$) of the treatments and their interaction. A Tukey type multiple comparison "Nemenyi" test was used to evaluate if there were statistically significant differences between each treatment. These tests were done using the software package R (R Developmental Core Team, 2005).

For measurement of the species diversity we determinate the abundance, dominance and Shannon indexes (Krebs, 1989; Ryan et al., 1995). The percentage abundance of each taxon was calculated according to the following formula: percentage abundance of taxon A = occurrence of taxon A × 100/occurrence of all taxa. Shannon $H = -\Sigma ni/n \ln(ni/n)$, where ni is number of individuals of the taxon i and n is the total number of individuals. This is a diversity index and can vary to 0 for communities with only a single taxon to high values for communities with many taxa, each with few individuals. Dominance $D = \text{sum}((ni/n)2)$, where ni is number of individuals of the taxon i and n is the total number of individuals. The dominance index ranges from 0 (all taxa are equally present) to 1 (one taxon dominates the community completely). All results were obtained with 95% confidence and bootstrap values calculated from 1.000 iterations. In this study we considered only nonsingletons, that is, species represented by more than two isolates. The calculations were carried out using the computer program PAST version 1.90 (Ryan et al., 1995).

Table 1
Number of arbuscular mycorrhizal spores in the rhizosphere of *Myrtus communis*.

Fungal family	Number of spores (g^{-1} soil)
Glomeraceae	6.3b
Acaulosporaceae	0
Gigasporaceae	0
Unknown black spores	1.5a

Column values followed by the same letter are not significantly different as assessed by the Tukey test ($P < 0.05$).

Table 2
Root length colonization of *Myrtus communis*.

Fungi	% Root length colonization
Arbuscular mycorrhizal	37.6b
Septate fungi	0.9a
Microsclerotial formation	0.6a

Column values followed by the same letter are not significantly different as assessed by the Tukey test ($P < 0.05$).

3. Results

The chemical properties of soil used in this study were: K was 2.4 g kg^{-1}, P was 0. 237 g kg^{-1}, Fe was 6.8 g kg^{-1}, Ca was 129.6 g kg^{-1}, Mn was 0.132 g kg^{-1}, Na was 0.474 g kg^{-1}, Mg was 0.071 mg kg^{-1}, Cu was 0.013 g kg^{-1} and Zn was 0.043 g kg^{-1}.

The number of AM fungal spores found in the rhizosphere of *M. communis* grown in the Natural Park is described in Table 1. Spores belonging to the *Glomeraceae* family were the most frequent AM fungal spores; spores belonging to the *Acaulosporaceae* (sessile spores) and *Gigasporaceae* families (spores with bulbous subtending hyphae) were not found. Unknown black spores were also present. Roots of *M. communis* was mainly colonized with AM fungi and reached a 37.6 ± 5.4% of root length colonization. Septate fungi and microsclerotial formation in the roots of *M. communis* were also observed but reached less than 1% of its root length (Table 2).

A total of 150 isolates of endophytic fungi were obtained and 36 isolates were represented by only one isolate (hereafter, singletons) while 114 were grouped into 25 different morphospecies. One isolate from each group that presented lowest dominance and highest abundance and Shannon index were randomly selected for the greenhouse experiments (Table 3) and were represented by eight groups. Molecular identification of these fungi revealed seven different taxa, all belong to the Ascomycota phylum (Table 3). Six endophytic fungi species showed 97–99% sequence similarity with the ones deposited in GenBank and were identified at the species level. The most frequent genera isolated associated with *M. communis* were *Phomopsis columnaris*, *Cylindrocarpon destructans*, *Bionectria ochroleuca* 1, *Bionectria ochroleuca* 2, *Phoma schachtii*, *Eucasphaeria* sp., *Cylindrocarpon pauciseptatum* and *Bisporella* sp. (Table 3). Two endophytic fungi showed high divergence in the ITS region when compared with fungal sequences of other fungi deposited in the GenBank database and were identified at the genus level. *Bisporella* sp. and *Eucasphaeria* sp. had 90% identity with *Bisporella citrina* (GenBank accession number GQ411507) and *Eucasphaeria capensis* CBS 120027 (GenBank accession number EF110619), respectively. Two endophytic fungal taxa, *Phomopsis* and *Phoma*, isolated from *M. communis* roots were dark septate endophyte.

Among all fungi tested, only *P. schachtii* and *C. destructans* significantly increased the shoot dry weight of sorghum plants grown in sterilized soil (Fig. 1).

Table 3
Identification and diversity indexes of endophytic fungi associated with *Myrtus communis*.

Closest related species	Maximum identities (%)	Identification	Abundance (%)	Dominance *D*	Shannon *H*
Phomopsis columnaris (FN394688)	99	*Ph. columnaris*	29	0.29 (0.13/0.21)	1.3 (1.7/2.02)
Cylindrocarpon destructans (AM419063)	99	*C. destructans*	13	0.76 (0.5/1.0)	0.48 (0/0.86)
Bionectria ochroleuca ATCC 48395 (GU256754)	98	*B. ochroleuca* 1	4	0.37 (0.37/1.0)	1.04(0/1.04)
Bionectria ochroleuca ATCC 48395 (GU256754)	99	*B. ochroleuca* 2	2	0.23 (0.17/0.5)	1.3 (0.87/1.79)
Phoma schachtii CBS 502.84 (FJ427066)	97	*Phoma schachtii*	2	0.5 (0.5/1)	0.69 (0/0.69)
Eucasphaeria capensis CBS 120027 (EF110619)	90	*Eucasphaeria* sp.	2	0.5 (0.5/1)	0.69 (0/0.69)
Cylindrocarpon pauciseptatum CBS 100819 (EF607090)	98	*C. pauciseptatum*	2	0.5 (0.5/1)	0.69 (0/0.69)
Bisporella citrina (GQ411507)	90	*Bisporella* sp.	2	0.5 (0.5/1)	0.69 (0/0.69)

The numbers in parenthesis represent the lower and upper diversities values, respectively, with 95% of confidence and bootstrap values calculated from 1000 iterations.

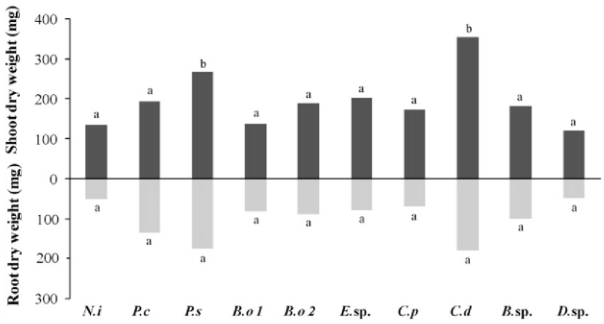

Fig. 1. Shoot and root dry weight of *Sorghum vulgare* cultivated in sterilized soil with different fungi. *N.i.*, non inoculated; *P.c.*, *Phomopsis columnaris*; *P.s.*, *Phoma schachtii*; *B.o.* 1, *Bionectria ochroleuca*-1; *B.o.* 2: *Bionectria ochroleuca*-2; *E.* sp., *Eucasphaeria* sp.; *C.p.*, *Cylindrocarpon paucisepatatum*; *C.d.*, *Cylindrocarpon destructans*; *B.* sp., *Bisporella* sp.; *D.* sp., *Drechslera* sp. The significance of differences between treatments was tested by the Tukey test and same letters above bars indicate lack of statistical significance ($P < 0.05$).

None endophytic fungi tested in this study, except *Drechslera* sp. colonized the root of sorghum. The percentage of root length colonization of sorghum by the fungus *Drechslera* sp. was $12.2 \pm 2.1\%$.

Ph. columnaris, *P. schachtii*, *Eucasphaeria* sp. and *C. pauciseptatum* increased the shoot and root dry matter of sorghum plants grown in unsterilized soil (Fig. 2). *B. ochroleuca*, *Bisporella* sp., *C. destructans*, *Drechslera* sp. did not increase the shoot and root dry weight of sorghum grown in unsterilized soils. The percentage of AM colonization of sorghum was increased significantly when *Ph. columnaris*, *P. schachtii*, the two strains of *B. ochroleuca*, *Eucasphaeria* sp. and *C. pauciseptatum* were applied to unsterilized soil (Fig. 3). However, no effect of *C. destructans*, *Bisporella* sp. and *Drechslera* sp. on the increase of the percentage of AM root length colonization was observed.

