SABRINA FELICIANO OLIVEIRA

DIVERSIDADE DE FUNGOS MICORRÍZICOS E ENDOFÍTICOS ASSOCIADOS A ORQUÍDEAS AMEAÇADAS DO BIOMA MATA ATLÂNTICA

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2012

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T
O48d
2012

Oliveira, Sabrina Feliciano, 1985-
Diversidade de fungos micorrízicos e endofíticos associados a orquídeas ameaçadas do bioma Mata Atlântica / Sabrina Feliciano Oliveira. – Viçosa, MG, 2012.
vii, 72f. : il. (algumas col.) ; 29cm.

Inclui anexos.
Texto em português e inglês.
Orientador: Maria Catarina Megumi Kasuya.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Micorriza. 2. Simbiose. 3. Sequenciamento de nucleotídeo. 4. Orquídea - Raízes. 5. *Sebacina*. 6. Biodiversidade. 7. Fungos. 8. Quadrilátero Ferrífero (MG). 9. Mata Atlântica. 10. *Tulasnella*. I. Universidade Federal de Viçosa. II. Título.

CDD 22. ed. 579.51785

SABRINA FELICIANO OLIVEIRA

DIVERSIDADE DE FUNGOS MICORRÍZICOS E ENDOFÍTICOS ASSOCIADOS A ORQUÍDEAS AMEAÇADAS DO BIOMA MATA ATLÂNTICA

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

APROVADA: 16 de julho de 2012.

Cynthia Canêdo da Silva

Marlon Corrêa Pereira

Olinto Liparini Pereira
(Coorientador)

Maria Catarina Megumi Kasuya
(Orientadora)

AGRADECIMENTOS

À Universidade Federal de Viçosa e ao Departamento de Microbiologia pelo aprendizado e pela imprescindível contribuição para minha formação pessoal e acadêmica.

Á CAPES, à FAPEMIG e American Orchid Society pelo apoio financeiro.

À professora Maria Catarina Megumi Kasuya, pelos ensinamentos, amizade, pela verdadeira orientação e dedicação durante todos esses anos, cumprindo de forma fantástica seu papel de formadora de recursos humanos. Espero conseguir, um dia, ser um pouco da excelente pessoa e pesquisadora que ela é.

À professora Denise Mara Soares Bazzolli, pela atenção, carinho, aprendizado, e a infinita disposição em ajudar e me incentivar por varias vezes durante essa jornada.

Aos demais professores do Departamento de Microbiologia, pelos ensinamentos.

Ao professor Olinto Liparini Pereira, por aceitar prontamente o convite para compor a banca examinadora deste trabalho.

Ao professor Marlon Correa Pereira pela amizade, inúmeros ensinamentos, apoio, além da participação na banca examinadora.

À Cynthia Canedo da Silva, pelas valiosas contribuições e pela participação na banca examinadora.

À Melissa Faust Bocayuva, pela amizade, paciência e constante presença em minha vida, contribuindo imensamente para minha formação. Sem a qual, este trabalho seria impossível!

Ao Tomás, pela amizade, paciência e dedicação, realizando com perfeccionismo implacável todas as atividades que lhe são designadas.

Aos amigos do Laboratório de Associações Micorrízicas pela agradável convivência, aprendizado, apoio e pelos divertidos “cafezinhos”.

Aos amigos Bruno, Marliane, Glaúcia e Andréia por compartilharem as minhas angústias e alegrias.

Aos amigos do Laboratório de Genética de Microrganismos pelo acolhimento, e apoio durante a realização deste trabalho.

Aos funcionários do DMB, em especial a Nilcéa, Danilo e Evandro pela atenção, carinho e disposição em ajudar!

Ao Everton, agradeço por todo amor, compreensão e a imensa paciência.

Aos meus grandes amigos de Abaeté, que mesmo distantes, demonstram o que é uma amizade verdadeira.

Aos meus pais e familiares, pelo amor incondicional, apoio e torcida em todos os momentos de minha vida. Parte essencial para essa conquista.

A Deus por sempre me privilegiar da companhia de pessoas maravilhosas e me proporcionar experiências e aprendizados inesquecíveis!

BIOGRAFIA

Sabrina Feliciano Oliveira, filha de Valdir Antônio de Oliveira e Maria Luiza Feliciano Oliveira Alves, nasceu em Abaeté, Minas Gerais, no dia 23 de novembro de 1985. Em maio de 2006 ingressou na Universidade Federal de Viçosa em Viçosa, Minas Gerais, graduando-se, julho de 2010, em Ciências Biológicas. Em agosto de 2010, iniciou o curso de mestrado no Programa de Pós-Graduação em Microbiologia Agrícola, na Universidade Federal de Viçosa.

SUMÁRIO

RESUMO

OLIVEIRA, Sabrina Feliciano, M.Sc., Universidade Federal de Viçosa, julho de 2012. **Diversidade de fungos micorrízicos e endofíticos associados a orquídeas ameaçadas do bioma Mata Atlântica.** Orientadora: Maria Catarina Megumi Kasuya. Coorientadores: Denise Mara Soares Bazzoli e Olinto Liparini Pereira.

O Brasil possui uma grande biodiversidade de orquídeas, principalmente de espécies epífitas. Dentre essa diversidade ressaltam-se as espécies *Hadrolaelia jongheana, Hoffmannseggella cinnabarina*, *Hoffmannseggella caulescens* presentes em diferentes formações vegetacionais da Floresta Atlântica. As espécies pertencentes à família Orchidaceae associam-se com fungos micorrízicos para promover a germinação de sementes e o estabelecimento do protocormo em seu ambiente natural. O conhecimento da diversidade da comunidade microbiana com qual a orquídea mantém essa associação é de extrema relevância para a compreensão da ecologia e complexidade das associações simbióticas. O presente trabalho teve como objetivo estudar a diversidade genética dos fungos associados às orquídeas *H. jongheana, H. cinnabarina, H. caulescens*, utilizando-se abordagens moleculares, como a contrução de bibliotecas de clones e amplificação do DNA diretamente das raízes. Os valores do índice de diversidade de Shannon-Wiener para *H. cinnabarina*, coletada em distintas áreas, foram diferentes, sugerindo que os fatores locais influenciam na diversidade. Observamos que as comunidades fúngicas das três espécies de orquídeas analisadas apresentaram alta diversidade, sendo a composição dessas comunidades estruturalmente diferentes entre si de acordo com as análises do LIBSHUFF. A construção de bibliotecas de clones permitiu identificar táxons de fungos basidiomicetos e ascomicetos associados às raízes dessas plantas. Os fungos basidiomicetos encontrados, em sua maioria, são potenciais candidatos a fungos micorrízicos de orquídeas. Ao passo que, os ascomicetos identificados são, possivelmente, fungos endofíticos dessas plantas. Além disso, verificamos que essas orquídeas tropicais são generalistas em suas associações micorrízicas com fungos rizoctonióides sebacinóides e tulasnelóides. Estes resultados são relevantes à medida que são informações importantes para traçar estratégias de conservação para espécies da flora brasileira ameaçadas de extinção e endêmicas de um importante e ameaçado *hotspot*.

ABSTRACT

OLIVEIRA, Sabrina Feliciano, M.Sc., Universidade Federal de Viçosa, July, 2012. **Diversity of mycorrhizal and endophytic fungi associated with endangered orchids from the Atlantic Forest biome.** Adviser: Maria Catarina Megumi Kasuya. Co-advisers: Denise Mara Soares Bazzolli and Olinto Liparini Pereira.

Brazil has a huge orchid biodiversity, mostly epiphytic species. Among this we emphasize *Hadrolaelia jongheana*, *Hoffmannseggella cinnabarina*, and *Hoffmannseggella caulescens* species occur in different vegetation formations of the Atlantic Forest. The species belonging to the family Orchidaceae are associated with mycorrhizal fungi to promote seed germination and establishment of protocormo in their natural environment. The knowledge of the microbial community diversity which the orchids maintain association is extremely important for understanding the ecology and complexity of symbiotic associations. The present work aimed to study the genetic diversity of fungi associated with orchids *H.jongheana*, *H. cinnabarina*, *H. caulescens*, using molecular approaches, such as the clone library construction and DNA amplification directly from the roots. The values of diversity index Shannon-Wiener for *H. cinnabarina*, collected in different areas were different, suggesting that local factors influence the diversity.We observed that the fungal communities of those three orchid species analyzed showed high diversity, and the composition of these communities are structurally different from each other according to the LIBSHUFF analysis. The clones libraries construction allowed to identify Basidiomycetes and Ascomycetes taxa associated with the roots of these plants. Basidiomycete fungi found, mostly, are potential candidates for orchid mycorrhizal fungi. While the identified Ascomycete fungi found are possibly endophytes of these plants. Furthermore, we found that these tropical orchids are generalists in their mycorrhizal associations with sebacnioid and tulasnelloid Rhizoctonia-like fungi. These results are relevant as they are important information to develop conservation strategies to threaten and endemic Brazilian orchid species from an important and threatened hotspot.

1. INTRODUÇÃO GERAL

Orchidaceae é mais diversa entre todas as outras famílias de angiospermas, com o estimado entre 17.000 a 35.000 espécies (Dressler, 1993; Cribb et al., 2003). As orquídeas compreendem cinco subfamílias, Apostasioideae, a mais basal, seguida pela Vanilloideae, Cypripedioideae, e as duas subfamílias mais ricas em número de espécies, Orchidoideae e Epidendroideae, sendo que todas reúnem aproximadamente 870 gêneros (Dixon et al., 2003). Essas plantas ocupam uma variedade de habitats na natureza, estando presentes em ilhas da Antártida, em elevações acima de 4000 metros, e até mesmo dentro de regiões urbanas altamente desenvolvidas (Brown, 2002; Chase et al., 2003). Aproximadamente 73 % das espécies de orquídeas são epifíticas ou litofíticas, utilizando outras plantas ou estruturas similares como suporte (Roberts & Dixon, 2008). A ampla distribuição e diversidade das espécies de orquídeas são, em partes, devido à associação com fungos micorrízicos (Rasmussen, 1995) e à grande variedade de estratégias de polinização (Tremblay et al., 2005).

As orquídeas produzem as menores sementes, tanto pelo tamanho (0.05-6 mm) como pela massa (0.31-24 µg), em relação a todas as outras plantas, sendo essas quase que completamente desprovidas de reservas (Roberts & Dixon, 2008). A produção de um grande número de pequenas sementes favorece altas taxas de dispersão, fecundidade vegetal e a expressão da variabilidade genética através de fronteiras geográficas e ecológicas, minimizando o investimento parental por semente (Batty et al., 2002; Zettler et al., 2003). Entretanto, devido às reservas limitadas de alimentos, as sementes de orquídeas possuem uma dependência completa de nutrientes fornecidos pela associação micorrízica durante a germinação e as fases de estabelecimento do protocormo (Rasmussen, 1995). Além disso, algumas espécies podem substituir ou adquirir novos simbiontes micorrízicos dependendo da maturidade da planta (Bidartondo & Read, 2008; Waterman et al., 2011).

A associação micorrízica em orquídeas é caracterizada principalmente pela presença de densos novelos de hifas intracelulares ramificadas, colonizando as células corticais do hospedeiro (Currah & Zelmer, 1992; Andersen & Rasmussen, 1996; Pereira et al., 2002). Essas estruturas em forma de novelos de hifas, conhecidas como *pelotons* provê uma grande área de superfície comum entre a célula e o fungo, permitindo uma eficiente troca de nutrientes (Zimmerman & Peterson, 2007). Vários

autores têm sugerido que as orquídeas exploram carboidratos, recursos hídricos e minerais do micobionte, sem oferecer qualquer benefício para o fungo (Batty et al., 2002; Julou et al., 2005), enquanto outros têm documentado que as interações micorrízicas envolvem um intercâmbio de nutrientes recíproco entre a planta e o simbionte fúngico (Cameron et al., 2006). De modo geral, a relação entre orquídeas e fungos é mais bem descrita como mutualista, com a planta fornecendo açúcares, vitaminas do complexo B e um abrigo seguro para o fungo, enquanto este fornece à orquídea água, sais minerais e até 85 % das necessidades da planta de carbono (Roberts & Dixon, 2008).

Os principais fungos que formam as micorrizas em orquídeas fotossintetizantes pertencem ao filo Basidiomycota, não produzem esporos na fase assexuada (*Mycelia Sterilia*) e, no estágio anamórfico são identificados como fungos *Rhizoctonia*-like ou rizoctonióides. Os principais fungos rizoctonióides micorrízicos de orquídeas pertencem às famílias Tulasnellaceae, Sebacinaceae e Ceratobasidiaceae (Muller et al., 1998). Entretanto, o conhecimento a cerca da freqüência, da diversidade e do papel ecológico de fungos endofíticos de orquídeas permanece, em grande parte, obscuro (Bayman & Otero, 2006). Gêneros de fungos tais como *Xylaria*, *Guignardia*, *Colletotrichum*, *Acremonium*, *Fusarium*, *Trichoderma*, *Rhizoctonia* e *Alternaria* spp. são alguns dos fungos não micorrízicos já identificados como endofíticos de orquídeas, presentes nas folhas e raízes dessas plantas (Bayman & Otero, 2006; Yuan et al., 2009). Baseado no conhecimento de endofíticos em outras plantas é provável que as orquídeas possuam uma grande comunidade de fungos endofíticos, os quais são importantes componentes da biodiversidade fúngica, muitas vezes, subestimados (Tao et al., 2008). Além disso, não é descartada a possibilidade de que fungos, até então considerados apenas endofíticos, possam também estar envolvidos nas interações mutualistas (Vendramin et al., 2010).

A distribuição e abundância das orquídeas são direcionadas para os trópicos e variam entre os continentes e dentro das regiões, estando presentes também em áreas denominadas de *hotspots* (Swarts & Dixon, 2009). *Hotspots* são caracterizados como regiões de biodiversidade reconhecida pela sua riqueza de espécies, altos níveis de endemismo e que não apresentam uma adequada conservação a essas espécies ali confinadas (Myers et al., 2000). Atualmente, essas áreas compreendem 1,4 % da superfície terrestre, sendo que muitas espécies dentro dessas regiões já estão extintas

ou ameaçadas de extinção (Brooks et al., 2002), além disso, a Orchidaceae, mais do que qualquer outra família de plantas, têm uma elevada proporção de espécies ameaçadas (Swarts & Dixon, 2009). O Domínio da Floresta Atlântica, nessa conjuntura, é um complexo de ecossistemas de extrema importância, uma vez que, abriga uma parcela significativa da biodiversidade do Brasil e do mundo (Stehmann et al., 2009). Composta por um mosaico de 10 formações vegetacionais distintas, a Floresta Atlântica ocupou originalmente uma área de 1.314.460 Km^2, cerca de 15 % do território brasileiro, atualmente está reduzida a cerca de 7,9 % da cobertura inicial (Lino & Simões, 2011). Os altos níveis de riqueza e endemismo, associados à destruição sofrida no passado, incluíram a Floresta Atlântica definitivamente no cenário mundial como um dos 34 *hotspots* de biodiversidade (Mittermeier et al., 2004). Em um recente levantamento, foi apresentado que o Domínio Atlântico compreende cerca de 5 % da flora mundial, estimada, atualmente, em 300.000 espécies de plantas (Judd et al., 2009), sendo também considerada como o quinto *hotspot* mais rico em endemismo (Stehmann et al., 2009). A família Orchidaceae é considerada como uma das mais diversas presente nesse bioma, destacando-se tanto em termos de riqueza absoluta, com 1.257 espécies, como em endemismos, com 791 espécies exclusivas desse domínio (Stehmann et al., 2009).

Dentre a grande diversidade de espécies de orquídeas, ressalta-se a espécie *Hadrolaelia jongheana* (Rchb. f.) Chiron, que se encontra entre as orquídeas brasileiras mais raras, sendo originária das zonas montanhosas de Minas Gerais (Decker, 1941). Essa espécie está preste a desaparecer em poucos anos devido a coleta intensa no seu habitat natural por alguns colecionadores (Badini, 1943), a exportação e a destruição das matas que reduziram a sua existência a raros exemplares na flora mineira (dados não publicados, 2009). *Hadrolaelia jongheana* é uma planta encontrada em áreas de Floresta Ombrófila Densa, na Floresta Estacional Semidecidual e no Cerrado a uma altitude média de 1392 metros, possuindo hábito epifítico. Em Minas Gerais é relatada a ocorrência da espécie em Unidades de Conservação, regidas pelo Instituto Estadual de Florestas (IEF). Dentre elas, o Parque Estadual da Serra do Brigadeiro (PESB) que se encontra situado no Maciço da Serra da Mantiqueira, totalmente inserido na Zona da Mata de Minas Gerais (Caiafa & Silva, 2005).

Apresentando, ainda, uma grande proporção inserida no ecossistema da Floresta Atlântica, pode-se considerar a riqueza também presente nos campos

rupestres da Cadeia do Espinhaço (Giulietti et al., 2009). Os campos rupestres ocorrem a partir de uma altitude de 900 m acima do nível do mar e estão em grande parte associados à Cadeia do Espinhaço, nos Estados de Minas Gerais e Bahia (Giulietti et al., 1987). Dentre as áreas de grande representatividade em campos rupestres, ressalta-se o Quadrilátero Ferrífero, que se localiza no centro-sul de Minas Gerais, ocupando uma área de 7000 Km^2, dos quais 14,2% são de formações ferríferas. Essa região é considerada uma das mais importantes províncias minerais do mundo (Spier et al., 2003), pois sua área abriga 60% das reservas brasileiras do minério de ferro (DNPM, 2008). Entre a vegetação típica desses domínios, são encontradas áreas de afloramentos quartzítico, granítico e hematítico, constituindo o topo das elevações que compõem a Cadeia do Espinhaço. Os depósitos superficiais de hematita constituem, por sua vez, os solos denominados de *canga* ferruginosa, que se caracterizam como um dos mais importantes tipos de habitats (Jacobi *et al*., 2008). Devido à grande diversidade florística desses campos ferruginosos e o endemismo de parte de sua flora, essa região é considerada "Área de Importância Biológica Extrema" (Costa et al., 1998), "Prioritária para a Conservação da Biodiversidade em Minas Gerais" (Drumonnd et al., 2005). A família Orchidaceae, por sua vez, contribui fortemente para essa diversidade florística (Jacob et al., 2008). Dentre as espécies de orquídeas presentes nesta região, ressalta-se *Hoffmannseggella cinnabarina* (Bateman ex Lindl.) H.G. Jones e *Hoffmannseggella caulescens* (Lindl.) H.G. Jones que estão entres as plantas que apresentam adaptações morfológicas e fisiológicas importantes para sobrevivência em um ambiente de condições como, solos pobres em nutrientes, baixo conteúdo de água, bem como altas concentrações de metais pesados (Jacobi et al., 2007). O desmatamento, a crescente urbanização, mineração e o turismo são considerados as principais ameaças antrópicas no Quadrilátero Ferrífero (Costa et al., 1998). Diante de tais ameaças, a partir do final da década de 1980, diversas metodologias foram empregadas no resgate da flora em regiões potencialmente mineradoras. A produção de mudas, formação de coleções, a translocação e reintrodução dessas plantas em novas áreas vegetacionais são alguns dos exemplos (Santos, 2010). Neste contexto, para resgate das orquidáceas nessas áreas de intensa mineração, deve-se considerar não só os fatores morfológicos e fisiológicos, mas também, são de extrema importância as interações micorrízicas estabelecidas por estas plantas.

Os primeiros estudos sobre a especificidade entre orquídeas e seus fungos micorrízicos foram principalmente baseados em métodos de cultivo ou testes de germinação em condições laboratoriais, sendo encontrada, nesses casos, uma considerável amplitude filogenética de fungos associados (Curtis, 1939). Pelos padrões atuais, a especificidade não é definida pelo número de espécies de fungos com os quais uma planta pode se associar, mas sim pela amplitude filogenética dos simbiontes (McCormick et al., 2004), sendo que a diversidade de fungos compatíveis (grau de especificidade) é esperada influenciar na competição, na sobrevivência e na distribuição de uma espécie de orquídea (Stark et al., 2009).

Em geral, pouco se sabe sobre os fatores determinantes da diversidade e da composição das comunidades fúngicas associadas a essas plantas (Stark et al., 2009). Se por um lado, os micro-organismos são considerados onipresentes, por outro, há vários fatores ambientais, tanto bióticos e abióticos, que influenciam a comunidade microbiana, como por exemplo, fatores extrínsecos tais como tipo de habitat, geografia ou fatores intrínsecos como a diferenciação genética (Shefferson et al., 2008). Além disso, tem sido sugerido que as orquídeas podem se associar com múltiplos simbiontes, considerando um mesmo *peloton* (Kristiansen et al., 2001; McKendrick et al., 2002), uma única raiz (Taylor et al., 2003), um única planta (Bougoure et al., 2005) ou dentro de uma mesma população (McKendrick et al., 2002).

Deve-se considerar, também, a possibilidade de que diferentes micro-organismos, incluindo fungos e bactérias simbiontes e não simbiontes, podem ser isolados das orquidáceas (Currah et al., 1990). Neste contexto, conhecer a diversidade de simbiontes fúngicos com os quais uma determinada orquídea pode se associar, considerando ainda a existência da sucessão nas associações simbióticas, é de extrema importância para traçar estratégias de conservação e reintrodução dessas plantas em seu ambientes (Zettler et al., 2003). Para acessar essa diversidade pode se utilizar diferente técnicas moleculares baseadas na extração do DNA total dos fungos a partir das amostras ambientais (Rasmussen, 2002), permitindo, dessa forma, uma maior compreensão da ecologia e especificidade dessa associação em condições naturais (Zettler et al., 2003). A construção de bibliotecas de clones, apesar de ser trabalhosa e apresentar alto custo, permite uma acurada descrição da comunidade fúngica (Dunbar et al., 2002), detectando grupos de micro-organismos presentes em menor abundância. Principalmente, quando construída a partir da região ITS,

considerada como *barcode* universal para fungos (Schoch et al., 2012). Dessa forma, pode-se obter uma maior compreensão sobre a complexidade da comunidade e permitir uma maior detecção de grupos fúngicos diferentes daqueles que são observados em culturas sob condições laboratoriais (Vainio & Hantula, 2000).

Referências Bibliográficas

ANDERSEN, T.F.; RASMUSSEN, H.N. **The mycorrhizal species of Rhizoctonia**. In: Sneh, B.; Jabaji-Hare, S.; Neate, S.; Dijst, G (eds). Rhizoctonia species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Kluwer Academic Publishers, 379-390, 1996.

BADINI, J. *Laelia jongheana* Reichb.f. **Orquídea** 6:55-56, 1943.

BATTY, A. L.; DIXON, K. W.; BRUNDRETT, M. C.; SIVASITHAMPARAM, K. **Orchid conservation and mycorrhizal associations. Microorganisms in plant conservation and biodiversity**. The Netherlands: Kluwer Academic Publishers, 2002.

BAYMAN, P.; OTERO, J.T. **Microbial endophytes of orchid roots**. In: Schulz B, Boyle C, Sieber T (eds). Microbial root endophytes. New York: Springer, 153–173, 2006.

BIDARTONDO, M. I.; READ, D. J. Fungal specificity bottlenecks during orchid germination and development. **Molecular Ecology** 17: 3707–3716, 2008.

BROOKS, T. M.; MITTERMEIER, R. A.; MITTERMEIER, C. G. Habitat loss and extinction in the hotspots of biodiversity. **Conservation Biology**, 16: 909–923, 2002.

BROWN, P. **Wild Orchids of Florida**. University of Florida Press, 2002.

BOUGOURE, J. J.; BOUGOURE, D. S.; CAIRNEY, J. W.; DEARNALEY, J. D. ITS-RFLP and sequence analysis of endophytes from Acianthus, *Caladenia* and *Pterostylis* (Orchidaceae) in southeastern Queensland. **Mycological Research,** 109: 452–460, 2005.

CAIAFA, A. N.; SILVA, A. F. Composição florística e espectro biológico de um campo de altitude no Parque Estadual da Serra do Brigadeiro, Minas Gerais – Brasil. **Rodriguésia** 56:163-173, 2005.

CAMERON, D. D.; LEAKE, J. R.; READ, D. J. Mutualistic mycorrhiza in orchids: evidence from plant–fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid Goodyera repens. **New Phytologist**, 171: 405–416, 2006.

CHASE, M.; CAMERON, K.; BARRETT, R.; FREUDENSTEIN, J. DNA data and **Orchidaceae systematics: a new filogenetic classification**. Orchid conservation. Natural History publications, Kota Kinabalu, 69-89, 2003.

CRIBB, P. J.; KELL, S. P.; DIXON, K. W.; BARRETT, R. L. **Orchid conservation: a global perspective**. Orchid conservation. Natural History Publications, Kota Kinabalu, 1–24, 2003.

COSTA, C. M.; HERMANN, G.; MARTINS, C. S. **Biodiversidade em Minas Gerais – um atlas para sua conservação**. Belo Horizonte: Fundação Biodiversitas, Governo de Minas Gerais, IEF e Conservação Internacional, 94p, 1998.

CURRAH, R. S.; SMRECIU, E. A.; HAMBLETON, S. Mycorrhizae and mycorrhizal fungi of boreal species of *Platanthera* and *Coeloglossum* (Orchidaceae). **Canadian Journal of Botany**, 68: 1171–1181, 1990.

CURRAH, R. S.; ZELMER, C. A. Key and notes for the genera of fungi mycorrizhal with orchids and a new species in the genus *Epulorhiza.* **Reports of the Tottori Mycological Institute,** 30: 43-59, 1992.

CURTIS, J.T. The relation of specificity of orchid mycorrhizal fungi to the problem of symbiosis. **American Journal of Botany**, 26: 390–399, 1939.

DECKER, J.S. *Laelia jongheana* var. *alba***. Orquídea** 4:1 p. 28-29, 1941.

DEPARTAMENTO NACIONAL DE PRODUÇÃO MINERAL-DNPM. **Sumário Mineral**, 2008. Ministério de Minas e Energia, Brasília, 2008.

DRESSLER, R. L. **Phylogeny and classification of the orchid family**. Cambridge: University Press Cambridge, 1993.

DRUMMOND, G. M.; MARTINS, C. S.; MACHADO, A. B. M.; SEBAIO, F. A.; ANTONINI, Y. Biodiversidade em Minas Gerais – um atlas para sua conservação. Belo Horizonte: **Fundação Biodiversitas,** 222p, 2005.

DIXON, K. W.; CRIBB, P. J.; KELL, S.P.; BARRETT, R. L. **Orchid conservation.** Natural History Publications, Kota Kinabalu, 2003.

DUNBAR, J.; BARNS, S. M.; TICKNOR, L. O.; KUSKE, C. R. Empirical and theoretical bacterial diversity in four Arizona soils. **Applied Environmental Microbiology**, 68:3035-3045, 2002.

GIULIETTI, A. M.; MENEZES, N. L.; PIRANI, J. R.; MEGURO, M.; WANDERLEY, M das G. **Flora da Serra do Cipó, Minas Gerais: Caracterização e Lista das Espécies.** Boletim de Botânica da Universidade de São Paulo, v-9, p1-151, 1987.

GIULIETTI, A. M.; RAPINI, A.; ANDRADE, M. J. G.; QUEIROZ, L. P.; SILVA, J. M. C. **Plantas Raras do Brasil**. Belo Horizonte, MG: Conservação Internacional, 496p, 2009.

JACOBI, C. M.; CARMO, F. F.; VINCENT, R. C.; STEHMANN, J. R. Plant communities on ironstone outcrops: a diverse and endangered Brazilian ecosystem. **Biodiversity and Conservation**, 16: 2185-2200, 2007.

JACOBI, C. M.; CARMO, F. F.; VINCENT, R. C. Estudo fitossociológico de uma comunidade vegetal sobre canga como subsídio para reabilitação de áreas mineradoras no Quadrilátero Ferrífero, MG. **Revista Árvore**, Viçosa, 32:345-353, 2008.

JUDD, W. S.; CAMPBELL, C. S.; KELLOGG, E. A.; STEVENS, P. F.; DONOGHUE, M. J. **Sistemática vegetal: um enfoque filogenético**. 3 ed. Porto Alegre: Artmed, 632p, 2009.

JULOU, T.; BURGHARDT, B.; GEBAUER, G.; BERVEILLER, D.; DAMESIN, C.; SELOSSE, MA.. Evolution of mixotrophy in orchids: insight from a comparative study of green and achlorophyllous *Cephalanthera damasonium*. **New Phytologist**, 166, 639–653, 2005.

KRISTIANSEN, K. A.; RASMUSSEN F. N.; RASMUSSEN H. N. Seedlings of *Neuwiedia* (Orchidaceae subfamily Apostasioideae) have typical orchidaceous mycotrophic protocorms. **American Journal of Botany**, 88:956–959, 2001.

LINO, C. F.; SIMÕES, L. L. **Avaliação do cumprimento das metas globais e nacionais de biodiversidade 2010 para a Mata Atlântica**. WWF-Brasil em parceria com a Reserva da Biosfera da Mata Atlântica, 2011.

MCCORMICK, M .K.; WHIGHAM, D. F.;O'NEILL, J. Mycorrhizal diversity in photosynthetic terrestrial orchids. **New Phytologist,** 163: 425-438; 2004.

MCKENDRICK, S.L.; LEAKE, J.R.; TAYLOR, D.L.; READ, D.J. Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp. **New Phytologist**, 154:233-247, 2002.

MITTERMEIER, R. A.; GIL, P. R.; HOFFMANN, M.; PILGRIM, J.; BROOKS, T.; MITTERMEIER, C. G.; LAMOREUX, J.; FONSECA, G. A. B. **Hotspots revisited Earth's biologically richest and most endangered terrestrial ecoregions**. Cemex, Mexico, 2004.

MYERS, N.; MITTERMEIER, R. A.; MITTERMEIER, C. G.; DA FONSECA, G. A. B.; KENT, J. 2000. Biodiversity hotspots for conservation priorities. **Nature** 403: 853–858, 2000.

MULLER, W.H.; STALPERS, J.A.; VAN AELST, A.C.; VAN DER KRIFT, T.P.; BOEKHOUT. T. Field emission gun-scanning electron micro-scopy of septal pore caps of selected species in the Rhizoctonia s.1. complex. **Mycologia**, 90: 170–179, 1998.

NYLANDER, J.A.A., 2004, **MrModeltestv2**. Program distributed by the auhor. Evolutionary Biology Centre, Uppsala University, Sweden.

PEREIRA, O. L.; ROLLEMBERG, C.L.; KASUYA, M.C.M. Associações micorrízicas em orquídeas: perspectivas e utilização em programas de propagação simbiótica. **Orquidário**, 6:2:40-44, 2002.

RASMUSSEN, H. N. **Terrestrial orchids: from seed to mycotrophic plant**. Cambridge: Cambridge University Press, 444p, 1995.

RASMUSSEN, H. N. Recent developments in the study of orchid mycorrhiza. **Plant and Soil,** 244: 149-163, 2002.

ROBERTS, D. L.; DIXON, K. W. Orchids. **Current Biology**, l18(8):325-329, 2008.

SANTOS, L. M. Restauração de Campos Ferruginosos mediante resgate de flora e uso de topsoil no Quadrilátero Ferrífero, Minas Gerais. Belo Horizonte, UFMG. (**Tese de Doutorado**), 2010.

SCHOCH, C.L.; SEIFERT, K.A.; HUHNDORF, S.; ROBERT, V.; SPOUGE, J.L.; LEVESQUE, C.A.; CHEN, W. Fungal Barcoding Consortium. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. **Proceedings of National Academy of Sciences,** 109:6241 – 6246, 2012.

SPIER, C. A.; BARROS, S. M.; ROSIERE, S. A. Geology and geochemistry of the Águas Claras and Pico Iron Mines, Quadrilátero Ferrífero, Minas Gerais**,** Brazil. **Mineralium Deposita**, 38: 751-774, 2003.

STARK, C.; BABIK, W.; DURKA, W. Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea*. **Mycological Research**, 952-959, 2009.

STEHMANN, J. R.; FORZZA, R. C.; SALINO, A.; SOBRAL, M.; COSTA, D. P.; KAMINO, L. H. Y. **Plantas da Floresta Atlântica**. Rio de Janeiro: Jardim Botânico do Rio de Janeiro, 516p, 2009.

SWARTS, N. D.; DIXON, K. W. Terrestrial orchid conservation in the age of extinction. **Annals of Botany**, 104: 543–556, 2009.

TAO, G.; LIU, Z.Y.; HYDE, K.D.; YU, Z.N. Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochraceae* (Orchidaceae). **Fungal Diversity** 33: 101-122, 2008.

TAYLOR, D.L.; BRUNS, T.D.; SZARO, T.M.; HODGES; S.A. Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a

nonphotosynthetic desert orchid. **American Journal of Botany** 90: 1168–1179, 2003.

TREMBLAY, R. L.; ACKERMAN, J. D.; ZIMMERMAN, J. K.; CALVO, R. N. Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. **Biological Journal of the Linnean Society**, 84: 1–54, 2005.

VAINIO, E.J.I.; HANTULA, J. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. **Mycological Research,** 104:927–936, 2000.

VENDRAMIN, E.; GASTALDO, A.; TONDELLO, A.; BALDAN, B.; VILLANI, M.C.; SQUARTINI, A. Identification of Two Fungal Endophytes Associated with the Endangered Orchid *Orchis militaris* L. **Journal of Microbiology and Biotechnology**, 20(3), 630–636, 2010.

YUAN, Z.; CHEN, Y.; YANG, Y. Diverse non-mycorrhizal fungal endophytes inhabiting an epiphytic, medicinal orchid (*Dendrobium nobile*): estimation and characterization. **World Journal Microbiology Biotechnology**, 25:295–303, 2009.

WATERMAN, RJ.; BIDARTONDO, M.I.; STOFBERG, J.; COMBS, J.K.; GEBAUER, G.; SAVOLAINEN, V.; BARRACLOUGH, T.G.; PAUW, A. The Effects of Above- and Belowground Mutualisms on Orchid Speciation and Coexistence. **The American Naturalist**, 177: 2, 2011.

ZETTLER, L. W.; SHARMA, J; RASMUSSEN, F. N. **Mycorrhizal diversity**. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ, eds. Orchid conservation. Kota Natural History Publications, 205–226, 2003.

ZIMMERMAN, E.; PETERSON, R.L. Effect of a dark septate fungal endophyte on seed germination and protocorm development in a terrestrial orchid. **Symbiosis,** 43:45-52, 2007.

2. ENDOPHYTIC AND MYCORRHIZAL COMMUNITIES ASSOCIATED WITH ROOTS OF ENDANGERED NATIVE ORCHIDS FROM THE BRAZILIAN ATLANTIC FOREST

Sabrina Feliciano Oliveira, Melissa Faust Bocayuva, Tomás Gomes Reis Veloso, Denise Mara Soares Bazzolli, Cynthia Canedo da Silva, Olinto Liparini Pereira, Maria Catarina Megumi Kasuya

Abstract

In this study, we characterized the fungal communities associated with three tropical orchid species, *Hadrolaelia jongheana*, *Hoffmannseggella caulescens* and *Hoffmannseggella cinnabarina*, found in different vegetation formations of the Atlantic Forest. All three are included in the Brazilian Flora Species Threatened List. The composition and diversity of fungal communities were determined by constructing clone libraries and by applying diversity and richness indices. The influence of ecological aspects was also evaluated. Our results demonstrated the presence of Basidiomycetes and Ascomycetes fungi associated with the roots of these plants. Among the Basidiomycetes found, Sebacinales (81.61%) and Cantharellales (12.10%) were the dominant orders. The Ascomycetes included the Helotiales (29.31%), Capnodiales (18.10%), and Sordariales (10.34%), among others. A Shannon-Wiener diversity index (H`) analysis showed higher fungal community diversity associated with *H. caulescens* and *H. cinnabarina* roots in ferruginous formations, which differed from the H` values of *H. cinnabarina* from granitic inselbergs, suggesting that local factors influence this diversity. The epiphytic *H. jongheana* orchid also showed high fungal community diversity. The Simpson diversity index values showed that all of the libraries presented dominant species, and LIBSHUFF analysis showed that the fungal communities were structurally different from each other. We can conclude that the three orchid species studied present a high diversity of fungal communities. This study generates

important information for the development of conservation strategies for threatened and endemic Brazilian flora species in an important and threatened hotspot.