The number of CFUs of inoculated fungi in the rhizosphere of sorghum grown in sterilized and unsterilized soil is described in Table 4. Only the population of *B. ochroleuca*-1 increased at the end of the experiment either in sterilized or in unsterilized soils.

We did not found significant effect of the endophytic fungi isolated from *M. communis* on shoot and root P, K, Ca, Na, Mg, Cu and Zn concentrations of plants grown in sterilized or unsterilized soils (Tables 5 and 6). Only the inoculation with *Eucasphaeria* sp. increased the shoot P concentration of sorghum growth in unsterilized soil compared with non inoculated control plants (Table 6).

Table 4
Population density (CFUs g^{-1} soil) of fungi in the rhizosphere of *Sorghum vulgare* cultivated in sterilized and unsterilized soil.

Fungal species	CFUs $\times 10^4$ g^{-1} soil			
	Sterilized soil		Unsterilized soil	
	2 wk	5 wk	2 wk	5 wk
Phomopsis columnaris	1.98	2.00	4.87	5.02
Cylindrocarpon destructans	5.43	5.38	7.79	7.87
Bionectria ochroleuca-1	3.41	23.4*	4.01	93.0*
B. ochroleuca-2	2.72	2.93	3.34	3.49
Phoma schachtii	1.52	1.48	2.26	2.23
Eucasphaeria sp.	1.15	1.13	2.23	2.28
C. pauciseptatum	1.89	1.96	4.12	4.07
Bisporella sp.	2.34	2.5	4.96	4.90

Within sterilized and within unsterilized soil treatments, values with asterisk were significantly different as assessed by the Tukey test ($P < 0.05$).

4. Discussion

The results obtained in this work confirm the occurrence of AM fungi within the Myrtaceae and indicated that the species associated with *M. communis* roots, as happened in other Mediterranean areas, are *Glomus* species (Matosevic et al., 1997). The information about the other endophytic fungi isolated from *M. communis* roots is scarce.

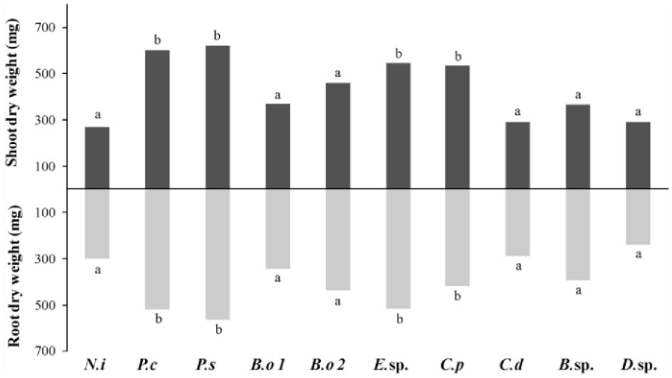

Fig. 2. Shoot and root dry weight of *Sorghum vulgare* cultivated in unsterilized soil with different fungi. *N.i.*, non inoculated; *P.c.*, *Phomopsis columnaris*; *P.s.*, *Phoma schachtii*; *B.o.* 1, *Bionectria ochroleuca*-1; *B.o.* 2, *Bionectria ochroleuca*-2; *E.* sp., *Eucasphaeria* sp.; *C.p.*, *Cylindrocarpon paucisepatatum*; *C.d.*, *Cylindrocarpon destructans*; *B.* sp., *Bisporella* sp.; *D.* sp., *Drechslera* sp. The significance of differences between treatments was tested by the Tukey test and same letters above bars indicate lack of statistical significance ($P < 0.05$).

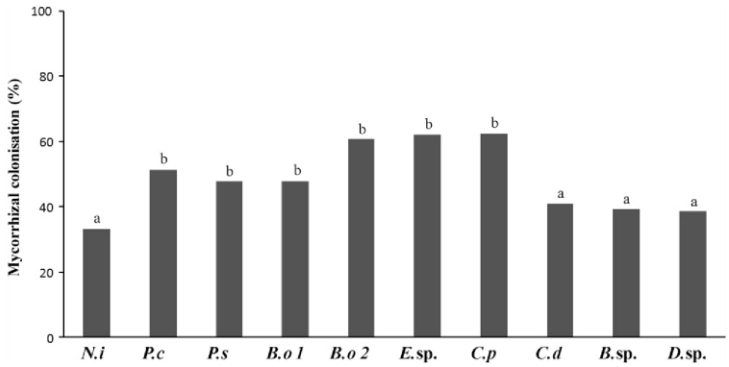

Fig. 3. Percentage of AM root colonization of *Sorghum vulgare* cultivated in non sterile soil with different fungi. *N.i.*, non inoculated; *P.c.*, *Phomopsis columnaris*; *P.s.*, *Phoma schachtii*; *B.o.* 1, *Bionectria ochroleuca*-1; *B.o.* 2, *Bionectria ochroleuca*-2; *E.* sp., *Eucasphaeria* sp.; *C.p.*, *Cylindrocarpon paucisepatatum*; *C.d.*, *Cylindrocarpon destructans*; *B.* sp., *Bisporella* sp.; *D.* sp.: *Drechslera* sp. The significance of differences between treatments was tested by the Tukey test and same letters above bars indicate lack of statistical significance ($P < 0.05$).

Species of the genera *Phomopsis* and *Phoma* are known as plant pathogens with a widespread occurrence (Sutton, 1980; Aveskamp et al., 2009) and are considered a generalist species with dark septate structures (Girlanda et al., 2002; Jumpponen and Trappe, 1998). *Ph. columnaris* were isolated from dying stem of field-grown plants of *Vaccinium vitis*-idaea (Farr et al., 2002). However, the only information about *P. schachtii* is as parasitizing cysts of the nematode *Heterodera schachtii* (Aveskamp et al., 2009). The species of *Cylindrocarpon* and *Bionectria* also are previously reported as a soil saprobe fungi, occur on leave and dead plats and can act as weak pathogen of various hosts (Domsch et al., 1980; Schroers, 2001). *C. pauciseptatum* have been isolated from partly decayed root still

Table 5
Mineral content ($g\,kg^{-1}$) of shoot of *Sorghum vulgare* grown in sterilized soil inoculated with different fungi.

Fungal species	P	K	Fe	Ca	Mn	Na	Mg	Cu	Zn
Non inoculated	0.762	17.079	0.157	4.894	3.827	0.235	3.827	0.011	0.025
Phomopsis columnaris	0.802	18.124	0.110	4.734	3.902	0.197	3.673	0.009	0.023
Phoma schachtii	0.952	19.969	0.090	5.030	4.060	0.165	4.060	0.008	0.026
Bionectria ochroleuca-1	0.775	18.452	0.098	4.678	3.923	0.172	3.365	0.007	0.024
Bionectria ochroleuca-2	0.784	13.495	0.118	4.281	3.201	0.190	3.201	0.008	0.026
Eucasphaeria sp.	0.832	20.448	0.084	4.872	3.835	0.107	3.835	0.007	0.024
Cylindrocarpon paucisepatatum	0.876	18.233	0.092	4.652	3.703	0.185	3.436	0.008	0.022
Cylindrocarpon destructans	0.765	19.105	0.107	4.776	3.912	0.193	3.521	0.007	0.026
Bisporella sp.	0.802	18.521	0.136	4.671	3.879	0.204	3.465	0.008	0.023
Drechslera sp.	0.798	17.832	0.142	4.901	3.918	0.181	3.902	0.007	0.024

Table 6
Mineral content (g kg^{-1}) of shoot of *Sorghum vulgare* plants grown in unsterilized soil inoculated with different fungi.