Keywords: Fungal diversity, *Hadrolaelia jongheana*, *Hoffmannseggella caulescens*, *Hoffmannseggella cinnabarina*, ITS clone library, hotspot

Introduction

It has been suggested that orchids can be associated with multiple symbionts (Huynh *et al*., 2009). However, little is known about the diversity and composition of fungal communities associated with orchids (Stark *et al*., 2009). The availability of compatible fungi in certain habitats can determine successful growth and influence the survival, competition and distribution of the species.

The greatest orchid diversity is found in the Neotropics (Pridgeon, 1995). The Atlantic Forest biome is a complex ecosystem of extreme importance because constitutes a significant portion of the biodiversity in Brazil and the world (Stehmann *et al*., 2009). It is recognized as one of 34 biodiversity hotspots in the known world (Mittermeier *et al*., 2004). Occurring in this hotspot are *Hadrolaelia jongheana* (Rchb. f.) Chiron, which is among the rare Brazilian orchids (Decker, 1941), and the genus *Hoffmannseggella*, which comprises species that are strictly rupicolous (that grow on rocks) and are endemic to southeastern Brazil, such as *Hoffmannseggella cinnabarina* (Bateman ex Lindl.) HG Jones and *Hoffmannseggella caulescens* (Lindl.) HG Jones. Because 47% of all *Hoffmannseggella* species are derived from a single population that, in many cases is found near an expanding industrial complex area outside legal protection (Verola *et al*., 2007), this genus is one of the most threatened plant taxa in Brazil (Antonelli *et al*., 2010).

Among the vegetation formations presenting a high species richness of Orchidaceae in the Atlantic Forest are the “campo rupestre” (rupestrian fields)

(Giulietti *et al*., 2009), consisting of the ferruginous formations in "Quadrilátero ferrífero" (Ferruginosous Quadrilateral) and located in Central-Southern Minas Gerais State. This region occupies an area of 7000 km^2, 14.2% of which contains iron formations, and is considered one of the most important mineral provinces in the world (Spier *et al.*, 2003). Due to high floristic diversity and endemism, this region is considered an "Extreme Biological Importance Area" (Costa *et al*., 1998). However, it is a rapidly developing region, where anthropogenic activities such as mining and tourism represent a serious threat to endemic biota (Costa *et al*., 1998).

To better understand the diversity of fungi associated with different orchid species and the influence of different ecological aspects (due to the geographic location), the fungal communities associated with three species of native orchids from the Atlantic Forest were evaluated. It is expected that those orchids with a greater diversity of fungal interactions than others can be introduced into new habitats more easily and are more tolerant to environmental disturbances. The diversity of the fungal community associated with orchids was assessed through the construction of clone libraries using primers that amplify the internal transcribed spacer (ITS) region of rDNA of Ascomycota and Basidiomycota rDNA in the roots of these plants. Therefore, the information generated here is important to infer the capacity of these plants to grow in other habitats, which contributes to the success of reintroduction programs.

Materials and methods

Plant material and study sites

All of the orchid species analyzed in this study are included in the Brazilian Flora Species Threatened List published by the Biodiversity Foundation (2008). The

orchid taxonomic names follow the nomenclature proposed by Barros et al. (2012). The samples were collected at two sites in the Atlantic Forest biome.

Root system samples from *H. jongheana* and *H. cinnabarina* were collected in Serra do Brigadeiro State Park (SBSP) (Figure 1). According to Köeppen`s classification (1948), the climate is mild mesothermic (Cwb). The average annual precipitation is 1,300 mm, and the average annual temperature is 18 °C (Engevix, 1995).

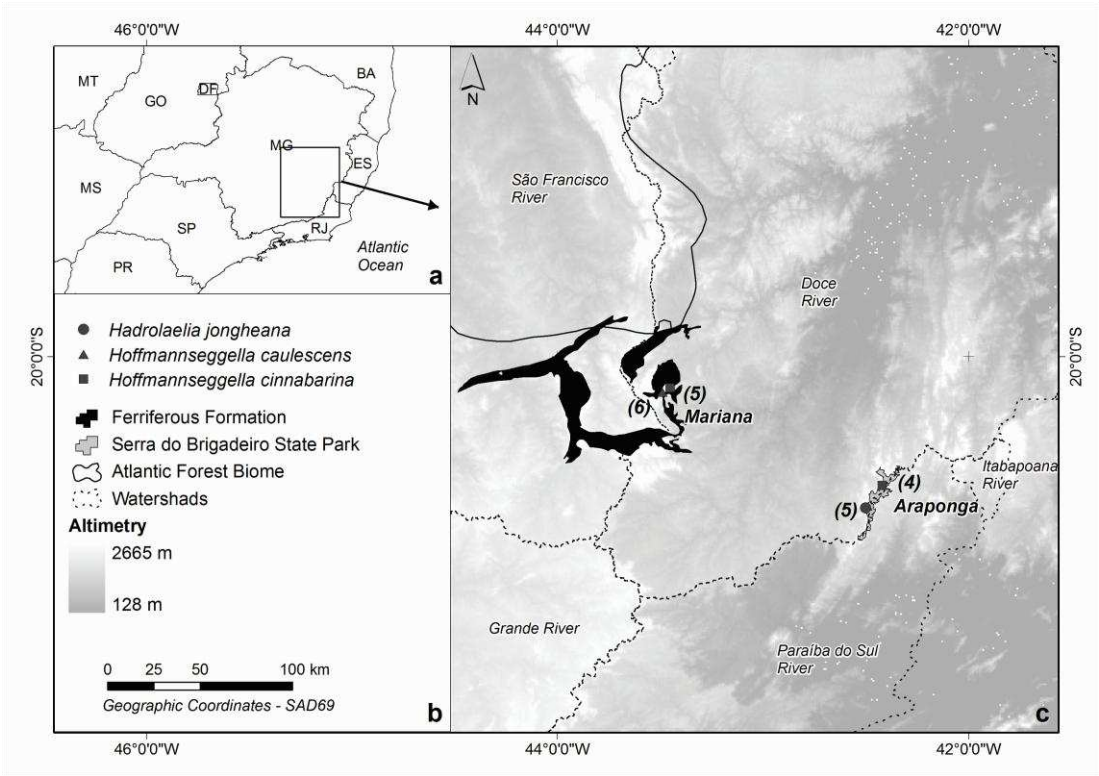

Figure 1 Map of the study site and four sampling locations. (b) Orchid species analyzed. (c) The number in parentheses represents the number of different individuals of each species from which root system samples were obtained to construct the clone libraries.

The SBPS vegetation comprises secondary fragments of the Seasonal Semideciduous Forest (Veloso *et al*., 1991), the Upper Montane Formation (Oliveira-Filho and Ratter, 1995) and the "Campos de Altitude" (Ferri, 1980), which encompasses variable extensions of rock outcrops (Paula, 1998), mainly granite and gneiss formations (Benites *et al*., 2003). *Hadrolaelia jongheana* root samples were collected

from tree trunks in the Seasonal Semideciduous Forest, while *H. cinnabarina* root samples were collected on granitic rock outcrops called inselbergs.

Hoffmannseggella caulescens and *H. cinnabarina* root samples were collected on rupestrian fields in the "Quadrilátero ferrífero" region (Figure 1). According to Köeppen`s classification (1948), the climate is Cwb (Strahler, 1963) and is characterized by a mild summer and low average annual temperature (17.4 to 19.8 °C). The "Quadrilátero Ferrífero", which is characterized as one of the most important habitat types (Jacobi *et al*.,2008), is composed of complex gneiss metamorphic rocks that form exposed blocks of ferruginous rock or ''canga'' (Viana and Lombardi, 2007).

The roots were carried to the laboratory under refrigeration and surface sterilized by the method proposed by Stewart & Zettler (2002). The root fragments containing pelotons were lyophilized for further molecular analysis. Samples were collected over a period of 12 months (from January 2011 to January 2012) with the permission of IEF/Brazil (Instituto Estadual de Florestas).

Soil Analysis

Eleven soil samples, six from the ferriferous formations and four from granitic inselbergs (SBSP) (Figure 1), were collected from the orchid rhizosphere, placed in labeled plastic bags, air dried, passed through a 2-mm mesh sieve, and subjected to routine chemical analysis at the Soils Analysis Laboratory of the Soils Department, Federal University of Viçosa (UFV) (Table 1). Chemical characteristics were interpreted following the recommendation for fertilizer use in Minas Gerais State, 5th Approximation (Ribeiro *et al*., 1999).

Table 1 Chemical properties of the soil samples

Origins of Soil	pH H_2O	P[a]	BS[b]	V[c]	m[d]	OM[e]
		mg dm^{-3}	$cmol_c$ dm^{-3}	%		dag kg^{-1}
Ferriferous formation	4. 25 ± 0.3	6. 13 ± 1.0	1.33 ± 0.4	9.1 ± 4.2	33.73 ± 24.2	30.62 ± 6.7
Granitic Inselberg	4.61 ± 0.1	4.5 ± 1.5	0.59 ± 0.03	3.42 ± 0.5	73,37 ± 4.3	31.28 ± 8.3

Data are indicated as mean ±SE, *n*=6 for Ferriferous formation; *n*= 4 for Granitic Inselberg. [a]Phosphorus. [b]Bases Sum. [c]Index of base saturation. [d]Aluminum saturation index. [e]Organic matter.

DNA extraction and PCR amplification

Total DNA extraction of the twenty orchid root systems (one of each individual) was performed using a Spin Kit ® Plant Mini Invisorb (Invetek) according to manufacturer's instructions. DNA integrity was evaluated on a 0.8% (w/v) agarose gel, and the concentration measurement was performed by spectrophotometry. All of the samples were subjected to PCR amplification. The reactions were prepared according to the manufacturer's recommendations Go TaqTM (Promega, Madison, WI, USA) and used 25 ng of DNA in each reaction. To assess the diversity of the fungal community, DNA from the roots was subjected to amplification of the ITS rDNA region using the universal primers for Ascomycota and Basidiomycota taxa, specifically the ITS1F (Gardes and Bruns, 1993) and ITS4 primers (White *et al*., 1990). The cycling scheme was 95 ºC for 2 min, followed by 39 cycles at 95 ºC for 1 min, 50 ºC for 1 min, 72 ºC for 1 min and final extension step at 72 ºC for 7 min. Every PCR included a control PCR mix without DNA template. The success of the PCR amplification was tested in 1.5% agarose that was stained in a solution of ethidium bromide (0.5 µg ml^{-1}).

Clone library construction and sequencing

For the ITS library construction, PCR products from successful reactions using the primers ITS1F/ITS4 were cloned into a pGEM T-easy Vector System (Promega) according to the manufacturer's instructions and transformed into *E. coli* DH5α ultracompetent cells. Recombinant clones were detected by blue/white screening, and white colonies picked from plates were used directly as templates in PCR with the standard sequencing primers M13F (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and M13R (5'-TCACACAGGAAACAGCTATGAC-3') (Invitrogen, 1999). The PCR reactions were prepared according to the manufacturer's recommendations Go TaqTM (Promega). The cycling scheme was 94 °C for 3 min, followed by 34 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, and final extension for 10 min at 72 °C. The PCR products of positive clones were sequenced by Macrogen Inc., South Korea, using BigDye TM and a 3730xl Automatic Sequencer (Applied Biosystems, Weiterstadt, Germany).

Diversity analyses

All of the sequences of each library were edited using Sequencher (version 4.1.4) (Genes Codes, Ann Arbor, MI, USA) and were aligned using MEGA 5 software (Tamura *et al*., 2011). ITS1-5.8S-ITS2 rRNA gene sequences were then clustered as operationl taxonomic units (OTUs) at an overlap identity cutoff of 97% using MOTHUR software (version 1.10) (Schloss *et al.*, 2009). Richness and diversity statistics, including the nonparametric richness estimators ACE, Chao1, and the Simpson and Shannon-Wiener diversity index, were also calculated using

MOTHUR. The sampling effort and community overlap were determined using a rarefaction curve and a Venn diagram, respectively.

Statistical analyses

Based on the sequence alignment, a distance matrix was constructed using DNAdist from PHYLIP (version 3.6) (Felsenstein, 2005), and pairwise comparisons of each clone library were performed using LIBSHUFF software (version 0.96; http://www.mothur.org/wiki/Libshuff).

Taxonomic assignments and phylogenetic analyses

The ITS sequences were queried against GenBank using BLASTn (http://www.ncbi.nlm.nih.gov/genbank/). All sequences for which the ITS1 and ITS2 regions matched unrelated sequences (i.e., different genera) were excluded from subsequent analyses. The taxonomic placement of the sequences with high similarity to anamorphic fungi were determined either by looking at the best BLASTn match for a teleomorph or by consulting phylogenetic studies that included that species. Phylogenetic analyses were performed using representatives of each OTU found within the libraries (at a distance level of 3% according to MOTHUR) and with closely related sequences that were recovered from the GenBank database. Bayesian likelihood was used to estimate the phylogenetic relationships. For the Bayesian approach, we used MrBayers 3.0 (Huelsenbeck and Ronquist, 2001). MrModeltest (Nylander, 2004) was used to estimate the DNA substitution models using the akaike information criterion (AIC). In this analysis, five independent runs with four Markovian Monte Carlo (MCMC) chains were turned by 15 million generations, and the trees were sampled and held at the end of the process. The first 1 million samples of trees were discarded in the burning phase, and the trees that remained were summarized to generate a consensus tree. The ITS region sequences obtained in the

present study were submitted to GenBank under accession numbers JX317084–JX317525, and they are listed in Supplementary Table 1.

Results

Soil chemical properties

Both ferriferous formations and granitic inselbergs contained acidic soils. Significant differences in other chemical parameters existed between soils from the two regions (Table 1). In general, the soils were characterized as dystrophic.

ITS sequences analysis

Twenty ITS clone libraries were generated in this study. Ten clone libraries were generated from *H. cinnabarina* roots (four from granitic inselberg and six from rupestrian field), five were generated from *H. jongheana* roots (Seasonal Semideciduous Forest), and the remaining five were generated from *H. caulescens* (rupestrian field) (Figure 1c). From these libraries, we obtained 535 sequences, and 93 poor-quality sequences were removed from further analysis. The remaining sequences comprised 118 OTUs, defined at 97% similarity using MOTHUR. For the subsequent analyses, the twenty libraries were grouped into four major clone libraries, one library for each orchid species and study site (Table 2). Among the four libraries examined, a larger number of OTUs was observed in the HCBc library, followed by the HCC, HJ and HCBg libraries (Table 2).

Table 2 Species richness estimates and diversity of ITS1-5.8S-ITS2 rDNA sequences as determined by MOTHUR software

Host species	Locality	Library	N°.of clones	N°. of OTUS[c]	ACE[d]	Chao1	Shannon-Wiener	Simpson (1/D)
H.cinnabarina	SBSP[a]	HCBg	110	19	34	47	2.44	9
H.cinnabarina	FF[b]	HCBc	123	42	205	112	3.17	17
H.caulescens	FF	HCC	107	36	137	82	3.03	15
H.jongheana	SBSP	HJ	102	21	30	30	2.6	11

Abbreviation: OTU, operational taxonomic unit. [a]SBPS- Serra do Brigadeiro State Park. [b]FF- Ferriferous Formation. [c]Number of unique OTUs defined by using the furthest neighbor algorithm in MOTHUR at 97% similarity. [d]Abundance based coverage estimator (ACE).

Composition of fungal communities

The taxonomic analysis of the 442 fungal sequences allowed their classification into Genus to Phylum. Of the 223 sequences distributed in the phylum Basidiomycota, 29.14% belonged to the HCBg library, 26.45% belonged to the HCBc library, 23.76% belonged to the HCC library, and 20.62% belonged to the HJ library. Of the 116 sequences distributed in the phylum Ascomycota, 24.13% belonged to the HCBg library, 32.75% belonged to the HCBc library, 26.72% belonged to the HCC library, and 16.37% belonged to the HJ library. One hundred three (23.30%) clone sequences were identified as non-culturable fungi. A total of 5.38% and 1.06% of sequences, distributed in Basidiomycota and Ascomycota, respectively, were identified only at the phylum level. However, most of the clones could be identified to the order level, some at the genus level (Figure 2), and minority at the class and family levels. Thirty-one clones, twenty-eight from HCBc, two from HCBg, and one from HCC, were related to the Leotiomycete class. Only one clone from the HCBg library was identified in the Sebacinaceae family. Other

families were also identified, such as Stereaceae (a single clone in the HCBg library), Helotiaceae (two clones, one from HCBg and one from HCC), and Hyaloscyphaceae (one clone in each of the four libraries).

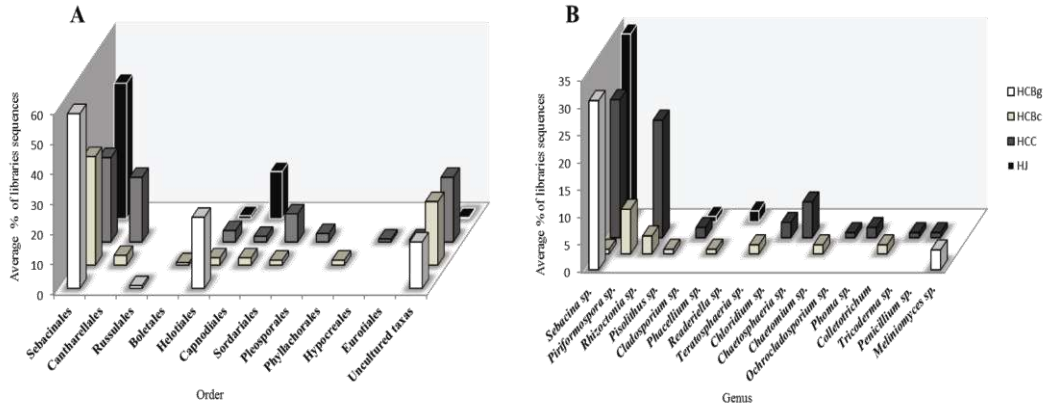

Figure 2 Proportion of ITS1-5.8S-ITS2 rDNA sequences from clone libraries that were classified at the order (a) and genus levels (b). Sequence classification was based on BLASTn results. HCBg - *Hoffmannseggella cinnabarina* from the Serra do Brigadeiro State Park (granitic inselberg); HCBc - *Hoffmannseggella cinnabarina* from a Ferriferous Formation (*canga*)*;* HCC - *Hoffmannseggella caulescens* from a Ferriferous Formation (*canga*)*;* HJ - *Hadrolaelia jongheana* from the Serra do Brigadeiro State Park (Semideciduous Seasonal Forest).