Fungal species	P	K	Fe	Ca	Mn	Na	Mg	Cu	Zn
Non inoculated	1.263	16.388	0.130	4.667	3.711	0.102	3.711	0.006	0.016
Phomopsis columnaris	1.190	17.752	0.102	3.647	3.281	0.090	3.193	0.007	0.015
Phoma schachtii	1.110	15.563	0.088	3.238	2.690	0.078	2.690	0.005	0.012
Bionectria ochroleuca-1	1.222	17.766	0.097	3.477	3.632	0.081	3.537	0.006	0.016
Bionectria ochroleuca-2	1.293	19.944	0.090	3.535	3.267	0.069	3.267	0.006	0.013
Eucasphaeria sp.	1.463*	18.268	0.075	3.850	3.473	0.068	3.473	0.007	0.013
Cylindrocarpon paucisepatatum	1.178	18.354	0.099	3.681	3.902	0.077	3.596	0.007	0.015
Cylindrocarpon destructans	1.280	18.665	0.110	3.476	3.731	0.091	3.362	0.008	0.014
Bisporella sp.	1.257	17.952	0.096	3.701	3.486	0.089	3.642	0.009	0.013
Drechslera sp.	1.245	18.710	0.116	3.902	3.620	0.101	3.731	0.008	0.014

Values with asterisk were significantly different as assessed by the Tukey test ($P < 0.05$).

living but badly shooting *Vitis* sp. (Schroers et al., 2008). *C. destructans* have been shown to be pathogenic towards apple seedlings causing root rot and a reduction in plant biomass (Tewoldemedhin et al., 2011). The genus *Eucasphaeria* was recently described as microfungi associated with *Eucalyptus* sp. (Myrtaceae) and the species *Eucasphaeria capensis* was isolated from living leaves and leaf litter from these plants (Crous et al., 2007). The species *Bisporella citrina* was isolated from aerial part from *Nothofagus solandri* var. *cliffortioides* (Nothofagaceae) (Fukami et al., 2010). Most of the fungal species obtained as endophytic fungi in *M. communis* have already been described as endophytic fungi of other plants species. This opens the possibility to find interesting species of fungi for application in plants of agricultural interest.

Communities of endophytic fungi may be ubiquitous or have host specificity (Schulz and Boyle, 2005). The endophytic fungi isolated from *M. communis* may be considered ubiquitous, but none of them, even the DSE *Ph. columnaris* and *P. schachtii*, were able to colonized sorghum roots. However, the DSE *Drechslera* sp. used as a model of endophytic fungus was able to colonize the root of sorghum, but did not affect the shoot and root dry weight of sorghum. *Drechslera* sp. is not considered as plant growth promoting microorganisms of several plants (Navarro, 2008), and the low level of root length colonization reached by this endophyte in the root of sorghum may explain its absence of beneficial effect on the plant dry matter. However, among the endophytic fungi isolated of *M. communis*, *P. schachtii* and *C. destructans* increased the shoot dry weight of sorghum. *Phoma* sp. has been reported to be beneficial to several crops by promoting their growth (Ravnskov et al., 2006) and the fungus *C. destructans* is considered as root pathogen (Tewoldemedhin et al., 2011). It is known that some roots endophytic fungi can act as saprobe fungi and get in the rhizosphere great nutritional benefit from organic and inorganic compounds released from living roots and sloughed cells (Scervino et al., 2009).

AM fungi interact with soil fungal communities at physical, metabolic and functional levels (Finlay, 2008). In general, interactions between AM and endophytic fungi as DSE are well known (Jumpponen and Trappe, 1998). Beneficial effects of DSE *Drechslera* sp. on the presymbiotic stage of development of the AM fungi *Gigaspora* have been described (Scervino et al., 2009). However, in our experiments no effect of *Drechslera* sp. neither on the increase of the percentage of root colonization nor on the dry weight of sorghum was found. It is known that the effect of microorganisms on AM development varies with the plant and the specie of AM fungus, most of the AM fungal spores found in the *M. communis* rhizosphere soil belong to *Glomus* genus and no *Gigaspora* spores were described. On the other hand, information about interactions between AM fungi and the endophytic fungi isolated from *M. communis*, such *Clonostachys*, *Phomopsis* and *Phoma* is scarce, and no information of interaction between AM and *Eucasphaeria*, *Cylindrocarpon* and *Bisporella* have been found (Girlanda et al., 2002; Ravnskov et al., 2006). The effect of the AM symbiosis and other soil microorganisms on the saprobe population of soil is important and seems not to be dependent of the saprobe fungal specie (McAllister et al., 1997; Martinez et al., 2004). However, in our experiments only beneficial effect of soil microorganisms on the number of CFUs of the strain *B. ochroleuca*-1 but not on the number of CFUs of *B. ochroleuca*-2 were found. This result indicates that the effect of soil microorganisms on saprobe fungal population may be completely different, even considering strains of the same species. This fact has to be taking into account in restoration of soil fungi population especially in recovering degraded soils (Godeas et al., 1999). On the other hand, although the fungi isolated from root of *M. communis* did not behave as endophytic fungi of sorghum roots, *Ph. columnaris*, *P. schachtii*, *Eucasphaeria* sp. and *C. pauciseptatum* behave as some saprobe fungi with synergistic effects on AM root colonization and plant dry matter. Several mechanisms implicated in the interactions between AM and saprobe fungi have been described (Fracchia et al., 2000; Arriagada et al., 2010). Some saprobe fungi are able to mobilize nutrients and to promote a more efficient use of the nutrients by AM colonized plants (Fracchia et al., 2000). In our experiments, the increase of shoot dry matter of sorghum inoculated with *Eucasphaeria* sp. coincided with an increase in shoot P concentration of AM colonized sorghum plants. However, both strains of *B. ochroleuca* increased AM root colonization but not plant dry matter. There are indications that saprobe fungi benefits AM symbiosis mainly by increasing AM fungal development in plant root more than to increase plant dry matter through nutritional effect (Aranda et al., 2007). Nevertheless, *Ph. columnaris*, *P. schachtii*, and *C. pauciseptatum* increased plant dry matter but did not increase nutrient concentration of sorghum suggesting that their beneficial effect on plant dry matter is through other mechanisms such as phytohormone production and will be investigated.

5. Conclusions

The root endophytic fungi isolated from *M. communis*, some of them belonging to DSE species, did not colonized sorghum root as did the DSE *Drechslera* sp. However, some of these fungi increased AM root length colonization and plant dry matter of sorghum. The mechanism by which these fungi increase sorghum dry weight varied with different fungi. The increase of P is not the only mechanism involved in the beneficial effect of saprobe fungi on the effectiveness of AM fungi on plant growth.

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Capítulo 5

Diversity of fungal endophytes in leaves of *Myrtus communis* studied by traditional isolation and cultivation-independent DNA-based method

Artigo em fase final de preparação

Diversity of fungal endophytes in leaves of *Myrtus communis* studied by traditional isolation technique and by denaturing gel gradient electrophoresis

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Abstract

The diversity of endophytic community of *Myrtus communis* in a Mediterranean ecosystem in south Spain has been evaluated by classical isolation from leaves fragments and denaturing gel gradient electrophoresis (DGGE). A total of 113 fungal isolates were obtained and identified by sequencing the ITS region of the rRNA gene. Of these, four genotypes were represented by only one sequence (singletons). Molecular identification revealed seven different endophytic taxa; all belonged to the phylum Ascomycota. The most frequent endophytic fungal genus isolated from *M. communis* was *Mycosphaerella*. We used the distance decay approach to analyze and compare the biogeography of endophytic fungal community. It was found that the similarity of endophytic fungal communities did not decrease with increasing geographical distance ($p=0.974$). The results obtained in this work suggests that the biogeographical patterns observed in endophytic fungi communities are fundamentally different from those observed in well studied plant and animal communities that have provided the foundation for biogeographical theory to date.

Keywords: Fungal endophytes, DGGE, Distance-decay.

Introduction

Fungal endophytes inhabit healthy plant tissues during at least one stage of their life cycle without causing any apparent symptom of disease or negative effects on their host plants (Petrini, 1992). Endophytes have been isolated from all plants previously studied, including bryophytes (U'Ren *et al.*, 2010), pteridophytes (Petrini *et al.*, 1992), gymnosperms (Soca-Chafre *et al.*, 2011), and both monocotyledonous (Pinruan *et al.*, 2010) and dicotyledonous angiosperms (Vaz *et al.*, 2009). These fungi colonise on all available tissues, such as roots, stems, leaves, bark, fruits, seeds, and floral organs (Petrini *et al.*, 1992; Rodriguez *et al.*, 2009; Vaz *et al.*, 2009). Most endophytic fungi isolated are ascomycetous, and *Sordariomycetes* and *Dothideomycetes* classes contain the majority of foliar fungal endophyte species (Arnold and Lutzoni, 2007). Several studies have studied the endophytic fungal diversity from angiosperms from temperate zones (Saikkonen *et al.*, 1998; Stone *et al.*, 2000) and these hosts may harbor dozens of endophytic fungi (Saikkonen *et al.*, 1998, Higgins *et al.*, 2007, Arnold & Lutzoni 2007). However, the biodiversity of fungi remains far little explored considering the small proportion of fungi that have been identified to date, and more recent estimates has been suggested that as many as 5.1 million fungal species exist (Blackwell 2011), which makes the fungi among the most diverse groups of organisms in our planet.

It is estimated that 99% of microorganisms observable in nature typically are not cultivated using cultivation-dependent methods (Amann *et al.* 1995), which have been traditionally used for the analysis of the community structure of endophytic fungi (Arnold *et al.*, 2001; Bussaban *et al.*, 2001; Wilberforce *et al.*, 2003). These methods have many inherent problems and the endophytic fungal communities will be biased towards faster growing fungi that are capable of growing rapidly on the media used (Hyde & Soytong 2008). Moreover, fungi in a latent/quiescent stage or with special growth requirements are often not recovered (Götz *et al.*, 2006). Then, these methods may provide incomplete data concerning endophyte biodiversity and molecular-based techniques, such as denaturing gradient gel electrophoresis (DGGE), may provide new insights into the endophytic fungal diversity (Muyzer *et al.*, 1993; Vainio *et al.*, 2005). DGGE has been successfully applied to document fungal communities (Götz *et al.*, 2006; Vainio *et al.*, 2005; Duong *et al.*, 2006) and is able to detect known and abundant fungi as well as unknown endophytic fungi (Duong *et al.*, 2006).

Furthermore, we used the results obtained by cultivation and molecular-based techniques to study the distribution of fungal endophytes across a host tree transect. The similarity between two observations often decreases as the distance between them increase, a pattern long observed in communities from all domains of life and once called "the first law of biogeography" (Nekola

& White, 1999; Green *et al.*, 2004). Although microorganisms are perhaps the most diverse and abundant types of organisms on Earth the distribution of microbial diversity is poorly understood (Fierer & Jackson, 2006). Little is known about how endophyte communities vary across a geographic range. In the present study, we combined cultivationdependent methods, DGGE and phylogenetic analysis to investigate the fungal endophyte communities of leaves of *Myrtus communis.* These results were used to compare the endophytic fungal diversity along a south to north transect of twenty host trees of *Myrtus communis* in a Mediterranean ecosystem. In addition we investigate the fungal endophyte diversity composition in different fragments leaves of *M. communis.*

Material and Methods

Site description and fungal isolation

The study site was in Sierra Tejeda, Almijara y Alhama Natural Park (36º51´N, 3º41´W, and elevation of around 360 m above sea level). The Park is located in the south of Andalucia, nearby Mediterranean Sea, between Malaga and Granada and consists of several dolomitic mountain ranges. Five apparently healthy leaves were collected from each of 20 individuals of all Myrtaceae species that occur in the studied sites. The trees were spaced approximately 5 m apart. All the leaves were stored in sterile plastic bags, and fungal isolation was performed on the same day of the collection. The leaves were surface-sterilised via successive dipping in 70% ethanol (1 min) and 2% sodium hypochlorite (3 min), followed by washing with sterile distilled water (2 min). After the leaf surface sterilisation, six fragments (approximately 4 mm^2) were cut from each leaf: one from the base, two from the middle vein, one from the left margin, one from the right margin and one from the tip (6 leaf fragments/leaf; 30 leaf fragments/tree; 600 leaf fragments/site; 3,000 leaf fragments overall) . All the fragments were plated onto potato dextrose agar (PDA, Difco, USA) supplemented with 100-µg/ml chloramphenicol (Collado *et al.*, 1996). The plates were incubated at 15°C for up to 60 days. To test the effectiveness of the surface sterilisation, 100 µl of the water used during the final rinse was plated on PDA to test for epiphytic microbial contaminants. Individual colonies were purified on PDA, and their morphologies were documented and photographed. For filamentous fungi, long-term preservation of mycelial samples was carried out in sterile distilled water at room temperature and in 30% sterile aqueous glycerol solution at –80°C. All fungal isolates were deposited in the Culture Collection of Microorganisms and Cells of the Universidade Federal of Minas Gerais.

DNA extraction and fungal identification of cultured fungi

The protocol for DNA extraction of filamentous fungi was adapted from Girlanda *et al.* (2002). Approximately 500 mg of mycelia of 7-day-old cultures were frozen in liquid nitrogen, ground with a prechilled mortar and pestle, transferred to 2-ml eppendorf tubes containing 600 µl of cetyltrimethylammonium bromide buffer [2% CTAB, 0.1 mM Tris-HCl pH 9, 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 0.2% β-mercaptoethanol, 1% polyvinylpyrrolidone]. The tubes were vortex-mixed and incubated at 60ºC for 60 min. After a 10-min centrifugation at 19.000 g, the aqueous phase was removed to a new tube and extracted twice with one volume of chloroform: isoamyl alcohol (24:1) and after maintained 30 min at 0ºC. Nucleic acids were precipited from the aqueous phase by addition of one volume of cold isopropanol and overnight incubated at - 4ºC. DNAs were collected by centrifugation at 19000 g for 20 min and the pellets were washed with 70% ethanol, dried briefly and resuspended in 50 µL of sterile water. The DNA extractions were diluted 1:150 and used as template for PCR amplification. The internal transcribed space (ITS) domains of rRNA gene were amplified using the universal primers ITS1 (5´- TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´- TCCTCCGCTTATTGATATGC-3´). Amplification of ITS and sequencing protocols were performed as described to Vaz *et al.* (2009) and subsequently sequenced using an automatic DNA sequencer (ABI-PRISM 310; Applied Biosystem, Inc.)

DNA extraction and fungal identification of uncultured fungi

Three leaf of each individual host tree was surface sterilized, grind in liquid nitrogen and 100 mg were used to DNA extraction with DNeasy Plant mini kit (Qiagen) following the manufacturer´s instruction. The primer pair NS1 (GTAGTCATATGCTTGTCTC) and GCfung (ATTCCCCGTTACCCGTTC) was used to amplify the variable V1 and V2 regions of 18S rDNA of fungi (Das *et al.*, 2007). NS1 had at its 5´ end a GC-rich clamp, the sequence of which has been reported by May *et al.* (2001). The total reaction mixture of the PCR consisted of 25 µl with the following ingredients: 2 µl of extracted DNA (approx. 10 ng), 2 µM primer NS1, 2 µM primer GCfung, 25 mM of $MgCl_2$, 2 mM of each dNTP, 1 U of Taq DNA polymerase (BIOLINE), 5X of PCR buffer (160 mM $(NH_4)_2$ SO_4, 670 mM Tris-HCl) and sterile Mili-Q water to a final volume. The PCR conditions were: initial denaturation at 95 ºC for 5 min; 35 cycles of amplification, each at 94 ºC for 30 s, then 55 ºC for 30 s and 72 ºC for 1 min, followed by a final extension step at 72 ºC for 5 min. PCR products from three parallel amplifications were pooled, separated in 1.5% (w/v) agarose gel and stained with ethidium bromide.

DGGE analysis

The INGENYphorU-2 system for DGGE (Ingeny International BV, Goes, NL) was used. Samples were loaded onto 8% polyacrylamide–bisacrylamide (37.5:1) gels. For the separation of amplified products from 18S, denaturing gradients were from 20% to 35%, [where 100% corresponds to 7 M urea and 40% (v/v) deionized formamide]. Gels were run at 85 V in 0.5x TAE (20 mM Tris acetate, 10 mM sodium acetate, 0.5 mM Na-EDTA at pH 7.4) at 60º C for 16 h, then stained with SYBR Gold (Invitrogen, Carlsbad, CA) in 1x TAE for 45 min at room temperature and visualized under UV illumination. DGGE banding patterns were digitized and processed using the InfoQuestTM FP (version 4.5; Bio-Rad Laboratories, Hercules, CA) and manually corrected for further analyses. An unweighed pair group method with arithmetic means (UPGMA) dendrogram was generated from a similarity matrix based on common band positions between lanes and calculated using the Jaccard coefficient (Li & Moe, 2004).