Diversity of fungal communities

In this study, the rarefaction curves for estimated species (distance = 0.03) tended toward saturation for the HCBg and HJ libraries, indicating that the sampling effort was enough to capture a substantial proportion of the species richness of the fungal communities associated with *H. cinnabarina* and *H. jongheana* collected at SBSP (Figure 3). On the other hand, for the HCBc and HCC libraries, the tendency toward plateau formation was not observed, suggesting that species richness was underestimated; further sampling of both libraries may reveal greater fungal species richness associated with the ferriferous formation orchids (Figure 3). Nonparametric

richness estimators, such as ACE and Chao 1, were used to estimate the total number of OTUs needed to sample all of the species in the fungal communities in root samples (Table 2). In the four libraries, the estimated species number was greater than the observed number; however, this difference was smaller in the HJ library (Table 2).

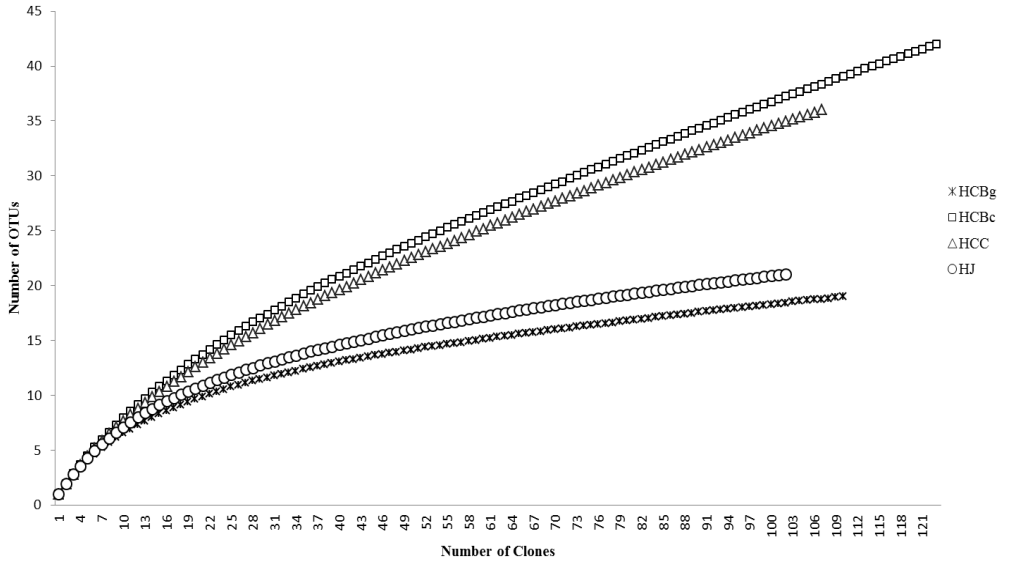

Figure 3 Rarefaction curves for clone libraries based upon the ITS1-5.8S-ITS2 rDNA sequences. HCBg - *Hoffmannseggella cinnabarina* from the Serra do Brigadeiro State Park (granitic inselberg); HCBc - *Hoffmannseggella cinnabarina* from a Ferriferous Formation (*canga*)*;* HCC - *Hoffmannseggella caulescens* from a Ferriferous Formation (*canga*)*;* HJ - *Hadrolaelia jongheana* from the Serra do Brigadeiro State Park (Semideciduous Seasonal Forest).

The highest values of the Shannon-Wiener and Simpson (1/D) index were observed for the HCBc library, followed by the HCC, HJ and HCBg libraries (Table 2). These results indicated that the fungal community diversity is very high in the root systems of the orchids growing in the ferriferous formation (HCBc and HCC) (Table 2). The LIBSHUFF program used for multiple comparisons of the libraries showed that all of the libraries were significantly different, with P values ($P < 0.0001$) below the threshold (0.0041) for a significant difference at 5% probability. The Venn diagram supports the above analysis because it reveals a low OUT overlap between

the libraries (Figure 4). The HCBg and HCBc libraries shared only five OTUs. The HCBg and HCC libraries shared just one OTU, and the HCC library shared two OTUs with the HJ library.

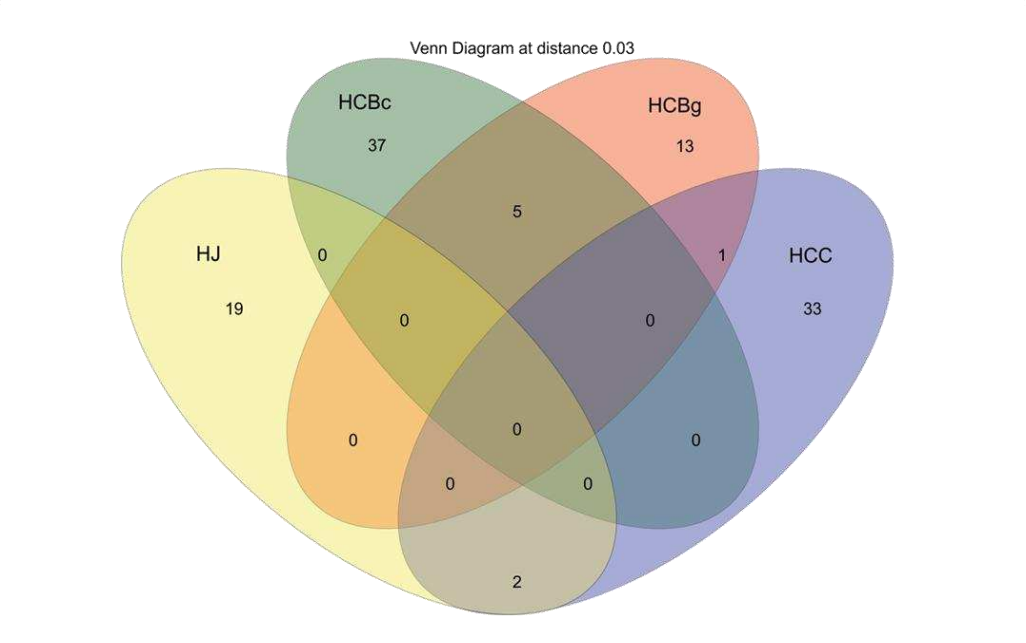

Figure 4 Venn diagram of fungal OTUs clustered with a 3% distance threshold, showing the number of OTUs shared by the four ITS gene libraries. HCBg - *Hoffmannseggella cinnabarina* from the Serra do Brigadeiro State Park (granitic inselberg); HCBc - *Hoffmannseggella cinnabarina* from a Ferriferous Formation (*canga*)*;* HCC - *Hoffmannseggella caulescens* from a Ferriferous Formation (*canga*)*;* HJ - *Hadrolaelia jongheana* from the Serra do Brigadeiro State Park (Semideciduous Seasonal Forest).

Phylogenetic analysis

The phylogenetic trees allowed the identification of the evolutionary relationships of the representative OTUs from the four libraries, separated into the main phyla, Basidiomycota (Figure 5) and Ascomycota (Figure 6). The evolutionary relationships of representative OTUs identified as non-culturable fungi were also elucidated (Supplementary Figure 1). According to the phylogenetic analysis, the OTUs classified as belonging to the Basidiomycota were grouped into two major orders (Figure 5). It was observed that some OTUs were related to known sequences

deposited in the GenBank database (NCBI); however, most other OTUs formed a distinct group unrelated to any known sequence deposited in the database (clade A). The same pattern was observed for the OTUs classified as belonging to the Ascomycota: clade A comprised OTUs that formed a distinct group unrelated to any known sequence (Figure 6). The other clone sequences were divided into several subgroups represented by classes (Figure 6).

Discussion

In this study, we characterized, for the first time, the diversity of the fungal communities associated with three tropical orchid species found in the Atlantic Forest. The clone library construction identified Basidiomycota and Ascomycota taxa associated with the roots of these plants. The high fungal community diversity observed in this work and the knowledge of the composition of this community are essential information for *in vitro* propagation of these species. Furthermore, such information contributes decisively to the success of reintroduction programs for these orchids in other habitats. The purpose of this study was to generate important information to develop conservation strategies for threatened endemic Brazilian flora species in an important and threatened hotspot. Thus, this information will help conserve global biodiversity. Considering the richness of unidentified fungal species present in the roots of these orchids, we see a need to explore this diversity, which may be underestimated, to increase the possibility of discovering new species.

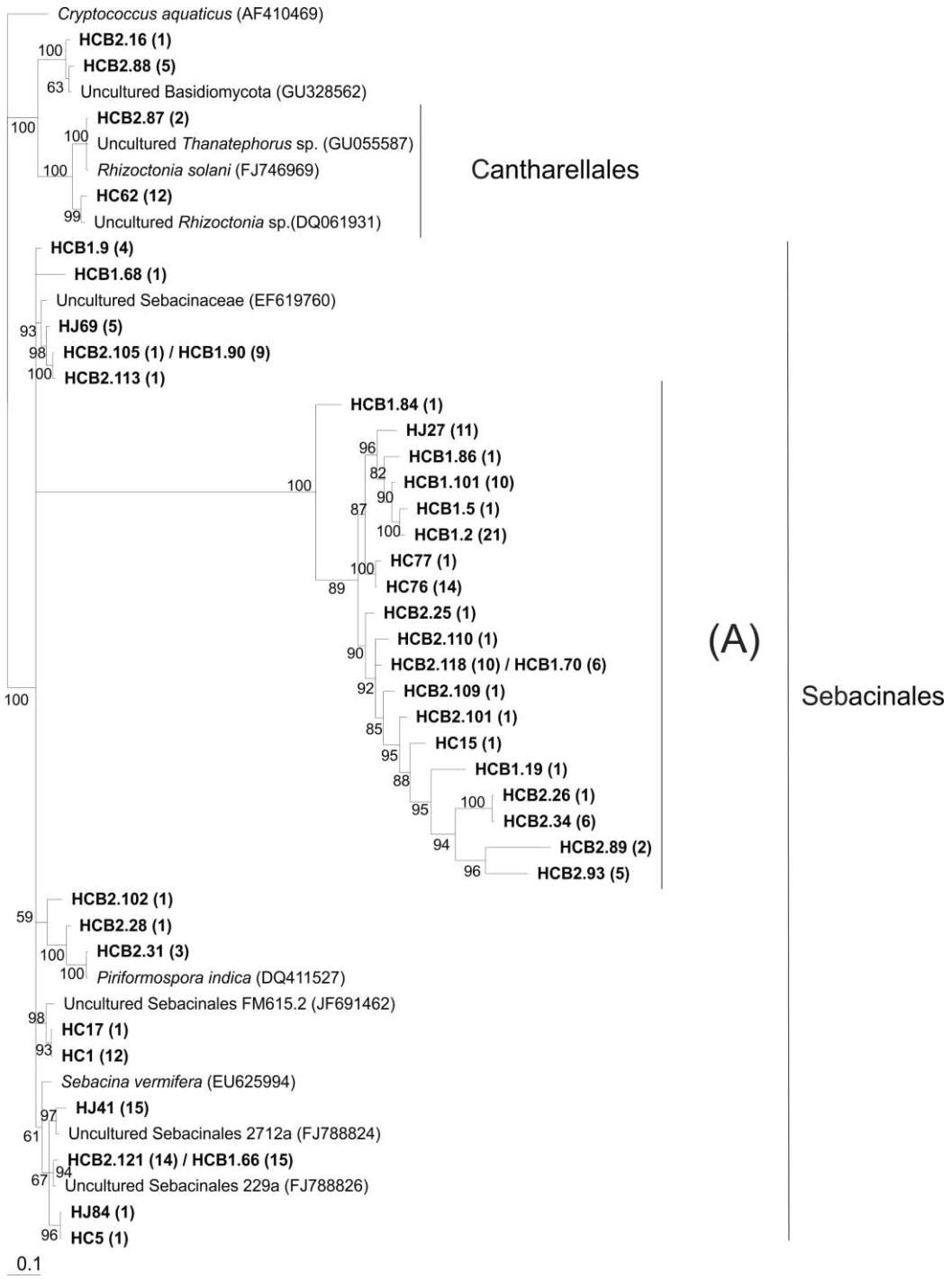

Figure 5 Phylogenetic relationships of Basidiomycota ITS sequences from orchid species roots. The numbers on the branches indicate the *a posteriori* probability obtained by Bayesian likelihood (only values exceeding 50% are shown). The tree was rooted with *Cryptococcus aquaticus*. HCB1 - clones obtained from the HCBg library; HCB2 - clones from HCBc; HC - clones from HCC; HJ - clones from HJ. The numbers in parentheses after the clone names represent the sequence number for this specific clone library. GenBank accession numbers are listed after species names. The letters A refers to the fungi clade, which comprise only clone sequences obtained in this study.

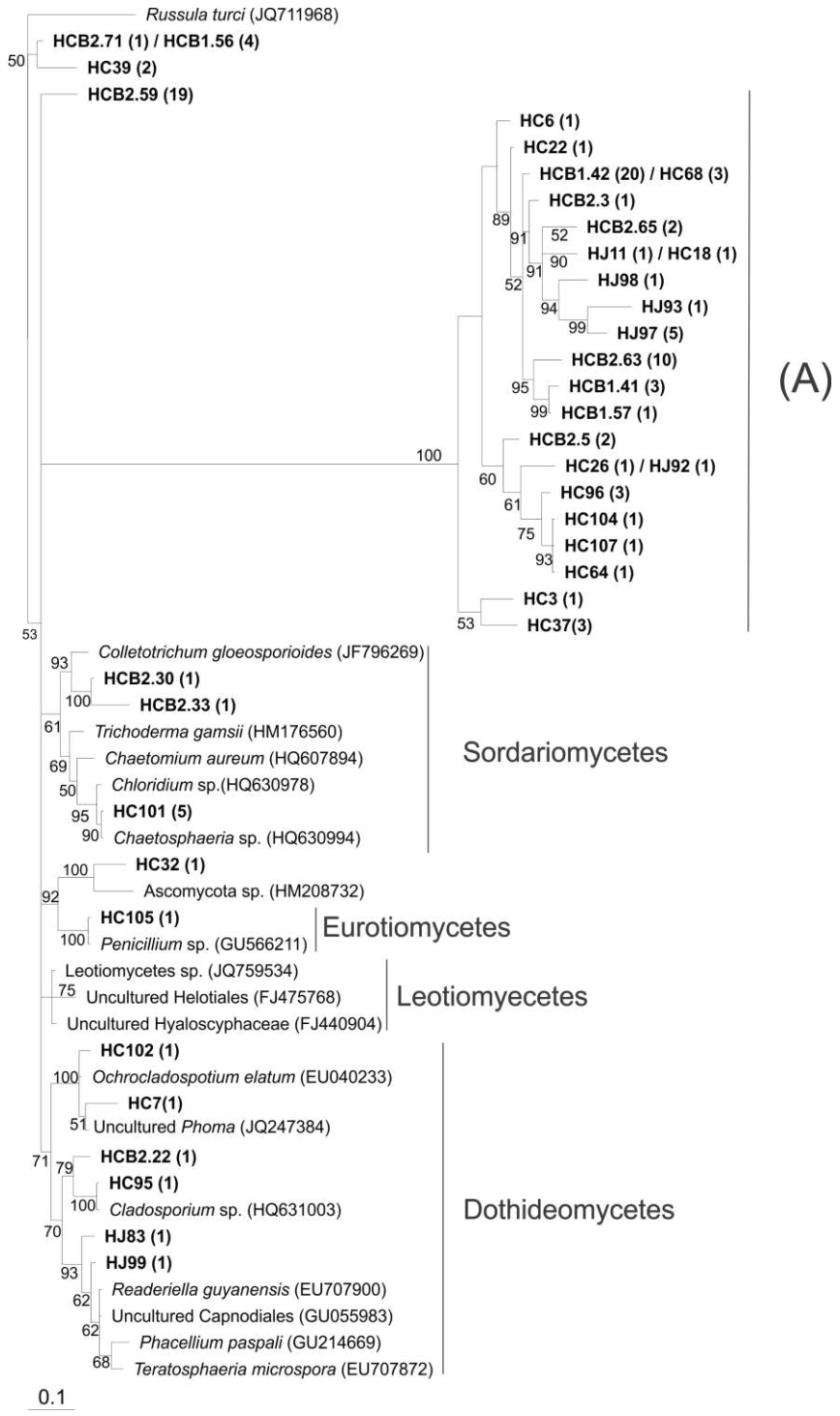

Figure 6 Phylogenetic relationships of Ascomycota ITS sequences from orchid species roots. The numbers on the branches indicated the *a posteriori* probability obtained by Bayesian likelihood (only values exceeding 50% are shown). The tree was rooted with *Russula turci*. HCB1 - clones obtained from the HCBg library; HCB2 - clones from HCBc; HC - clones from HCC; HJ - clones from HJ. The numbers in parentheses after the clone names represent the sequence number for this specific clone library. GenBank accession numbers are listed after species names. The letters A refers to the fungi clade, which comprise only clone sequences obtained in this study.

Since the last century, it has been widely accepted that the main orchid mycorrhizal fungi are saprophytic or pathogenic basidiomycetes belonging to the *Rhizoctonia*-like group, a polyphyletic group that includes the orders Cantharellales, Sebacinales and Tulasnellales (Roberts, 1999). Cantharellales and Sebacinales, but not Tulasnellales, were identified in this study (Figure 2). We observed a wide distribution and dominance of the Sebacinales order in the four libraries. This order includes the Sebacinaceae family that is involved in various mycorrhiza types: ectomycorrhiza, ericoides, jungermannioid, being well known to the potential of some mycorrhizal taxa associated with orchids, such as the *Sebacina* genus fungi (Selosse *et al*., 2002, Taylor *et al*., 2003). Another genus belonging to that order which occurred in one of the libraries was *Piriformospora* sp. (Figure 2). It is known that this fungi genus is associated with the terrestrial orchid roots, such as species of the genus *Dactylorhiza* sp. (Blechert *et al*., 1995), however, we reported for the first time the occurrence of this fungus in the tropical orchid roots.