Analysis of ecological data

The isolation rate of the fungal endophytes was calculated by the following formula: total number of isolates/ total number of samples in a trial X 100, and expressed as percentage. The rates of colonization were calculated as follows: Total number of samples ≥1/ total number of samples in a trial. The data in most cases did not fit the assumptions for parametric statistics and this was confirmed by a Shapiro-Wilk test in our study. Therefore, non-parametric statistical tests were used throughout. A Kruskal-Wallis test was performed to evaluate whether the numbers of endophytic fungal species were statistically different among the host tree species. A Tukey type multiple comparison "Nemenyi" test was used to evaluate if there were the statistically significances between each two host tree species. These tests were done using the software package R (R Development Core Team, 2005).

The diversity was estimated by Shannon (H`) index ($H = -\Sigma\ ni/n\ ln\ (ni/n)$, where *ni* is number of individuals of the taxon *i* and *n* is the total number of individuals). The Shannon index is usually between 1.5 and 3.5, 1.5 representing the lowest diversity and 3.5 the highest (Maggurran, 2004; Gazis & Chaverri, 2010). The species accumulation curves were used to determine whether enough samples were taken of each study site. Species accumulation curves were generated for each host tree species using EstimateS, version 8.0 (Colwell 2005). For calculation purposes and statistical analysis, each individual fragment was considered a sample unit and a total of 600 sample units were evaluated, 100 from each fragment.

We used the distance decay approach to analyze and compare the biogeography of endophytic fungal community. The rate of distance decay of the endophytic fungal

communitiesassumes that community similarities will decrease with increasing geographical distance and was calculated according to Nekola & White (1999). The dissimilarities among endophytic communities was calculated by using the Jaccard distance, which was used due to its simplicity, its widespread use and represents a more conservative measure of community similarity than ones based on species abundance data which are more sensitive to disturbance and local environmental differences (Nekola & White, 1999). Community dissimilarity were calculated and compared with pairwise distances among individual host tree using the function "mantel" in the Vegan R package (R development core team, 2010). Linear regression was used to calculate the effect of distance in endophytic fungi community composition. These tests were done using the software package R (R development core team, 2010).

Results

A total of 600 fragment samples were analysed and were obtained 113 endophytic fungal isolates in culture-dependent technique. The overall colonization rate (%) and isolation rate for the assemblages of endophytes recovered was 0.10 and 44.5%, respectively. The samples do not fit the assumptions of a normal distribution, which could be confirmed by the low P value in the Shapiro-Wilk test (P = 2.2 10-16). The Kruskall-Wallis test showed that there was no significant difference in the number of endophytic fungal species isolated from each individual host tree species (χ^2 =12, P = 0.44). The accumulation curves of endophytic fungal assemblage of cultured endophytes did not reach an asymptote which means the sampling effort was no statistically sufficient to capture the total species richness of cultivable endophytic fungi associated with leaves of M. *communis* (Figure 1). The Shannon index obtained from cultivable endophytic fungi was 2.5.

A total of 113 endophytic fungal isolates were isolated, grouped and 30% of representative fungal isolates were identified by sequencing the ITS region of the rRNA gene. Of these, four genotypes were represented by only one sequence (singletons). Molecular identification revealed thirteen different operational taxonomic units (OTUs); all belonged to the phylum Ascomycota (Table 1).The genera of fungi associated with *M. communis* levaes were: *Alternaria*, *Aspergillus*, *Ascochyta*, *Cladosporium*, *Diaporthe*, *Gnomoniopsis*, *Mycosphaerella*, *Neofusicoccum*, *Neonectria*, *Phaeosphaeria*, *Pseudocercospora.* The most frequent endophytic fungus genus isolated from *M. communis* was *Mycosphaerella*. A phylogenetic tree was constructed with *Mycosphaerella* because many isolates obtained in this work presented 99% similarity with different species of *Mycosphaerella* deposited in GenBank. The manually adjusted ITS alignment contained 53 sequences (including out group sequence) and 572 characters. Of the 572

characters used in the phylogenetic analysis, 132 were parsimony-informative, 113 were variable and parsimony-uninformative and 327 were constant. Neighbour-joining analysis using two substitution models (Maximum likelihood and Kimura-2) on the sequence alignment yielded trees with identical topologies to one another and support the same clades as obtained from the parsimony analysis (Figure 2). For the parsimony analysis, only the first 10 000 equally most parsimonious trees were retained, the first of which is shown in Figure 1 (TL = 500, CI = 0.72, RI = 0.91, RC = 0.65). The endophytic *Mycosphaerella* isolates obtained in this work did not produced spores when cultured on common mycological media such as potato dextrose, cornmeal, malt extract, potato dextrose, Sabouraud dextrose, and yeast extract sucrose agars. Pure cultures of the fungal isolates were grouped based on morphological characters including aerial mycelium form, colony colours, surface texture and margin characters. The isolates were identified at genus level based on morphological characteristics and phylogenetic analysis (Figure 2) and five *Mycospharella* groups were defined.

We used the distance decay approach to analyze and compare the biogeography of endophytic fungi community. A plot of community similarity versus geographic distance for each pairwise set of samples revealed that the distance decay curve of cultivable endophytic fungi did not differ from zero (P = 0.974) (Figure 3). The DGGE analysis suggested that the is no difference among the

Discussion

In this study the diversity of endophytic fungi in leaves from *M. communis* was assessed by means of traditional isolation techniques and cultivation-independent methods. Endophytic fungi were isolated by plating leaf fragments on potato dextrose agar, which is a common method for isolation of endophytic fungi (Vizcaíno *et al.*, 2005; Phongpaichit al., 2006; Vaz *et al.*, 2009). The accumulation curves of cultivable fungi did not reach an asymptote suggesting that further sampling would be needed to obtain an accurate notion of the cultivable endophytic fungal community diversity. The diversity index obtained from cultivable fungi were similar to other works with foliar endophytic fungi in temperate ecosystems, as those obtained to Lycopodiaceae, Rosacea and Pinaceae from Canada (Higgins *et al.*, 2007) and Cupressaceae from USA (Hoffman & Arnold, 2008).

Most of the fungal genera obtained as endophytes in *M. communis* have already been described as endophytes of plants from tropical and temperate ecosystems, e.g., *Alternaria*, *Ascochyta*, *Aspergillus*, *Cladosporium*, *Diaporthe*, *Mycosphaerella*, *Neonectria* and *Phaeosphaeria*. The most frequent endophytic fungus genus isolated from *M. communis* was

Mycosphaerella. *Mycosphaerella* species have been isolated as pathogens, endophytes or saprophytes and are commonly found on the leaves of Myrtaceae, many of which are defoliated by these pathogens (Crous *et al.*, 2007; Carnegie, 2007; Cheewangkoon *et al.*, 2009). While *Mycosphaerella* species are generally accepted to be host specific, several species now been shown to have wider host ranges than was commonly accepted (Crous *et al.*, 2008). Species of *Alternaria* and *Phaeosphaeria* also has been found as pathogens on numerous plants (Simmons 1999; Thomma 2003). Many of the fungi most commonly isolated as endophytes are considered typical epiphytic saprobes, as *Cladosporium* species, which are considered ubiquitous epiphytic but also are capable of internal colonization of healthy tissue (Stone 2000). *Gnomoniopsis idaeicola* (anamorph *Diaporthe idaeicola*) have been associated with necrosis of leaves and chestnut galls (Magro *et al.*, 2010) however there this is the first report of *G. idaeicola* species as endophyte. *Neofusicoccum* genera has been isolated from *Myrciaria floribunda* present in west of the Tocantins state, Brazil, in an ecotone formed by Cerrado, Amazon forest, and Pantanal ecosystems (Vaz *et al.*, 2012).

We found that the similarity of endophytic fungal communities did not decrease with increasing geographical distance and this pattern has been found to root-associated fungal (Queloz *et al.*, 2011), soil bacteria (Fierer & Jackson, 2006) and endophytic fungi associated with liverworths (Davis & Shaw 2008). Despite the high taxonomic resolution to identify the endophytic fungi in this work, we found no biogeographical pattern unlike observations for plants and animals (Gaston, 2001). Consequently the results obtained in this work suggests that the biogeographical patterns observed in endophytic fungi communities are fundamentally different from those observed in well studied plant and animal communities that have provided the foundation for biogeographical theory to date.