The Helotiales order was distributed among the four libraries (HCBg, HCBc, HCC and HJ), though in smaller proportions (Figure 2). The Helotiales order, an ecologically diverse group, includes pathogenic fungi, ectomycorrhizal fungi, endophytic plants, ericoid mycorrhizal fungi, and different saprophytic types (Wang *et al*., 2006; Stark *et al*., 2009). However, it is known that photosynthetic orchids are also associated with ectomycorrhizal fungi (Selosse *et al*., 2004). It has even been suggested that the replacement of *Rhizoctonia*-like fungi by common ectomycorrhizae fungi might be a strategy to ensure access to carbohydrates in places where the *Rhizoctonia*-like fungi are not available (Selosse *et al*., 2004; Waterman *et al*., 2011). These associations may be a possible explanation for the

moderate occurrence of this group in the analyzed orchid roots. This group of fungi has also been reported in the roots of the photosynthetic species *Gymnadenia conopsea* (L.) R Brown (Stark *et al*., 2009).

The Capnodiales order was distributed among three libraries (HCBc, HCC and HJ), though in smaller proportions as well the Sordariales and Pleosporales orders (Figure 2). These orders comprise potentially endophytic genus fungi, e.g., the genera *Chaetomium*, *Phoma* and *Cladosporium* have been reported as endophytic to several Chinese medicinal plant species (Huang *et al*., 2008). The orders Hypocreales, Phyllachorales and Eurotiales constitute the genera *Trichoderma* sp., which, along with *Colletotrichum* sp. and *Penicillim* sp., are some of the mycorrhizal fungi previously identified as orchid endophytes (Bayman and Otero, 2006). However, for these orders, it is more difficult to predict possible ecological roles in their association with orchid roots because a variety of relationships exist between fungal endophytes and their host plants; these relationships range from symbiotic or mutualistic to antagonistic or slightly pathogenic (Arnold, 2007). Data regarding the frequency, diversity and ecological role endophytes of orchids remain largely unavailable (Bayman and Otero, 2006). Thus, one must consider the possibility that different microorganisms, including symbiotic and non-symbiotic fungi and bacteria, can be isolated with Orchidaceae (Currah *et al*., 1990). Furthermore, it is possible that fungi hitherto considered merely endophytic may also be involved in mutualistic interactions (Vendramin *et al*., 2010).

The classification of ITS sequences obtained in this study based on similarity to sequences deposited in GenBank (BLASTn) or based on phylogenetic analyses did not result in similar outcomes (Figure 2, 5 and 6). Although the MOTHUR program identified different representative OTUs at the species level (cutoff 97%) in the

libraries, these taxa could not be identified when compared to reference sequences in the database of ITS sequences. Some were identified only at the phylum level, and the high proportion of non-culturable fungi (23.30%) that was observed may represent new species not isolated in pure culture or known fungal species with ITS sequence not deposited in the database. This finding was reinforced in the phylogenetic analysis of clade A (Figure 5 and 6), which clustered only the clones obtained in this study, without any affiliation with the known sequence. According to some authors, only fraction of the 1.5 to 5.1 million estimated fungal species existing in the world has been identified, and a very small fraction of the species that have been identified are represented in GenBank (O'Brien *et al*., 2005; Nagy *et al*., 2011).

Furthermore, an important result was observed when clone sequences distributed in different OTUs (cutoff 97%) by the MOTHUR program were classified, when compared to reference sequences deposited in the database (BLASTn), as belonging to the same species. This result most likely reflects the high number of errors in taxonomic assignments of sequences in the ITS region of rDNA gene deposits in international databases (Bueé *et al*., 2009). According to Bidartondo et al. (2008), more than 20% of fungal DNA sequences may have erroneous lineage designations in GenBank, given the difficulty and low-resolution phylogenetics inherent to fungal taxonomy. Strategies to improve the classification quality based on ITS sequences include expanding the reference database and improving existing annotations of taxonomic sequences (Porter and Golding, 2011).

Despite these limitations, Schoch et al (2012) proposed that the ITS sequence be used as a standard barcode for fungi because the ITS region combines a high resolution between closely related species with high success rates in PCR and sequencing rates. However, it is unlikely that a single barcode label system would be

able to identify every specimen or culture at the species level (Schoch *et al*., 2012). The combined application of ITS and large ribosomal subunit (LSU) rRNA gene sequences, particularly in studies involving environmental samples (Vandenkoornhuyse *et al*., 2002, O'Brien *et al.*, 2005; Klaubauf *et al*., 2010) has provided both species identification from the ITS and phylogenetic analysis from LSU.

The diversity of fungal communities differed among orchid species and between locations (Table 2). The Shannon-Wiener diversity index (H') values found for the HCBg and HJ libraries (Table 2) were comparable to those reported in the literature for the terrestrial orchid species *Gymnadeia conopsea* and higher than those found for the fungi associated with roots of the *Bletilla ochracea* Schltr. species (Stark *et al*., 2009; Tao *et al*., 2008). For HCBc and HCC, the H' value was considerably higher than those previously reported (Table 2). According to Magurran (2004), the Shannon index values obtained from empirical data are usually between 1.3 and 3.5 and rarely exceed 4. The high H' values found for the ferriferous formation species *H. cinnabarina* (HCBc) and *H. caulescens* (HCC) may be related to the area where these plants were collected (Table 2, Figure 1). The plants that constitute the "canga" vegetation are subject to many environmental stresses (Table 1). The soils are generally compact, thin, nutrient-poor, and highly acidic (Table 1) with low water content (Jacobi *et al*., 2007). Furthermore, the ferriferous formation soils have an additional stress factor: high levels of heavy metals (Jacobi *et al*. 2007). Given these adverse conditions, it is expected that plants in this location, in addition to presenting morphological and physiological adaptations, diversify their fungal associations, which favors the survival of these plants in a heavily disturbed area. Despite the oppressive environmental conditions, the vegetation in this area generally

occurs in niches surrounded by the surfaces of rocks, soil aggregates called "soil islands" (Conceição and Pirani, 2007). In these "soil islands" humic substances are prevalent and soil fertility is increased, which allows the support of a microbial community, providing an ecological niche for symbiotic fungi that may associate with the orchid roots (Matias *et al*., 2009).

Mycorrhizal associations can modify the interaction between soil and plants because the structure of filamentous fungi allows the exploration of tiny pores and surfaces of the aggregates not accessible by the root. This association is of particular importance in the transfer of nutrients to plants when nutrients are scarce (Smith and Read, 1997). Moreover, the mycorrhizae also have the ability to reduce the toxic effects of heavy metals, creating a protective barrier against potentially toxic elements. This protective effect has been reported in orchid mycorrhizal fungi. According to Turnaru et al. (2001), hyphal coils of *Epipactis atrorubens* (Hoffm.) Besser root, growing in mining areas in southern Poland, showed a higher enrichment of metals such as zinc and lead compared with root sections of the same species growing in areas distant from the tailings. Thus, it is possible that an association with a diverse fungal community influences the persistence and vulnerability of orchids to disturbed and constantly changing habitats as observed in this study. The “Quadrilátero ferrífero”, rich in heavy metals, presents adverse conditions that can potentially be exploited as an important source of fungal species that are resistant to environmental stress. Thus, these fungi, mycorrhizal or not, can be used in programs that reintroduce orchid species endangered by anthropogenic impacts.

The lowest H' value, found for *H. cinnabarina* (HCBg) (Table 2) collected in the granitic inselberg, can be explained by the peculiarities of the site (Figure 1). The

granitic inselberg shares several stressful features, such as high UV exposure, nutrient-poor soils (Table 1) with low water retention, daily temperature variations, low humidity and high evapotranspiration, with the "canga" ferruginous area (Scarano, 2002; Porembski and Barthlott, 2000). Furthermore, this soil is characterized by high levels of aluminum (Table 1), an element that can potentially be toxic to certain microorganisms. However, a major factor limiting the support of a diverse microbial community in this site is the shallow soil because soil occurrence in inselbergs is locally restricted (Bremer and Sander, 2000). Moreover, orchids constitute the vascular epilithic plants (rupicolous) in this habitat; growing directly on the rock causes difficulties for fixing seeds, seedlings and roots (Porembski, 2007). Another factor that can lower fungal community diversity at this site is the altitudinal gradient; these plants were collected at altitudes ranging from 1,600-1,700 m (Figure 1). Ruotsalainen et al. (2002) suggest that the benefits of mycorrhizal symbiosis may change as altitude increases, and the change of these benefits are be directly related to environmental constraints, particularly nutrient levels in the soil.

The *H. jongheana* (HJ) library also showed a lower value for H' in relation to the "canga" species (Table 2), which can be explained by the orchid's epiphytic habit. The epiphytic species are less dependent on mycobiont taxa when compared with terrestrial species and can be found in association with fungi only at the seed germination stage (Roberts and Dixon, 2008; Swarts and Dixon, 2009). Until recently, little has been known about the determinants of diversity and composition of fungal communities associated with orchids (Stark *et al*., 2009), mainly related to epiphytic orchids.

Based on the Simpson diversity index (1/D) values, we observed that all four libraries presented dominant species in the community (Table 2). According to Zhou

et al. (2002), values of less than 50 for the reciprocal Simpson index (1/D) may indicate dominance in the community profile.

Statistical analysis using LIBSHUFF showed that the fungal communities associated with the studied orchid species were different. Although the HCBg and HCBc libraries were from the same orchid species, they shared few fungal species (Figure 4); their fungal communities were structurally different. These differences in fungal composition may be explained by the difference in collection area (Figure 1). In addition to these sites having different soil types (Table 1), the floristic composition of these areas can potentially contribute to the differentiation of fungal composition diversity. Due to the severe soil conditions and microclimate, the granitic inselberg vegetation is clearly distinct from the flora of its surroundings and presents a high degree of endemism and greater geographic isolation relative to the ferruginous region (Porembski and Barthlott, 2000). These factors may contribute, as well as for lower diversity indices, for a fungal community is also specific and endemic. Thus, this regional difference in species composition suggests that local-scale factors may strongly affect the fungal communities' compositions and, therefore, diversity at the regional level. Furthermore, this wide taxonomic range found for the fungi associated with *H. cinnabarina* roots (HCBg and HCBc) may contribute to its ability to grow in different habitat types with characteristic fungal communities, including disturbed habitats (such as mines) and granitic outcrops.

In conclusion, we observed that the fungal communities of the three orchid species analyzed showed high diversity, indicating that these plants may have low specificity for certain fungal clades. The Basidiomycete fungi identified in this study are potential candidates for orchid mycorrhizal fungi. While the identified Ascomycetes include known potentially endophytic fungi. The high diversity of

fungal communities associated with these plants also offers greater flexibility in the plants' adaptation to changing environmental conditions and offers a great advantage to the reintroduction programs for those species. In addition, differences in environmental conditions, due to the different habitats where these orchids were collected, justified the distinct fungal communities associated with the roots. Understanding the diversity of fungal symbionts is crucial to the success of *in situ* conservation programs and the conservation of these plants through assisted migration. These approaches are considered promising for the conservation of global biodiversity, especially if we consider the constant anthropogenic threat suffered by Atlantic Forest over time.

Acknowledgments – We thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do estado de Minas Gerais (FAPEMIG), and the American Orchid Society (AOS) for their financial support. Special thanks to José Maria Rodrigues da Luz for their helpfulness and attention.

References

Antonelli A, Verola CF, Parisod C, Gustafsson ALS. (2010). Climate cooling promoted the expansion and radiation of a threatened group of South American orchids (Epidendroideae: Laeliinae). Biol J Linn Soc 100:597–607.

Arnold AE. (2007). Understanding the diversity of foliar endophytic fungi: Progress, challenges,and frontiers. Fungal Biol Rev 21:51–66.

Barros F, Vinhos F, Rodrigues VT, Barberena FFVA, Fraga CN, Pessoa EM. (2012). *Orchidaceae* in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. *http://floradobrasil.jbrj.gov.br/2012/FB066871*. Accessed 26 May 2012.

Bayman P, Otero JT. (2006). Microbial endophytes of orchid roots. In: Schulz, B (ed). Microbial root endophytes. Springer: New York, pp 153–173.

Benites VM, Caiafa AN, Mendonça ES, Schaefer CE, Ker JC. (2003). Solos e Vegetação nos Complexos Rupestres de Altitude da Mantiqueira e do Espinhaço. Floresta e Ambiente 10:76-85.

Bidartondo M, Bruns T, Blackwell M, Edwards I, Taylor A, Horton T *et al*. (2008). Preserving accuracy in GenBank. Science 319: 1616.

Blechert O, Kost G, Hassel A, Rexer KH, Varma A. (1995). Mycorrhiza Structure, Function, Molecular Biology and Biotechnology. Springer-Verlag: Berlin.

Bremer H, Sander H. (2000). Inselbergs: Geomorphology and geoecology. In: Porembski, S (ed). Inselbergs – biotic diversity of isolated rock outcrops in tropical and temperate regions. Springer-Verlag: Berlin, pp 7-35.

Bueé M, Reich M, Murat C, Morini E, Nilsson RH, Uroz S *et al*. (2009). 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytol 184:449-456.

Conceiçao AA, Pirani JR. (2007). Diversidade em quatro áreas de campos rupestres na Chapada Diamantina, Bahia, Brasil: espécies distintas, mas riquezas similares. Rodriguésia 58: 193–206.

Costa CM, Hermann G, Martins CS. (1998). Biodiversidade em Minas Gerais – um atlas para sua conservação. Fundação Biodiversitas, Governo de Minas Gerais, IEF e Conservação Internacional, Belo Horizonte, pp. 94.

Currah RS, Smreciu EA, Hambleton S. (1990). Mycorrhizae and mycorrhizal fungi of boreal species of *Platanthera* and *Coeloglossum* (Orchidaceae). Can J Bot 68: 1171–1181.

Decker JS. (1941). *Laelia jongheana* var. *Alba*. Orquídea 4:28-29.

Drummond GM, Machado ABM, Martins CS, Mendonça MP, Stehmann JR. (2008). Listas Vermelhas das espécies ameaçadas de extinção em Minas Gerais. Fundação Biodiversitas. 2ª edição. ISBN: 978-85-85401-23-8.

Engevix. (1995). Caracterização do meio físico da área autorizada para criação do Parque Estadual da Serra do Brigadeiro – Relatório técnico final dos estudos – 8296- RE-H4-003/94 "VER. 1". Instituto Estadual da Floresta, BIRD/PRÓ-FLORESTA/SEPLAN, pp. 34.

Felsenstein J. (2005). PHYLIP (Phylogeny Inference Package) Version 3.6, Department of Genome Science, University of Washington, Seattle, Wash, USA.

Ferri MG. (1980). Vegetação Brasileira. Editora da Universidade de São Paulo: São Paulo, pp.57.

Gardes M, Bruns TD. (1993). ITS primers with enhanced specificity for basidiomycets – application to the identification of mycorrhizae and rust. Mol Ecol 2:113–118.

Giulietti AM, Rapini A, Andrade MJG, Queiroz LP, Silva JMC. (2009). Plantas Raras do Brasil, Belo Horizonte, MG: Conservação Internacional, pp. 496.

Huang WY, Cai YZ, Hyde KD, Corke H, Sun M. (2008). Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers 33:61–75.

Huelsenbeck JP, Ronquist F. (2001). Mrbayes: Bayesian inference of phylogenetic trees. Bioinformatics,17: 754-755.

Huynh TT, Thomson R, Mclean CB, Lawrie AC. (2009). Functional and genetic diversity of mycorrhizal fungi from single plants of *Caladenia formosa* (Orchidaceae). Ann Bot-London 104: 757–765.

Invitrogen (1999). Instruction manual of original TA cloning kit.Invitrogen, Carlsbad.

Jacobi CM, Carmo FF, Vincent RC. (2008). Estudo fitossociológico de uma comunidade vegetal sobre canga como subsídio para reabilitação de áreas mineradoras no Quadrilátero Ferrífero, MG. Rev Árv 32:345-353.

Jacobi CM, Carmo FF, Vincent RC, Stehmann JR. (2007). Plant communities on ironstone outcrops: a diverse and endangered Brazilian ecosystem. Biodivers Conserv 16:2185–2200.

Jurkiewicz A, Turnau K, Mesjasz-Przybyłowicz J, Przybyłowicz W, Godzik B. (2001). Heavy metal localisation in mycorrhizas of *Epipactis atrorubens* (Hoffm.) Besser (Orchidaceae) from zinc mine tailings. Protoplasma 218:117-124.

Klaubauf S, Inselsbacher E, Zechmeister-Boltenstern S, Wanek W , Gottsberger R, Strauss J *et al*. (2010). Molecular diversity of fungal communities in agricultural soils from Lower Austria. Fungal Diversity 44: 65-75.

Koeppen W. (1948). Climatologia. Fondo Cultura Económica. México & Buenos Aires, pp. 478.

Magurran AE. (2004). Measuring Biological Diversity. Blackwell Publishing: Oxford.

Matias SR, Pagano MC, Muzzi FC, Oliveira CA, Carneiro AA, Horta S *et al*. (2009). Effect of rhizobia, mycorrhizal fungi and phosphate-solubilizing microorganisms in the rhizosphere of native plants used to recover an iron ore area in Brazil. Eur J Soil Biol 45:259–266.

Mittermeier RA, Gil PR, Hoffmann M, Pilgrim J, Brooks T, Mittermeier CG *et al*. (2004). Hotspots revisited Earth's biologically richest and most endangered terrestrial ecoregions. Cemex: Mexico.

Nagy LG, Petkovits T, Kovacs GM, Voigt K, Vagvolgyi C, Papp T. (2011). Where is the unseen fungal diversity hidden? A study of Mortierella reveals a large contribution of reference collections to the identification of fungal environmental sequences. New Phytol 191:789-794.

Nylander JAA. (2004.) MrModeltestv2 Program distributed by the auhor. Evolutionary Biology Centre, Uppsala University, Sweden.

O'Brien HE, Parrent JL, Jackson JA, Moncalvo J-M, Vilgalys R. (2005). Fungal community analysis by large-scale sequencing of environmental samples. Appl Environ Microbiol 71:5544–5550.

Oliveira – Filho AT, Ratter JA. (1995). A study of the origin of Central Brazilian Forests by the analysis of plant species distribution patterns. J Bot 52: 141-194.

Paula CC. (1998). Florística da família Bromeliaceae no Parque Estadual da Serra do Brigadeiro, MG – Brasil. Tese, Universidade Estadual Paulista.

Porembski S. (2007). Tropical inselbergs: habitat types, adaptive strategies and diversity patterns. Rev Bras Bot 30:579-586.

Porembski S, Barthlott W. (2000). Granitic and gneissic outcrop (inselbergs) as centers of diversity for desiccation-tolerant vascular plants. Plant Ecol 151:19–28.

Porter, TM, Golding GB. (2011) Are similarity- or phylogeny-based methods more appropriate for classifying internal transcribed spacer (ITS) metagenomic amplicons? New Phytol 192: 775–782.

Pridgeon A. (1995). The illustrated encyclopaedia of orchids. Timber Press: Portland.

Ribeiro, AC, Guimarães, PTG, Alvarez, VH. (1999). Recomendações para o uso de corretivos e fertilizantes em Minas Gerais 5ª aproximação. Comissão de Fertilidade do Solo do Estado de Minas Gerais - CFSEMG – Viçosa.

Roberts DL, Dixon KW. (2008). Orchids. Current Biology 18:325-329.

Roberts P. (1999). Rhizoctonia-forming Fungi: a taxonomic guide. Royal Botanic Gardens, Kew Publishing.

Ruotsalainen AL, Tuomi J, Väre H. (2002). A model for optimal mycorrhizal colonization along altitudinal gradients. Silva Fenn 36: 681–694.

Saitou N, Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.

Scarano FR. (2002) Structure, function and x oristic relationships of plant communities in stressful habitats marginal to the Brazilian Atlantic Rainforest. Ann Bot 90:517–524.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB *et al*. (2009). Introducing MOTHUR: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, et al. (2012). Fungal Barcoding Consortium. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. PNAS 109:6241 – 6246.

Selosse MA, Faccio A, Scappaticci G, Bonfante P. (2004). Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microbial Ecol 47: 416–426.

Selosse MA, Weiß M, Jany JL, Tillier A. (2002). Communities and populations of sebacinoid basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. Rich. and neighbouring tree ectomycorrhizae. Mol Ecol 11:1831–1844.

Smith SE, Read DJ. (1997). Mycorrhizal Symbiosis. Academic Press: New York.

Spier CA, Barros SM, Rosiere SA. (2003). Geology and geochemistry of the Águas Claras and Pico Iron Mines, Quadrilátero Ferrífero, Minas Gerais, Brazil. Mineralium Deposita 38:751-774.

Stark C, Babik W, Durka W. (2009). Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea*. Mycol Res 952-959.

Stehmann JR, Forzza RC, Salino A, Sobral M, Costa DP, Kamino LHY. (2009). Plantas da Floresta Atlântica, Rio de Janeiro: Jardim Botânico do Rio de Janeiro, pp. 516.

Stewart SL, Zettler LW. (2002). Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens*, *H. quinquiseta*, *H. macroceratilis*) from Florida. Aquat Bot 72:25-35.

Strahler AN. (1963). The Koppen climate classification system. In: (Ed.). Physical geography. 2nd ed. New York, John Willey, pp.185-188.

Swarts ND, Dixon KW. (2009). Terrestrial orchid conservation in the age of extinction. Ann Bot 104: 543–556.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011). "MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods". Mol Biol Evol 28: 2731–2739.

Tao G, Liu ZY, Hyde KD, Lui XZ, Yu ZN. (2008). Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochracea* (Orchidaceae). Fungal Diversity 33: 101-122.

Taylor DL, Bruns TD, Szaro TM, Hodges SA. (2003). Divergence in mycorrhizal specialization within Hexalectris spicata (Orchidaceae), a nonphotosynthetic desert orchid. Am J Bot 90: 1168–1179.

Vandenkoornhuyse P, Baldauf SL, Leyval C, Straczek J, Young JPW. (2002). Extensive fungal diversity in plant roots. Science 295: 2051.

Veloso HP, Rangel Filho ALR, Lima JCA. (1991). Classificação da vegetação brasileira, adaptada a um sistema universal. IBGE, Departamento de Recursos Naturais e Estudos Ambientais, Rio de Janeiro, pp. 123.

Vendramin E, Gastaldo A, Tondello A, Baldan B, Villani MC, Squartini A. (2010). Identification of Two Fungal Endophytes Associated with the Endangered Orchid *Orchis militaris* L. J Microbiol Biotechn 20:630–636.

Verola CF, Semir J, Antonelli A, Koch I. (2007). Biosystematic studies in the brazilian endemic genus *Hoffmannseggella* H. G. Jones (Orchidaceae: Laeliinae): a multiple approach applied to conservation. Lankesteriana 7: 419–422.

Viana PL, Lombardi JA. (2007). Florística e caracterizaçãoo dos campos rupestres sobre canga na serra da calcada, Minas Gerais, Brasil. Rodriguesia 58:159–177.

Wang Z, Binder M, Schoch CL, Johnston PR, Spatafora JW, Hibbett DS. (2006). Evolution of helotialean fungi (Leotiomycetes, Pezizomycotina): a nuclear rDNA phylogeny. Mol Phylogenet Ev 41: 295–312.

Waterman RJ, Bidartondo MI, Stofberg J, Combs JK, Gebauer G, Savolainen V *et al*. (2011). The Effects of Above- and Belowground Mutualisms on Orchid Speciation and Coexistence. Ame Naturalist177: 2.

White TJ, Bruns T, Lee S, Taylor JW. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand DH, Sninsky JJ, White T J (eds) PCR Protocols ± A guide to methods and applications. Academic Press, San Diego, pp. 315-322.

Zhou J, Xia B, Treves DS, Wu LY, Marsh TL, O'Neill RV, Palumbo AV *et al*. (2002). Spatial and resource factors influencing high microbial diversity in soil. Appl Environ Microbiol 61: 326 – 334.

3. TULASNELLOID AND SEBACINOID FUNGI ASSOCIATED WITH TROPICAL NATIVE ORCHIDS FROM THE BRAZILIAN ATLANTIC FOREST

Sabrina Feliciano Oliveira, Melissa Faust Bocayuva, Tomás Gomes Reis Veloso, Denise Mara Soares Bazzolli, Olinto Liparini Pereira, Maria Catarina Megumi Kasuya

Abstract

Three orchid species, *Hadrolaelia jongheana*, *Hoffmannseggella cinnabarina* and *Hoffmannseggella caulescens*, are among the species included in the List of Threatened Brazilian Flora Species. They are found in the Atlantic Forest, an important global hotspot constantly threatened by anthropogenic impacts. This study aimed to characterize and identify, via molecular methods, possible tulasnelloid and sebacinoid fungal symbionts associated with these orchid species in different Atlantic Forest vegetation formations. Our results show that tropical orchids can be considered generalists in their associations with sebacinoid and tulasnelloid mycorrhizal fungi, and the diversity of this association is influenced by factors inherent to the sites that these plants inhabit. The fungal symbionts identified showed wide distribution, as they were present in disturbed and undisturbed areas and established associations with both rupicolous and epiphytic orchids. However, it is likely that other *Rhizoctonia*-like fungi groups may be associated with the orchid roots because all of the roots analyzed containing fungal pelotons in root cortical cells, but there was no amplification with the specific primers used. Our results indicate that for efficient rehabilitation of these endangered species, it is necessary to use different tulasnelloid and sebacinoid species as symbionts of orchids. It is necessary for mycobionts to be carefully selected for *H. cinnabarina* reintroduction, particularly when this species inhabits areas characterized by endemic vegetation and geographical isolation, such as granitic rock outcrops.

Keywords: orchid mycorrhizal fungi, *Rhizoctonia*-like, hotspot, threatened orchid, molecular tools, phylogeny.

Introduction

Orchid seeds are almost completely devoid of nutritional reservations, which results in a dependency, at least in the first stage of development, on an external carbon source (Arditti 1992; Whigham et al. 2006). Thus, the species belonging to the family Orchidaceae are associated with mycorrhizal fungi to promote seed germination, protocorm formation and thus establishment in the natural environment (Andersen and Rasmussen 1996). The nutritional dependence on the mycorrhizal fungus is highly variable among the orchids. The epiphytic species have lower symbiont dependence compared with terrestrial species and can be in association with fungi only at the stage of seed germination, and adults may be independent of mycorrhizas for nutrition (Roberts and Dixon 2008; Swarts and Dixon 2009).

In Brazil, approximately 50% of the species richness of Orchidaceae is concentrated in the Atlantic Forest (Giulietti et al. 2009) that originally occupied an area of 1,314,460 km^2, approximately 15% of the territory of Brazil, but is now reduced to approximately 7.9% of the initial coverage (Lino and Simões 2011). Because of the greater richness and endemism associated with the destruction suffered in the past, the Atlantic Forest are considered to be one of the world's 34 biodiversity hotspots and the fifth richest environments in endemic species (Mittermeier et al. 2004; Stehmann et al. 2009).

The Orchidaceae family is regarded as one of the most diverse in this biome, especially in terms of absolute richness, with 1,257 species, and endemism, with 791 species unique to this biome (Stehmann et al. 2009). The *Hadrolaelia jongheana (*Rchb.f.) Chiron species, which is among the most rare Brazilian orchids, stands out

among the great diversity of orchid species (Decker 1941). The intense collection and export of these orchids and the destruction of their forest habitats have reduced their prevalence within the local flora (Bocayuva, pers. comm.). Another orchid genus, *Hoffmannseggella,* is endemic to Southeastern Brazil and comprises species, such as *Hoffmannseggella cinnabarina* (Bateman ex Lindl.) HG Jones and *Hoffmannseggella caulescens* (Lindl.) HG Jones, that are strictly rupicolous (growing on rocks). Because 47% of all *Hoffmannseggella* species are known to originate from a single population and most of them are found near the expanding industrial complex area and outside of the legally protected area (Verola et al. 2007), this genus is one of the most threatened plant taxa in Brazil (Antonelli et al. 2010).

Rhizoctonia-like fungi have been identified as the main group that forms mycorrhizas in photosynthetic orchids. This group of fungi belongs to the families Tulasnellaceae, Ceratobasidiaceae and Sebacinaceae (Muller et al. 1998). The family Tulasnellaceae includes the *Tulasnella* genus whose members are commonly described as saprophytes; however, many species are capable of forming ectomycorrhizae with birch and pine (Bidartondo et al. 2003). Furthermore, the fungi of this genus have been reported to be mycorrhizal in both epiphytes and terrestrial orchids in the tropics (Currah et al. 1997; Ma et al. 2003; Pereira et al. 2005). Therefore, the ecological range of these fungi must be wide because they occur in the soil and on tree bark (Porras-Alfaro and Bayman 2007). Fungi of family Sebacinaceae, in turn, have been isolated from a broad range of orchid species, including *Caladenia* spp., *Elythranthera* spp., *Eriochilus* spp., *Glossodia major*, *Microtis unifolia* and *Platanthera orbiculata* (Warcup and Talbot 1967; Currah et al. 1990), although some species are also considered ectomycorrhizal (Weiss et al. 2004). The identification of fungi associated with orchid roots is mainly performed

by isolation techniques on culture media (Rasmussen 2002). However, these techniques are limiting due to the inability of many mycorrhizal fungi to be cultivated in the culture medium or by favoring the growth of contaminants (Stark et al. 2009).

For the success of orchid conservation programs and programs to introduce these plants into their natural environments, the mycorrhizal associations within the Orchidaceae family as well as the diversity of the associated fungal symbionts must be considered (Bougoure et al. 2009, Swarts and Dixon 2009). This study aimed to characterize and identify, via molecular methods, possible tulasnelloid and sebacinoid fungal symbionts associated with orchid species in different vegetation formations of Atlantic Forest.

Materials and methods

Study site and plant material

All orchid species analyzed in this study are included in the Brazilian Flora Species Threatened List according to the Biodiversity Foundation (2008). The orchid taxonomic names follow the nomenclature proposed by Barros et al. (2012). The samples were collected in two sites inserted in the Atlantic Forest.

One of the sites where the orchids were collected is located in the Serra do Brigadeiro State Park (SBSP) (Fig. 1). According to Köeppen's classification (1948), the climate is mild mesothermic (CWB). The average annual precipitation is 1,300 mm, and the average annual temperature is 18 °C (Engevix 1995). The SBSP vegetation comprises secondary fragments of the Seasonal Semideciduous Forest (Veloso et al. 1991, Oliveira-Filho and Fontes 2000), the Upper Montane Formation (Oliveira-Filho and Ratter 1995) and "Campos de Altitude" (Ferri 1980) that

encompass variable extensions of rock outcrops (Paula 1998), mainly granite and gneiss rocks (Benites et al. 2003). In the SBSP, *H. jongheana* root samples were collected from tree trunks in Seasonal Semideciduous Forest, and *H. cinnabarina* root samples were collected on the granitic rock outcrops.

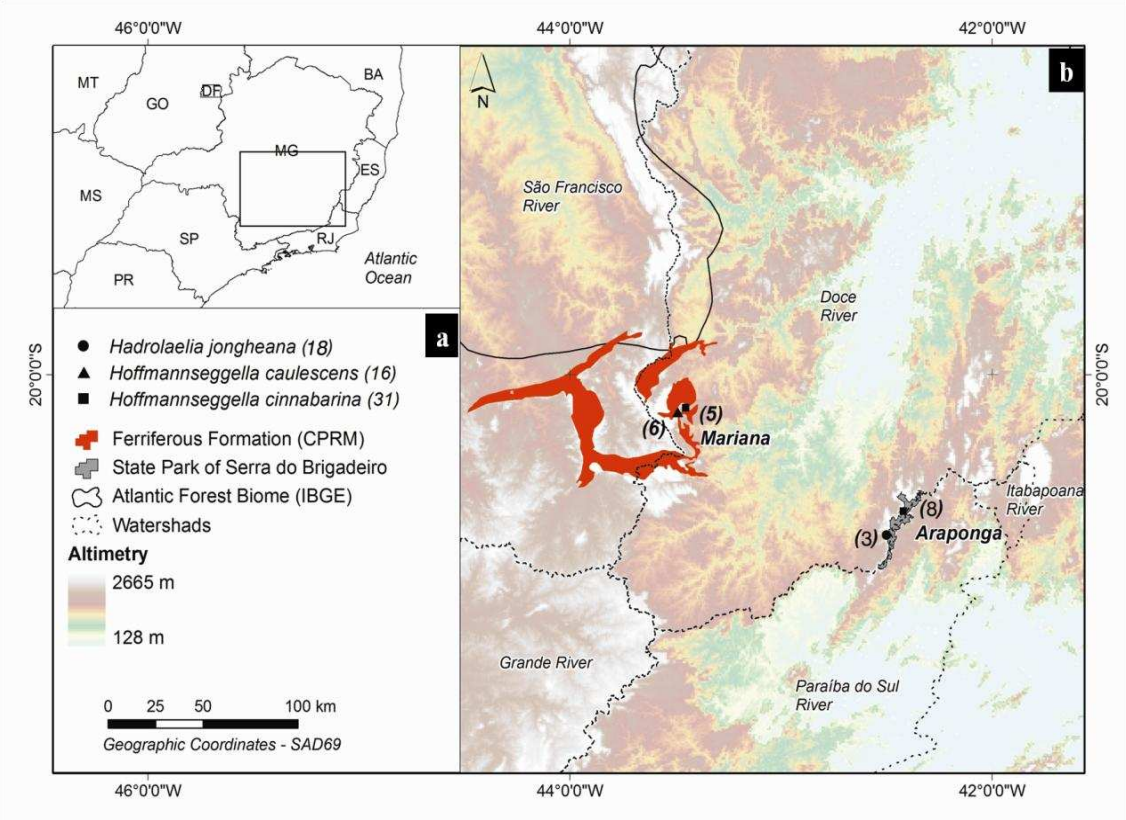

Fig. 1 Map of the study site and the four sampled locations. (a) The number in parentheses represents the number of individuals collected. (b) The number in parentheses represents the number of individuals of each orchid species used in subsequent analyses

The other study area encompass "campo rupestre" (rupestrian field) in the region of "Quadrilátero ferrífero", which is located in Central-Southern Minas Gerais State, occupying an area of 7,000 km^2, of which 14.2% are iron formations (Fig. 1). According to Köeppen's classification (1948), the climate is CWB (Strahler 1963). This climate is characterized by milder summers and lower average annual temperature (17.4 to 19.8 °C). The months of December, January and February record the highest precipitation; the average annual precipitation is 1,800 mm. The

“Quadrilátero Ferrífero” is composed of complex gneiss metamorphic rocks that form exposed rock blocks of Ferruginous rock or ‘‘canga’’ (Viana and Lombardi 2007). *Hoffmannseggella caulescens* and *H. cinnabarina* root system samples were also collected in “campo rupestre” on “canga” in a mining area belonging to the Brazilian multinational Vale SA (Fig. 1). Having been totally destroyed by mining activity, this region no longer exists.

The roots were carried to the laboratory under refrigeration and surface sterilized by the method described by Stewart & Zettler (2002). The root fragments that presented mycorrhizal association, which was previously detected by visualization of pelotons under a stereomicroscope, were stored under refrigeration. Next, these roots fragments were lyophilized for further molecular analysis. Samples were collected over a period of 12 months, which included January 2011 to January 2012.

DNA extraction, PCR amplification and sequencing

Total DNA extraction of the orchid root systems (58 individuals) was performed using Spin Kit ® Plant Mini Invisorb (Invetek) according to the manufacturer's instructions. The DNA integrity was evaluated on agarose gel 0.8% (w / v) and the measurement carried out by spectrophotometry. All samples were subjected to PCR amplification. The reactions were prepared according to the manufacturer's recommendations (Go TaqTM, Promega, Madison, USA), and 25 ng of DNA was used in each reaction. To evaluate the possible presence of Sebacinales mycorrhizal fungi, the DNA from roots was subjected to amplification targeting the 28S rDNA region LSU (Large Subunit of the Ribosome) using primers specific to this group, ITS3Seb (Setaro et al. 2006) and NL4 (White et al. 1990). The cycling scheme was 94ºC for 4 min, followed by 35 cycles at 94 ºC for 30 s, 53 ºC for 30 s, and 72 ºC for 30 s and a final

extension step at 72 ºC for 10 min. In the same way, to amplify the ITS1, 5.8S, and ITS2 regions and a portion of 28S rDNA (LSU) from possible tulasnelloid mycorrhizal fungi, we used the ITS1 (White et al. 1990) and ITS4Tul (Taylor 1997) primers. The cycling scheme was 96 ºC for 2 min, followed by 35 cycles at 94 ºC for 30 s, 54 ºC for 40 s, and 72 ºC for 1 min and a final extension step at 72 ºC for 10 min. In every PCR, a negative control including PCR mix without DNA template was included. As a positive control, we used DNA of fungi already identified as belonging to the genus *Tulasnella* and sebacinales order. Success of the PCR amplifications was tested on 1.5% agarose gels stained in a solution of 0.5 µg mL^{-1} ethidium bromide.