Conclusion

The results obtained in this work provided useful information on the taxonomy, diversity and distribution of endophytic fungal communities presents in surface-sterilized leaves of *M. communits*. Moreover, this work represents the first comprehensive survey of cultivable and unculturable endophytic fungi community of *M. communis* in Spain. We did not present the DGGE results because we are finishing this analysis.

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Table 1. Fungal endophytes obtained from *Myrtus communis* and closest related species according to similarity percentage of the ITS region of the rRNA gene by alignment with sequences of related species retrieved from the GenBank database.

Closest related species	Identification	e-value	Query cover	pb	Similarity %	Number of isolates
Alternaria alternata BASALT5-10 [HQ540552]	*A. alternata* [KC959211]	0	100	502	100	1
Ascochyta obiones CBS 786.68 [GU230753]	*Ascochyta* sp. [KC959210]	9e-157	90	422	93	1
Cladosporium oxysporium CBS 125.80 [AJ300332]	*C. oxysporium* [KC959209]	0	98	532	99	4
Diaporthe cynaroidis CBS 122676[EU552122]	*D. cynaroidis* [KC959207]	0	100	532	99	17
Gnomoniopsis idaeicola [GU320824]	*G. idaeicola* [KC959208]	0	100	538	99	3
Mycosphaerella fragariae CPC 4903 [GU214691]	*Mycosphaerella fragariae* [KC959216]	0	100	493	99	4
Mycosphaerella handelii CBS 113302 [EU167581]	*Mycosphaerella* sp.1 [KC959218]	0	100	484	96	6
Mycosphaerella coacervata [EU167596]	*Mycosphaerella* sp. 2 [KC959219]	0	100	497	100	16
Mycosphaerella buckinghamiae CBS 112175 [EU707856]	*Mycosphaerella* sp. 3 [KC959217]	0	100	495	100	35
Neofusicoccum australe CBS 115185 [FJ150696]	*Neofusicoccum austral* [KC959212]	0	100	546	99	6
Neonectria macrodidyma CBS 112601 [JF268765]	*Neonectria macrodidyma* [KC959213]	0	99	495	99	1
Phaeosphaeria pontiformis CBS 589.86 [AF439499]	*Phaeosphaeria pontiformis* [KC959214]	0	100	494	97	1
Pseudocercospora crousii CBS 119487 [GQ852756]	*Pseudocercospora* sp. [KC959215]	0	100	499	99	12

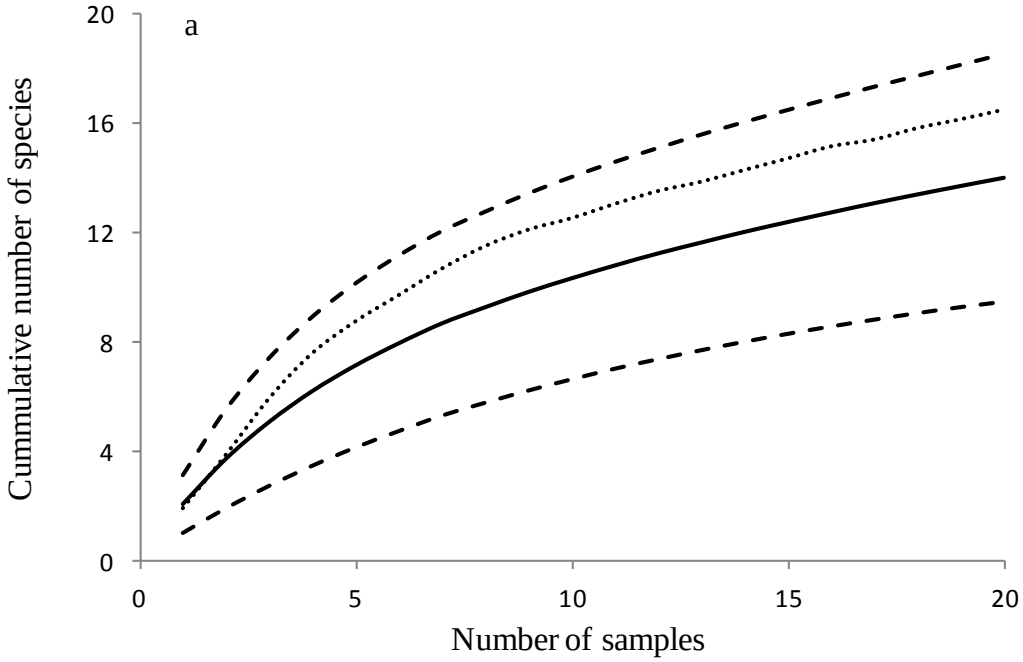

Figure 1. Species accumulation curves for fungal endophytes of leaves of *Myrtus communis* based on the Mao Tao estimator. Species groups were based on 97% similarity ITS rDNA sequence similarity. Solid black line indicates observed richness and solid gray line indicate 95% CI around the observed richness. Dashed black line indicates bootstrap estimates of total species richness inferred using EstimateS v.8.0.

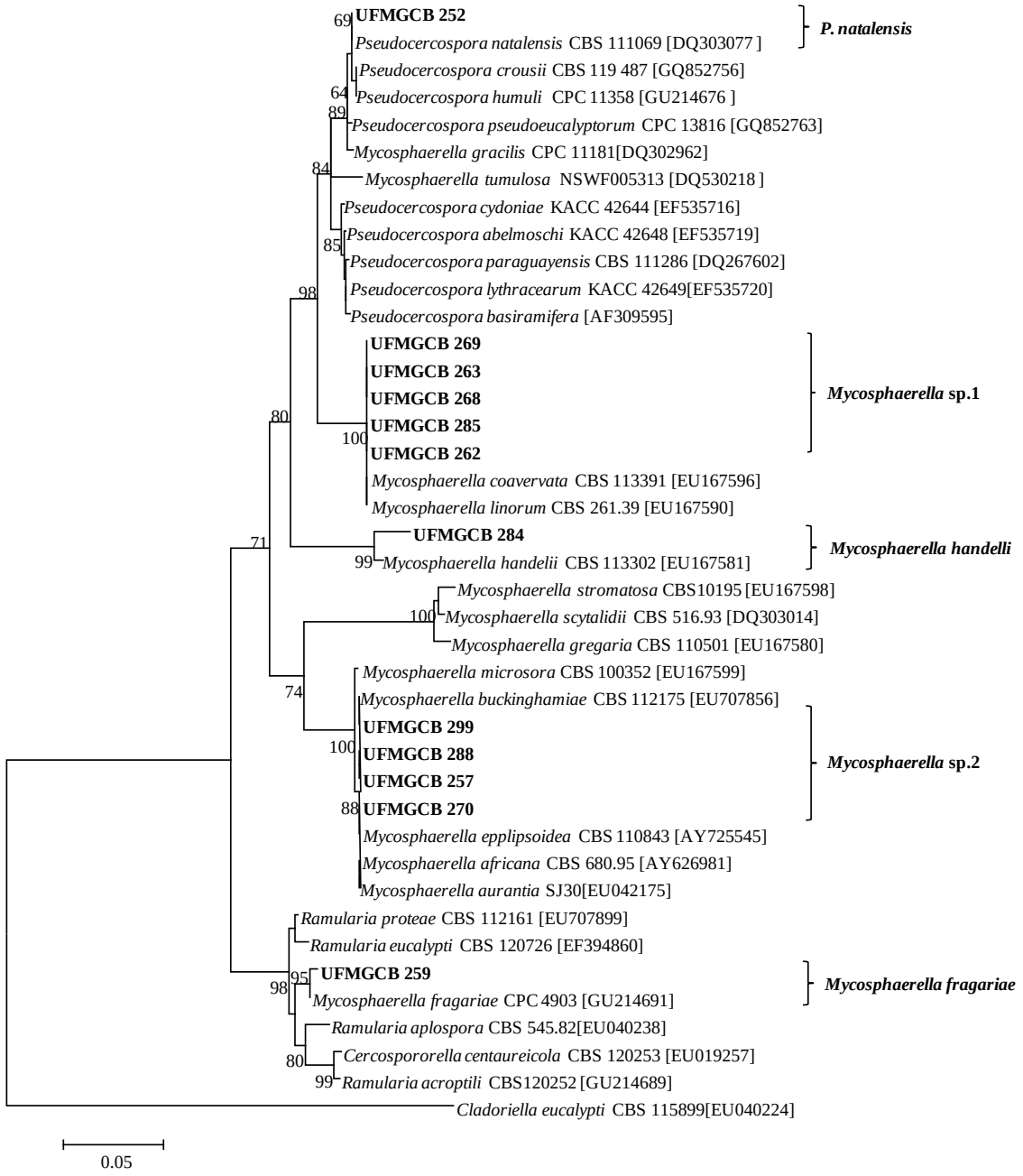

Figure 2. One of 10 000 equally most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment using PAUP v.4.0b 10. The scale bar shows 10 changes and bootstrap support values from 1000 fast stepwise replicates are shown at the nodes. The tree was rooted with *Cladoriella eucalypti* [EU040224] as the out-group.

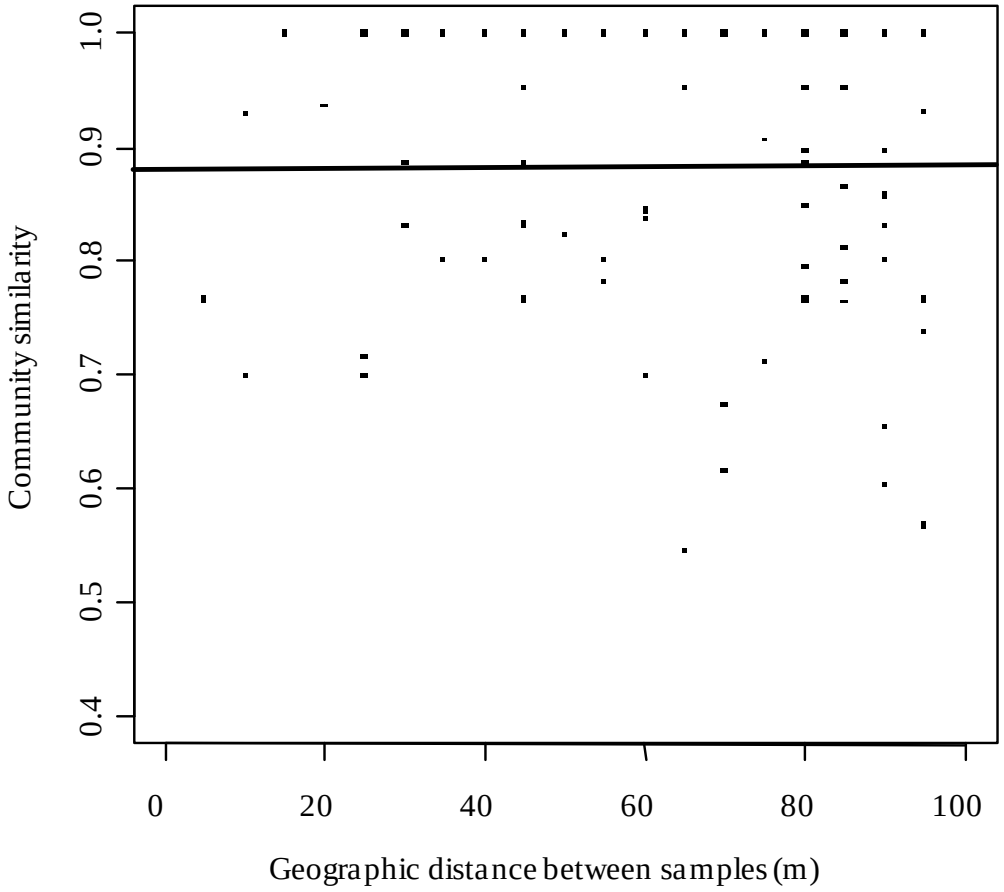

Figure 3. Distance decay relationship for cultivable fungal endophytes community of *Myrtus communis*. Pairwise community similarities were calculated using Jaccard index and plotted against the distance among study sites. The solid black lines denote the linear regression across all spatial scales and showed that are not statistical significance.

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Capítulo 6

Discussão integrada

Diversidade de fungos endofíticos

Vários trabalhos mostraram que a maioria das plantas em ecossistemas naturais é capaz de estabelecer associações simbióticas com fungos micorrizicos e/ou endofíticos (Petrini *et al.*, 1986). Apesar da variedade de estudos realizados com fungos endofíticos esta é ainda uma área com enorme potencial a ser explorado, visto que estimativas sugerem a existência de aproximadamente 5.1 milhões de espécies de fungos, dos quais somente cerca de 5% já foram descritos (Blackwell 2011). No presente trabalho foi obtido um total de 108 espécies de fungos endofíticos, sendo que deste valor 50% podem representar novas espécies sugerindo a importância deste grupo para as estimativas de fungos no planeta.

Biogeografia de fungos endofíticos

Relatos fósseis indicam que a associação endofítica existe a aproximadamente 400 milhões de anos quando os vegetais surgiram pela primeira vez no planeta (Krings *et al.*, 2007). Desde então, supõe-se que diversos tipos de interações entre vegetais e endófitos podem ter sido estabelecidas ao longo dos anos, incluindo-se relações de especificidade entre os micro-organismos e a planta hospedeira (Tan & Zou, 2001; Strobel, 2002) e que podem estar relacionadas com a distribuição dos hospedeiros (Arnold *et al.*, 2010). Micro-organismos foram considerados como cosmopolitas por muitos anos por possuírem tempos de geração curtos, grandes populações e terem a capacidade de serem dispersos por longas distâncias (Fenchel & Finlay, 2004; Queloz *et al.*, 2011) levando a hipótese de que os micro-organismos são aleatoriamente distribuídos no espaço (Martiny *et al.*, 2006). Entretanto esta hipótese tem sido muito discutida após o advento das metodologias moleculares para a taxonomia de micro-organismos, o que permitiu a identificação das espécies mais efetivamente (Taylor *et al.*, 2000). A primeira lei da biogeografia considera que a similaridade da comunidade frequentemente diminui com o aumento da distância geográfica, e este padrão tem sido amplamente observado para os macroorganismos (Nekola & White, 1999; Green *et al.*, 2004). Este padrão também foi observado neste trabalho, no capítulo 3, cujo objetivo foi avaliar o padrão de distribuição de fungos endofíticos associados a plantas presentes na Argentina e Brasil. A análise de escalonamento métrico não dimensional (NMDS) sugeriu que os fungos endofíticos agrupam-se a nível local. A análise de regressão múltipla de matrizes (MRM) permitiu avaliar a contribuição das variáveis ambientais e da distância na distribuição da comunidade de fungos endofíticos. Os resultados obtidos sugerem que a comunidade de fungos endofíticos é influenciada por fatores ambientais e distância geográfica dependendo da escala espacial utilizada. Além disso, não foi

encontrada correlação entre a similaridade da comunidade de fungos endofíticos e a proximidade filogenética entre os hospedeiros. No capítulo 5 o padrão de distribuição da comunidade de fungos endofíticos também foi avaliado, porém em menor escala. Nesta parte, foram amostrados indivíduos de *Myrtus communis* ao longo de um transecto, sendo cada indivíduo separado do seguinte em uma distância de 5 metros, compreendendo um total de 100 metros de distância entre o primeiro e último indivíduos. Para avaliar a influência da metologia de isolamento na determinação dos padrões de diversidade, foram utilizados métodos dependentes e independentes de cultivo. Entretanto as técnicas independentes de cultivo ainda estão sendo realizadas e, portanto conclusões a este respeito não foram abordadas. Considerando os dados obtidos dos fungos isolados em meio de cultura foi observado que a similaridade na comunidade de endófitos não diminui com o aumento da distância geográfica. Ao contrário dos resultados obtidos para as plantas descritas no capítulo três.

Métodos dependentes de cultivo apresentam alguns incovenientes, como por exemplo, fungos que apresentam crescimento rápido são capazes de crescer mais rápido nos meios de cultura tradicionalmente utilizados impedindo o crescimento de fungos de crescimento lento (Hyde & Soytong 2008). Além disso, fungos em estágios latentes, quiescente ou que necessitam de alguns requerimentos nutricionais frequentemente não são isolados por estas metodologias (Götz *et al.*, 2006). Os dados obtidos neste trabalho fornecem dados interessantes sobre a diferença nos padrões de diversidade e distribuição de fungos endofíticos ao longo de diferentes ecossistemas.