All positive PCR products from sebacinoid and tulasnelloid fungi were sequenced directly without cloning. Both strands were also sequenced using BigDye TM and 3730xl Automatic Sequencer (Applied Biosystems, Weiterstadt, Germany). Sequence editing was performed using Sequencher version 4.1.4 (Genes Codes Corporation, Ann Arbor, MI).

Data analysis

A BLASTn search was conducted, and all DNA sequences were compared with those available in GenBank (NCBI). Sequences from sebacinoid and tulasnelloid fungi were then manually aligned using the Mega 5.0 program (Tamura et al. 2011). Due to heterogeneity of ITS1 and ITS2 rDNA, we excluded considerable portions of this region. Bayesian likelihood and maximum likelihood were used to estimate the phylogenetic relationships. For the Bayesian approach, we used MrBayers 3.0 (Huelsenbeck and Ronquist 2001). MrModeltest (Nylander, 2004) was used to estimate DNA substitution models using the Akaike Information Criterion (AIC). In this analysis, five independent runs with four Markovian Monte Carlo (MCMC) chains were tuned with 10 million generations, and trees were

sampled and fixed at the end of the process. The first samples of trees, which, in this case, were 1 million trees, were discarded in the burning phase, and the trees that remain were summarized to generate a consensus tree of the majority. For the maximum likelihood method, heuristic search tree bisection and reconnection (TBR) was used. The program Modeltest 3.7 (Huelsenbeck and Crandall 1997; Posada and Crandall 1998) was used to establish a model of DNA evolution that best fits the data for the analysis of ML, and the tree is initiated with the ML Neighbor joining model (NJ).

Pairwise alignments were generated among all ITS sequences using the Needleman-Wunsch global alignment algorithm (Needleman and Wunsch 1970) as implemented in the program Needle in the EMBOSS software suite (Rice et al. 2000). This program calculates pairwise similarity values by dividing the number of matching nucleotides by the total length of the alignment. Operational taxonomic units (OTUs) were defined as a group of sequences in clades that generally have high support values in the phylogenetic analysis, and these sequences share at least 97% pairwise similarity.

Results

Plant material, DNA extraction and PCR amplification

This study investigated, for the first time, the presence of tulasnelloid and sebacinoid fungi associated with these orchid species through the application of molecular techniques directly on root samples.

We sampled and extracted DNA from 65 root systems from different orchid species individuals. The sample number for each orchid species was low because these orchids are endangered and it is difficult to access the mining area from which

the samples were obtained. In addition, there were problems with the DNA extraction because the roots oxidized quickly after collection. Thus, only 33 root system samples, from which DNA of satisfactory quality was obtained, were subjected to PCR with primers specific for the *Tulasnella* genus and Sebacinales order. Among these roots, only 10 produced positive PCR products for the Sebacinales order, which represents 30.3% of all samples, and only 12 produced positive PCR products for *Tulasnella* genus, which represents 36.3% of all samples (Table 1).

Table 1 Mycorrhizal fungi from tropical orchids sampled in the Atlantic Forest. All plants analyzed containing fungal pelotons in root cortical cells

Host species	Habitat	Ecological categories	Number sampled plants[a]	ITS3Seb/ NL4[b]	ITS1/ ITS4-Tul[c]
H. jongheana	Seasonal Semideciduos Forest	Epiphyte	18(6)	2	1
H. cinnabarina	*Campo rupestre* (canga)	Rupicolous	12(9)	2	3
	Campos de Altitude (granite outcrop)	Rupicolous	19(12)	3	5
H. caulescens	*Campo rupestre* (canga)	Rupicolous	16 (6)	3	3

[a] The number in parenthesis is the number of plants which produced adequate quality DNA for the subsequent analysis. [b,c] Number of positive PCR products

Sequencing and molecular identification

Twenty-two sequences corresponding to the ITS and 28S regions of mycorrhizal fungi were obtained. However, only eighteen sequences presented good quality and were subjected to molecular identification. The ITS and 28S region sequences obtained and analyzed in the present study and sequences of closely related taxa obtained from GenBank are shown in the Table 2.

Table 2 Sebacinoids and tulasnelloids fungi sequences obtained from orchids roots

Roots Code [a]	ITS3Seb/NL4 (Close relative from GenBank)	ITS1/ITS4-Tul (Close relative from GenBank)
A. *Hadrolaelia jongheana*		
Hj1; Hj3; Hj4	Hj3 - Uncultured Sebacinales clone 3EG1-3; Identity: 98%; GenBank code: HM451810; Country of origin: Ecuador; Source: Suarez et al., Unpublished. Hj4 - Uncultured Sebacinales clone FM731.2; Identity: 99%; GenBank code: FJ514086; Country of origin: França; Source: Martos,F. & Selosse,M.-A. Unpublished.	Hj1 - *Tulasnella tomaculum* specimen voucher KC 429; Identity: 85%; GenBank code: AY373296; Country of origin: USA; Source: McCormick et al., 2004.
B. *Hoffmannseggella cinnabarina* (Granitic rock outcrops)		
Cb1;Cb3; Cb4; Cb5; Cb6;	Cb4 - Uncultured Sebacinales clone 4EE2_6; Identity: 96%; GenBank code: HM451821; Country of origin: Ecuador; Source: Suarez et al., Unpublished. Cb5 - Uncultured Sebacinales clone 4EE2_6; Identity: 98%; GenBank code: HM451821; Country of origin: Ecuador; Source: Suarez et al., Unpublished. Cb6 - Uncultured Sebacinales clone 4EE2_6; Identity: 97%; GenBank code: HM451821; Country of origin: Ecuador; Source: Suarez et al., Unpublished.	Cb1 - Uncultured *Tulasnella* clone 1124a; Identity: 84%; GenBank code: FJ788890; Country of origin: England; Source: Waterman et al., 2011. Cb3 - Uncultured *Tulasnella* voucher C3.MN4; Identity: 85%; GenBank code: DQ178115; Country of origin: Ecuador; Suaréz et al., 2006. Cb4 -*Ceratobasidium* sp. UAMH 9846; Identity: 98%; GenBank code: DQ068770; Country of origin: USA; Sharma & Selosse, Unpublisehd.
C. *Hoffmannseggella cinnabarina* (Ferriferous formation)		
Cb9; Cb11; Cb13	Cb9 - Uncultured Sebacinales clone Seb109D; Identity: 96%; GenBank code: FJ514086; Country of origin: França; Source: Martos,F. & Selosse,M.-A. Unpublished. Cb13 - Uncultured Sebacinales clone FM731.2; Identity: 99%; GenBank code: JF691535.1; Country of origin: França; Source: Martos,F. & Selosse,M.-A. Unpublished.	Cb11 - *Epulorhiza* sp. ES4.1M; Identity: 99%; GenBank code: HQ127087; Country of origin:Brazil; Pereira & Kasuya, Unpublished. Cb13 - *Epulorhiza* sp. ES4.1M; Identity: 99%; GenBank code: HQ127087; Country of origin:Brazil; Pereira & Kasuya, Unpublished.
D. ***Hoffmannseggella caulescens***		
Cc1; Cc2; Cc3; Cc5; Cc6	Cc3 - Uncultured Sebacinales clone 4EE2_6; Identity: 98%; GenBank code: HM451821; Country of origin: Ecuador; Source: Suarez et al., Unpublished. Cc5 - Uncultured Sebacinales clone FM610.2; Identity: 97%; GenBank code: JF691455; Country of origin: França; Source: Martos,F. & Selosse, M.-A. Unpublished.	Cc1 - Uncultured Tulasnellaceae clone FM705.1; Identity: 98%; GenBank code: JF691505; Country of origin: França; Source: Martos,F. & Selosse,M.-A. Unpublished. Cc2 - Uncultured *Tulasnella* clone 9809; Identity: 87%; GenBank code: EU909334.1; Country of origin: Germany; Source: Krause et al, 2011. Cc6 - *Ceratobasidium* sp. UAMH 9846; Identity: 98%; GenBank code: DQ068770; Country of origin: USA; Sharma & Selosse, Unpublisehd.

[a] Each root code corresponding to a different individual.

Phylogenetic analysis of Sebacinales mycobionts

Portions of the LSU region sequences showed consistent results in phylogenetic analysis, presenting five distinct OTUs clustered into four clades represented by the letters A to D (Fig. 2). All clades generated by Bayesian analysis, in which lie the five sequence types, showed high support values with few value variations obtained by Maximum Likelihood analysis (Fig. 2). Furthermore, the sequences classified as the same OTU had pairwise similarity ranging from 98 to 100%. Only clade D comprised two OTUs, while the other clades consisted only of one OTU each. Symbionts of the same OTU were shared among orchid species, e.g., sequence OTU 1 and 2. The two sebacinales sequences from *H. jongheana* belonged to different OTUs, 1 and 5. The *H. cinnabarina* and *H. caulescens* sequences from ferriferous formation belonged to different OTUs, 1 and 2 and 2 and 4, respectively. Only the three *H. cinnabarina* sequences from granite outcrop belonged to the same OTU, 3.

Phylogenetic analysis of Tulasnelloid mycobionts

The 5.8 / ITS1-2 sequences and a portion of the LSU region sequence showed consistent results in the phylogenetic analysis, presenting five distinct OTUs clustered into four clades, represented by the letters A to D (Fig. 3). All clades generated by Bayesian analysis, which include the five OTUs, showed high support values, with few values variations obtained by maximum likelihood analysis (Fig. 3). Furthermore, the sequences classified as the same OTU had 100% pairwise similarity. Only clade D comprised two OTUs, while the other clades consisted only of one each. Only symbionts of the OTU 3 (clade C) were shared among two orchid species, *H. cinnabarina* and *H. caulescens*.

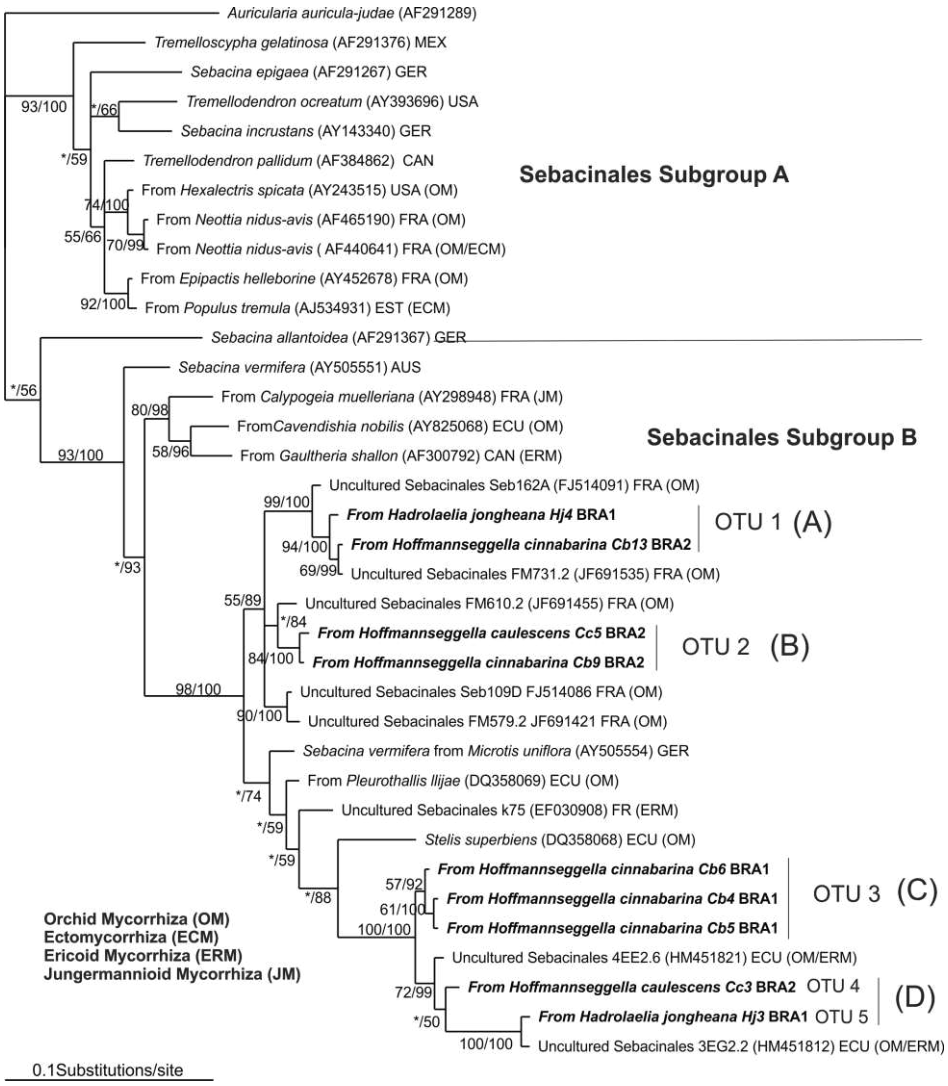

Fig. 2 Phylogenetic placement of Sebacinales sequences from the orchid species *Hadrolaelia jongheana*, *Hoffmannseggella caulescens*, and *Hoffmannseggella cinnabarina* by Bayesian likelihood analysis from an alignment of 5.8S-ITS/LSU sequences. Above each branch are shown bootstrap values generated by maximum likelihood and branch support Bayesian Markov chain Monte Carlo analysis (only values exceeding 50% are shown). The asterisk (*) is used when the branches did not have the same topology between the methods used and are considered values of the a *posteriori* probability obtained by the method of Bayesian likelihood. The tree was rooted with *Auricularia auricula-judae*. The letters A to D refer to clades. Sample provenances: AUS Australia, BRA Brazil (BRA1- State Park of Serra do Brigadeiro SBPS/BRA2- Ferriferous Formation), ECU Ecuador, EST Estonia, FRA France, GER Germany, MEX Mexico, and USA United States of America. GenBank accession numbers are listed after the species names.

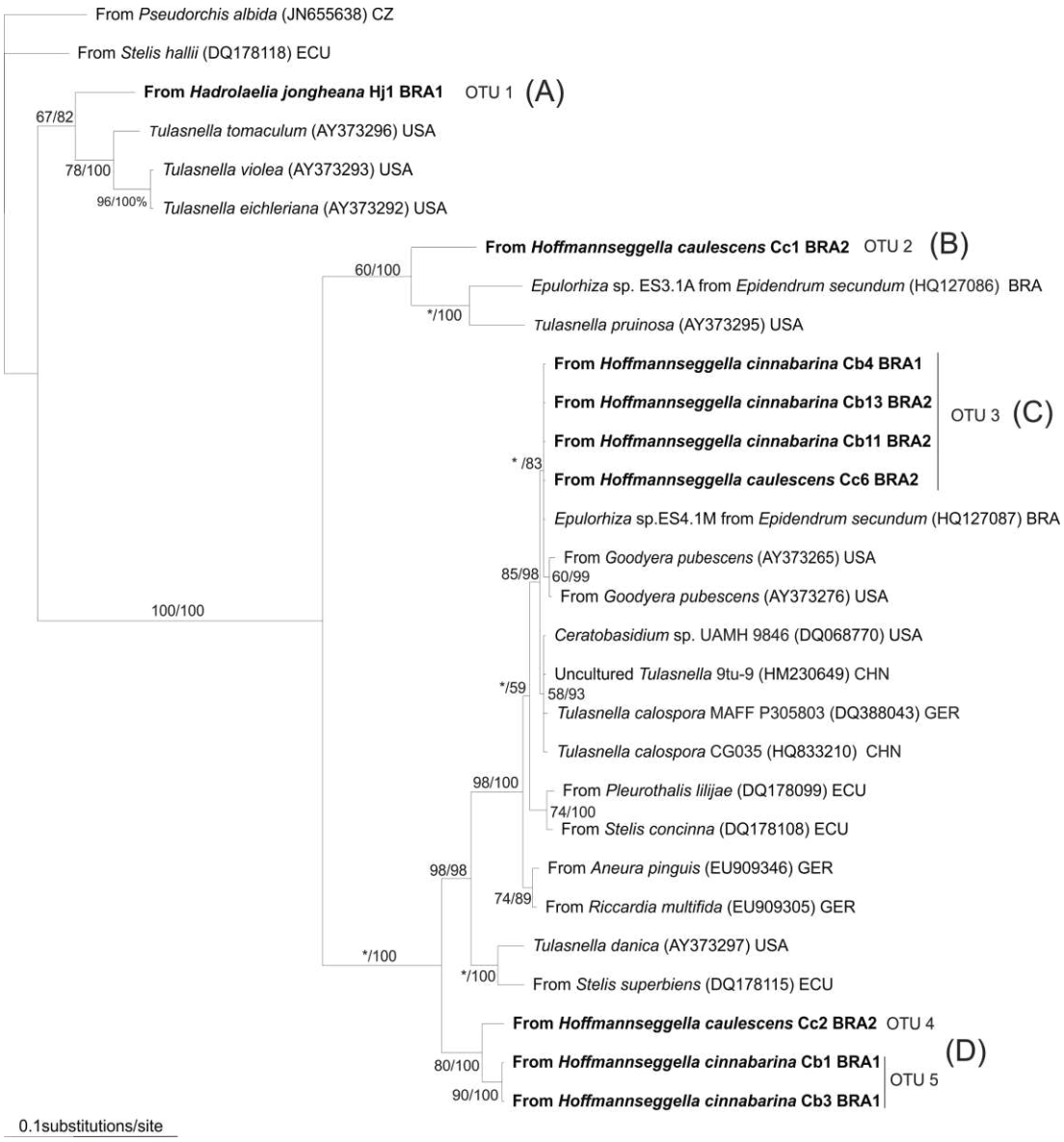

Fig. 3 Phylogenetic placement of *Tulasnella* sequences from the orchid species *Hadrolaelia jongheana*, *Hoffmannseggella caulescens*, and *Hoffmannseggella cinnabarina* by Bayesian likelihood analysis from an alignment of 5.8S-ITS/LSU sequences. Above each branch are shown bootstrap values generated by maximum likelihood and branch support Bayesian Markov chain Monte Carlo analysis (only values exceeding 50% are shown). The asterisk (*) is used when the branches did not have the same topology between the methods analyzed and are considered values of the a *posteriori* probability obtained by the method of Bayesian likelihood. The letters A to D refer to clades. Sample provenances: BRA Brazil (BRA1- State Park of Serra do Brigadeiro SBPS/BRA2- Ferriferous Formation), CHN China, CZ Czech Republic, ECU Ecuador, GER Germany, and USA United States of America. GenBank accession numbers are listed after the species names

The clade C comprised the tulasnelloid sequence from *H. cinnabarina* (Cb4) collected from a different area (BRA1) from the other individuals in this group. This sequence (Cb4) is phylogenetically distant from the other sequences Cb1 and Cb3 (clade D), which are from the same orchid species and were also collected in the same place (BRA1). The three sequences from *H. caulescens* belonged to different OTUs, present in clades B, C and D. The single tulasnelloid sequence obtained from *H. jongheana* (Hj1) roots presented as phylogenetically distant from the other sequences obtained in this study.