Potencial biotecnológico dos fungos endofíticos

Fungos endofíticos podem apresentar efeitos profundos na ecologia, adaptação e evolução das plantas (Brundrett, 2006) além de poder contribuir para a adaptação das plantas a estresses bióticos e abióticos (Giordano *et al.* 2009) fato que tem sido correlacionado com a produção de metabólitos naturais pelos fungos associados (Zhang *et al.* 2006; Suryanarayanan *et al.*, 2009; Aly *et al.*, 2010). Os resultados obtidos no presente trabalho confirmam o potencial na produção de metabólitos secundários pelos fungos, uma vez que 41% dos extratos metanólicos obtidos de fungos endofíticos associados a plantas presentes em ecossitemas de Cerrado e em uma área de ecótono entre Cerrado, Mata Amazônica e Pantanal (Capítulo 2) apresentaram atividade antimicrobiana contra diferentes micro-organismos testados. Os fungos *Emericellopsis donezkii* and *Colletotrichum gloesporioides* mostraram os melhores valores de concentração inibitória mínima (CIM), cujos valores foram menores ou similares à valores de CIM de drogas antifúngicas e antibacterianas conhecidas.

Outro aspecto importante e útil da utilização dos fungos endofíticos é o seu potencial efeito promotor do crescimento vegetal. No capítulo 4 foi avaliado o potencial da comunidade endofítica de raízes de *M. communis* na colonização e aumento do peso seco de plantas de *S. vulgare*. Apesar dos fungos mais frequentemente isolados de *M. communis* não terem a capacidade de infectar raízes de *S. vulgare* foi observado que alguns destes fungos aumentaram significativamente o peso seco da parte aérea e a porcentagem de colonização por micorrizas arbusculares, desta forma estes fungos atuaram como micro-organismos promotores do crescimento vegetal.

Este trabalho contribui para o conhecimento da riqueza da micota endofítica e o potencial biotecnológico destes micro-organismos. A bioprospecção realizada no capítulo dois mostrou que dois isolados de fungos endofíticos podem ser considerados como promissores em futuros estudos químicos para o isolamento de moléculas bioativas antimicrobianas. O estudo de interação entre micro-organismo e vegetal realizado no capítulo quatro também mostrou o potencial biotecnológico de isolados de fungos endofíticos. Estes fungos podem atuar como promotores do crescimento vegetal de plantas de interesse agronômico.

Capítulo 7

Conclusões

O estudo da biogeografia de fungos endofíticos associados à hospedeiros presentes em diversos ecossistemas é ainda muito escasso. Além disso, estes fungos constituem uma riqueza em termos de patrimônio genético e podem ser fontes de diferentes substâncias químicas úteis em processos biotecnológicos. Desta forma, considerando os resultados parciais deste trabalho foi possível inferir as sequintes conclusões:

- A análise molecular e filogenética dos mostrou que provavelmente 50% das espécies isoladas podem representam novas espécies de fungos. Isto mostra o potencial destas espécies vegetais em abrigar espécies de fungos ainda desconhecidas;

- Dois isolados de fungos pertencentes às espécies *Emericellopsis donezkii* and *Colletotrichum gloesporioides* podem ser considerados como promissores para estudos detalhados quanto ao isolamento do(s) metabólito(s) ativo(s);

- Os resultados obtidos da análise biogeográfica dos fungos endofíticos mostraram que a comunidade endofítica não é aleatoriamente distribuída. A análise de decaimento da distância confirmou que a composição de fungos varia ao longo do gradiente latitudinal estudado. A comunidade de fungos endofíticos é influenciada por fatores ambientais e pela distância geográfica e este efeito depende da escala espacial avaliada;

- Fungos endofíticos isolados de raízes de *Myrtus communis* não infectaram raízes de *Sorghum vulgare*, porém alguns fungos atuaram como promotores do crescimento vegetal quando inoculados na rizosfera de sorgo. Estudos mais detalhados devem ser feitos para determinar os mecanismos os mecanismos pelos quais estes fungos proporcionaram o aumento do peso seco e porcentagem de micorrização arbuscular de sorgo;

- A similaridade da comunidade de fungos endofíticos cultiváveis associados à *M. communis* não diminuiu com o aumento da distância geográfica;

- Este trabalho mostra o potencial biotecnológico dos fungos endofíticos como produtores de substâncias antimicrobianas e seu potencial como promotor do crescimento vegetal em plantas de interesse econômico. Os dados obtidos neste trabalho foram promissores em determinar padrões de distribuição da comunidade endofítica associada à um grupo de hospedeiros vegetais filogeneticamente associados. Além disso, o melhor entendimento da biogeografia microbiana é essencial para as pesquisas de novos produtos e processo de interesse econômico.

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Anexos

Produção científica durante o doutorado

Produção científica relacionada à tese

Artigos científicos publicados em revistas internacionais

Vaz ABM, Brandão LR, Vieira MLA, Pimenta RS, Morais PB, Sobral MEG, Rosa LH, Rosa CA (2012). Diversity and antimicrobial activity of fungal endophyte communities associated with plants of Brazilian savanna ecosystems. *African journal of microbiology research*, 6: 3173-3185.

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Artigos científicos submetidos para publicação

Vaz ABM, Fontenla S, Rocha FS, Brandão LR, Vieira MLA, Garcia V, Rosa CA. Diversity and biogeography of endophytic fungi associated with Myrtaceae species present in different ecosystems of Argentina and Brazil.

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Trabalho publicado em anais de congresso sob forma de resumo expandido

Vaz ABM, Gil VB, Vieira MLA, Mafra M, Sobral M, Pimenta RS, Moraes PB, Rosa LH, Rosa CA. Aplicações biotecnológicas de fungos endofíticos isolados do Cerrado e regiões ecotonais do Estado do Tocantins In: XI Encontro Nacional de Microbiologia Ambiental, 2008, Fortaleza. XI Encontro Nacional de Microbiologia Ambiental, 2008. p. 365 – 366.

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Prêmio

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Vaz ABM, Gil VB, Vieira MLA, Mafra M, Sobral M, Pimenta RS, Moraes PB, Rosa LH, Rosa CA. Aplicações biotecnológicas de fungos endofíticos isolados do Cerrado e regiões ecotonais do estado do Tocantins.

Produção científica relacionada à trabalhos desenvolvidos em colaboração durante a tese de doutorado

Artigos científicos publicados em revistas internacionais

Vieira MLA, Hughes AFS, Gil VB, **Vaz ABM**, Alvez TMA, Zani CL, Rosa CA, Rosa LH (2012). Diversity and antimicrobial activities of the fungal endophyte community associated with the traditional Brazilian medicinal plant *Solanum cernuum* Vell. (*Solanaceae*). *Canadian Journal of Microbiology*, 58: 54-66.

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Outras atividades

Expedições

Participação na Operação Antártica XXVII no período 17/12/2008 a 15/01/2009, exercendo função de pesquisadora do Projeto Vivian, na estação antártica Comandante Ferraz.

Cursos Internacionais

Curso de especialização em Edafologia e Biologia vegetal oferecido pela UNESCO na Estación Experimental Del Zaidin, Granada, Espanha. 1210 h. 2010-2011.

Aplicación de metodos moleculares y herramientas de análisis para el estúdio de la biodiversidad microbiana. Centro Brasileiro Argentino de Biotecnologia, CBAB, Argentina. Ministério Ciência e Tecnologia. 80h. 2010

International Course on Molecular Methods for Diagnosis, genomic analysis and Biotechnological applications of microorganisms. Universidad Nacional del Comahue, UNCo, Argentina. 60 h. 2009

Introducción al Analisis Multivarido. Universidad Nacional del Comahue, UNCo. Argentina. 60h. 2009.

Cursos nacionais

Modelagem estatística utilizando o programa R. 80h. 2012. ABG consultoria estatística.

Biotecnologia de Fungos Endofíticos da Amazônia. Universidade do Amazonas. UEA. Centro Brasileiro Argentino de Biotecnologia, CBAB, Brasil. Ministério Ciência e Tecnologia. 80h. 2009.

Treinamento Pré-Antártico. Centro de adestramento da Ilha da Marambaia. Marinha do Brasil, RJ. 40 h. 2007.

Estatística Aplicada utilizando o R. 48h. 2010. ABG consultoria estatística.