Discussion

The importance of the association of mycorrhizal fungi with orchids is already well established. Wahrlich (1886) and Janse (1897) cited by Zhu et al. (2008) first noted the occurrence of mycorrhizal fungi in the roots of temperate and tropical orchids. Thus, we expected to obtain molecular evidence of the presence of *Rhizoctonia*-like fungi samples. However, there was a large discrepancy between the number of roots analyzed and the number of positive PCR results for both the tulasnelloid and sebacinoid groups (Table 1). These results may suggest that in these negative cases, there was no colonization by these specific fungal groups. On the other hand, these results do not mean that no other fungal symbionts are associated with the plants because all roots analyzed contained fungal pelotons in root cortical cells, the structure that is characteristic of colonization by *Rhizoctonia*-like fungi. In our studies, we have observed that the diversity of fungi associated with the roots of these orchid species is high, mainly relating to sebacinoids mycorrhizal fungi (Oliveira et al. unpublished). Therefore, the low number of positive PCR products may be related to problems inherent in the molecular methodology, especially the limitations related to PCR with these specific primers. Another difficulty is

determining an appropriate cutoff value for the definition of sequence type or OTU so that there is a correspondence with the traditionally recognized species. McCormick et al. (2006) suggested that sequence differences of approximately 2% in 5.8-ITS are sufficient to distinguish pure cultures of *Tulasnella* sp. isolates. Suarez et al. (2008) used proportional nucleotide differences of <1% between LSU D1/D2 sequences to define sequence types for the Sebacinales order and genus *Tulasnella*. Despite these speculations, species definition based on sequence variation is not currently possible in sebacinales and tulasnellales. The cutoff value of 97% pairwise similarity was also used to separate possible arbuscular mycorrhizal fungi (AMF) from the divergence between amplicons of 550 bp (s) (Helgason et al. 2002).

Sebacinales mycorrhizal taxa, including ectomycorrhizae mycobionts, arbuscular, ericoides, jungermannioid and orchidoid mycorrhizae, are distributed between two subgroups. All sequence types obtained in this study belong to the Sebacinales subgroup B (Weiss et al. 2004) (Fig. 2). This subgroup also comprised sequences obtained from fungi associated with Ericaceae family plants, jungermannioid and other fungi from tropical orchids, as well as *Sebacina vermifera* sequences obtained from Australian and German orchids. Only the sebacinales sequences from *H. cinnabarina* orchids collected in granitic rock belonged to a single OTU (Fig. 2, Clade C). This tendency to observe a more specific association between the orchid and fungal group can be explained by the characteristics of the location where this species was sampled.

The granitic rock outcrop vegetation is distinct from the flora of its surroundings, and it is characterized by a high degree of endemism (Porembski and Barthlott 2000). These factors, as well as the greater geographical isolation of this area, may also result in endemic mycorrhizal associations. These fungal isolates are

closely related to each other (clade C, Fig. 2) and phylogenetically distant from fungi associated with the same orchid species but collected from another area, "campo rupestre" on canga. Therefore, regional differentiation, or in other words, local environmental factors, may have an effect on the association between this orchid species with that particular fungal group.

However, when we consider the association between *H. cinnabarina*, also collected in SBPS (BRA1), and tulasnelloid fungal symbionts (Fig. 3), the tulasnelloid fungi from this species were clustered into distinct clades. For the orchid species *H. jongheana* and *H. caulesce*ns, as well as for *H cinnabarina* from ferriferous formation (BRA 2), we did not observe a pattern of association with the sebacinoid fungi because other clades clustered fungal isolates from orchids collected in different areas and belonging to different species (Fig. 2). Furthermore, the sebacinoid mycobionts were identified as uncultured Sebacinales, and they may potentially be considered to be new fungal species.

Tulasnella was confirmed as a genus that forms mycorrhizal associations with a wide range of orchid species (Kristiansen et al. 2004, Ma et al. 2003, McCormick et al. 2004, Shefferson et al. 2005). In addition, the *Epulorhiza* genus (anamorph of *Tulasnella*) has been reported as being a major symbiont associated with tropical orchids in Brazil (Pereira et al. 2003, Nogueira et al. 2005, Pereira et al. 2005). Additionally, *Epulorhiza* sp. fungi have been characterized as having a widespread distribution; they can occur in both disturbed and undisturbed habitats (Bonnardeaux et al. 2007).

Regarding the tulasnelloid mycobionts obtained in this study, we observed that the clades clustered fungal isolates from orchids collected in different areas and different species. This fact leads us to suggest that this fungal group presents

widespread distribution because the isolates obtained from orchids from different locations were closely related.

The fact that these closely related fungal isolates are also from different orchid species suggests that these plants are generalists when in association with *Tulasnella* sp. Fungi belonging to clade C (Fig. 3) were closely related to *Epulorhiza* sp. fungi isolated from the Brazilian orchid *Epidendrum secundum* Jacq., which is also found in the Serra Brigadeiro State Park (BRA1). In this case, it is suggested that this fungal isolate in particular presents worldwide distribution, being common even in disturbed areas such as the mining region in "campo rupestre". Additionally, this clade is related to the *Tulasnella calospora* (Boud.) Juel species proposed by Hadley (1970) to be a universal orchid symbiont due to its ability to establish *in vitro* symbiotic associations with a wide variety of orchid species.

The clade D (Fig. 3), in turn, comprised symbionts closely related with *Tulasnella danica* Hauerslev, a fungal species associated with terrestrial orchids (McCormick et al. 2004). Reinforcing this trend of generalist mycorrhizal association, clade B (Fig. 3) includes one of the fungal symbionts from *H. caulescens* (CC1), which is related to *Tulasnella pruinosa* Bourdot & Galzin. The only tulasnelloid isolate obtained from an epiphytic *H. jongheana* orchid appeared phylogenetically distant from others, presenting high identity with th*e Tulasnella tomaculum* P. Roberts. Both species *T. pruinosa* and *T. tomaculum* are mycorrhizal fungi isolated from terrestrial orchids (McCormick et al. 2004).

In summary, our results show that tropical orchids can be considered generalists in their associations with sebacinoid and tulasnelloid mycorrhizal fungi, and the diversity of this association is influenced by factors inherent to the sites that these plants inhabit. However, it is likely that other *Rhizoctonia*-like fungi groups

may be associated with the orchid roots, which were observed to have pelotons but did not yield positive results with the specific primers used. In addition, the fungal symbionts analyzed showed a wide distribution, being present in disturbed and undisturbed areas and establishing associations with both rupicolous and epiphytic orchids. Thus, our analysis indicates that for efficient rehabilitation of these endangered orchids from the Atlantic Forest, it is necessary to use different tulasnelloid and sebacinoid species as orchid symbionts. It is necessary to carefully select mycobionts for *H. cinnabarina* reintroduction, particularly when this species inhabits an area characterized by endemic vegetation and geographically isolated, such as the granitic rock outcrop.

The data from this study support and complement the diversity found by Bocayuva et al. (unpublished) through the isolation method, which showed another type of mycorrhizal fungi associated with these three orchid species. This information is important for the conservation of these orchids, whose threatened condition is made worse by the fact that the *Hoffmannseggella* genus is endemic to the Atlantic Forest, a biome that suffers constantly from anthropogenic impacts.

Acknowledgments – We thank Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FAPEMIG (Fundação de Amparo à Pesquisa do estado de Minas Gerais), and AOS (American Orchid Society) for financial support.

References

Andersen TF, Rasmussen HN (1996) The mycorrhizal species of Rhizoctonia. In: Sneh B, Jabaji-Hare S, Neate S, Dijst G (Eds) *Rhizoctonia* species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Kluwer Academic Publishers, pp 379-390

Antonelli A, Verola CF, Parisod C, Gustafsson ALS (2010) Climate cooling promoted the expansion and radiation of a threatened group of South American orchids (Epidendroideae: Laeliinae). Biol J Linn Soc 100:597–607

Arditti, J (1992) Fundamentals of orchids biology. John Wiley & Sons, pp 691

Barros F,Vinhos F, Rodrigues VT, Barberena FFVA, Fraga CN, Pessoa EM (2012).Orchidaceae in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. (*http://floradobrasil.jbrj.gov.br/2012/FB066871*)

Benites VM, Caiafa AN, Mendonça ES, Schaefer CE, Ker JC (2003) Solos e Vegetação nos Complexos Rupestres de Altitude da Mantiqueira e do Espinhaço. Floresta e Ambiente 10:76-85

Bidartondo MI, Bruns TD, Weiss M, Sergio C, Read DJ (2003) Specialized cheating of the ectomycorrhizal symbiosis by an epiparasitic liverwort. Proc Royal Soc London, Biol Sci 270:835–842

Bonnardeaux Y, Brundrett M, Batty A, Dixon K, Koch J, Sivasithamparam K (2007) Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. Mycol Res 111:51–61

Bougoure J, Ludwig M, Brundrett M, Grierson P (2009) Identity and specificity of the fungi forming mycorrhizas with the rare mycoheterotrophic orchid *Rhizanthella gardneri*. Mycol Res 113:1097–1106

Currah RS, Smreciu EA, Hambleton S (1990) Mycorrhizae and mycorrhizal fungi of boreal species of *Platanthera* and *Coeloglossum* (Orchidaceae). Can J Bot 68: 1171–1181

Currah RS, Zettler WL, McInnis TM (1997). *Epulorhiza inquilina* sp. nov. from *Platanthera* (Orchidaceae) and a key to *Epulorhiza* species. Mycotaxon 61:335–342

Decker JS (1941) *Laelia jongheana* var. *alba*. Orquídea 4:28-29

Drummond GM, Machado ABM, Martins CS, Mendonça MP, Stehmann JR (2008) Listas Vermelhas das espécies ameaçadas de extinção em Minas Gerais. Fundação Biodiversitas. 2ª edição. ISBN: 978-85-85401-23-8

Engevix (1995) Caracterização do meio físico da área autorizada para criação do Parque Estadual da Serra do Brigadeiro – Relatório técnico final dos estudos – 8296- RE-H4-003/94 "VER. 1". Instituto Estadual da Floresta, BIRD/PRÓ-FLORESTA/SEPLAN., pp. 34

Ferri MG (1980). Vegetação Brasileira. Editora da Universidade de São Paulo, São Paulo, pp.157

Giulietti AM, Rapini A, Andrade MJG, Queiroz LP, Silva JMC (2009) Plantas Raras do Brasil. Conservação Internacional, Belo Horizonte, MG, pp.496

Helgason TJW, Merryweather J, Denison P, Wilson JPW, Young A, Fitter, H (2002) Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. J. Ecol. 90:371–384

Huelsenbeck, JP, Crandall KA (1997) Phylogeny estimation and hypothesis testing using maximum likelihood. Annual Review of Ecology and Systematics, 28: 437-466.

Huelsenbeck JP, Ronquist F (2001) Mrbayes: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755

Koeppen W (1948) Climatologia. Fondo Cultura Económica. México & Buenos Aires, pp 478

Kristiansen KA, Freudenstein JV, Rasmussen FN, Rasmussen HN (2004) Molecular identification of mycorrhizal fungi in *Neuwiedia veratrifolia* (Orchidaceae). Mol Phylogenet Evol 33: 251–258

Lino CF, Simões LL (2011) Avaliação do cumprimento das metas globais e nacionais de biodiversidade 2010 para a Mata Atlântica. WWF-Brasil em parceria com a Reserva da Biosfera da Mata Atlântica

Ma M, Tan TK, Wong SM (2003) Identification and molecular phylogeny of *Epulorhiza* isolates from tropical orchids. Mycol Res 107: 1041–1049

McCormick M, Whigham DF, Sloan D, O, Malley K, Hodkinson B (2006) Orchid-fungus fidelity: a marriage meant to last? Ecology 87:903–911

Mccormick MK, Whigham DF, O'Neill J (2004) Mycorrhizal diversity in photosynthetic terrestrial orchids. New Phytologist 163: 425-438

Mittermeier RA, Gil PR, Hoffmann M, Pilgrim J, Brooks T, Mittermeier CG, Lamoreux J, Fonseca GAB (2004) Hotspots revisited Earth's biologically richest and most endangered terrestrial ecoregions. Cemex, Mexico

Muller WH, Stalpers JA, Van Aelst AC, Van Der Krift TP, Boekhout T (1998) Field emission gun-scanning electron micro-scopy of septal pore caps of selected species in the Rhizoctonia s.1. complex. Mycologia 90:170–179

Needleman SB, Wunsch CD (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. 48:443–453

Nogueira RE, Pereira OL, Kasuya MCM, Lanna MC, Mendonça M (2005) Fungos micorrízicos associados e orquídeas em campos rupestres na Região do Quadrilátero Ferrífero, Minas Gerais, Brasil. Acta Bot Bras 3:417-424

Nylander JAA (2004) MrModeltestv2 Program distributed by the auhor. Evolutionary Biology Centre, Uppsala University, Sweden

Oliveira – Filho AT, Ratter JÁ (1995) A study of the origin of Central Brazilian Forests by the analysis of plant species distribution patterns. J Bot 52: 141-194

Oliveira Filho AT, Fontes MAL (2000) Patterns of floristic differentiation among Atlantic Forests in Southeastern Brazil, and the influence of climate. Biotropica 32: 793-810

Paula CC (1998) Florística da família Bromeliaceae no Parque Estadual da Serra do Brigadeiro, MG – Brasil. Tese, Universidade Estadual Paulista

Pereira OL, Rollemberg CL, Borges AC, Matsuoka K, Kasuya MCM (2003) *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. Mycoscience 44:153-155

Pereira OL, Kasuya MCM, Borges AC, Araújo EF (2005) Morphological and molecular characterization of mycorrhizal fungi isolated from neotropical orchids in Brazil. Can J Bot 83: 54–65

Porembski S, Barthlott W (2000) Granitic and gneissic outcrop (inselbergs) as centers of diversity for desiccation-tolerant vascular plants. Plant Ecol 151:19–28

Porras-Alfaro A, Bayman P (2007) Mycorrhizal fungi of *Vanilla*: diversity, specificity and effects on seed germination and plant growth. Mycologia 99:510–525

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Rasmussen HN (2002) Recent developments in the study of orchid mycorrhiza. Plant Soil 244: 149-163

Rice P, Longden I, Bleasby A (2000) EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet 16:276–277

Roberts DL, Dixon KW (2008) Orchids. Curr Biol 18:325-329

Setaro S, Weiss M, Oberwinkler F, Kottke I (2006) Sebacinales form ectendomycorrhizae with *Cavendishia nobilis*, a member of the Andean clade of Ericaceae, in the mountain rain forest of southern Ecuador. New Phytol 169:355–365

Shefferson RP, Weiss M, Kull T, Taylor DL (2005) High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. Mol Ecol 14: 613–626

Stark C, Babik W, Durka W (2009) Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea*. Mycol Res 952-959

Stehmann JR, Forzza RC, Salino A, Sobral M, Costa DP, Kamino LHY (2009) Plantas da Floresta Atlântica. Jardim Botânico do Rio de Janeiro, Rio de Janeiro, pp 516

Stewart SL, Zettler LW (2002) Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens*, *H. quinquiseta*, *H. macroceratilis*) from Florida. Aquat Bot 72:25-35

Strahler AN (1963) The Koppen climate classification system. In: (Ed.). Physical geography, John Willey, New York, pp.185-188

Swarts ND, Dixon KW (2009) Terrestrial orchid conservation in the age of extinction. Ann Bot-London 104: 543–556

Tamura K, Peterson D, Peterson N, Stecher G, Nei, M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Taylor DL, Bruns TD (1997) Independent, specialized invasions of ectomycorrhizal mutualism by two non-photosynthetic orchids. Proc. Natl Acad Sci USA 94:4510–4515

Veloso HP, Rangel Filho ALR, Lima JCA (1991) Classificação da vegetação brasileira, adaptada a um sistema universal. IBGE, Departamento de Recursos Naturais e Estudos Ambientais, Rio de Janeiro, pp. 123

Verola CF, Semir J, Antonelli A, Koch I (2007) Biosystematic studies in the Brazilian endemic genus *Hoffmannseggella* H. G. Jones (Orchidaceae: Laeliinae): a multiple approach applied to conservation. Lankesteriana 7: 419–422

Viana PL, Lombardi JA (2007) Florística e caracterizaçãoo dos campos rupestres sobre canga na serra da calcada, Minas Gerais, Brasil, Rodriguésia 58:159–177

Warcup JH, Talbot PHB (1967) Perfect states of rhizoctonias associated with orchids. New Phytol 66:631-641

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. Mycol Res 108:1003–1010

Whigham DF, O'Neill JP, Rasmussen HN, Caldwell BA, McCormick MK (2006) Seed longevity in terrestrial orchids potential for persistent *in situ* seed banks. Biol Conserv, 129:24–30

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols ± A guide to methods and applications. Academic Press, San Diego, pp 315-322

Zhu GS; Yu ZN, Gui Y, Liu ZY (2008) A novel technique for isolating orchid mycorrhizal fungi. Fungal Diversity, 33: 123-137

4. CONCLUSÕES GERAIS

Caracterizamos, pela primeira vez, a diversidade das comunidades fúngicas associadas às orquídeas *Hadrolaelia jongheana*, *Hoffmannseggella cinnabarina* e *Hoffmannseggella caulescens*, nativas da Floresta Atlântica.

A construção de bibliotecas de clones permitiu identificar taxa de fungos basidiomicetos e ascomicetos associados às raízes dessas plantas. Os fungos basidiomicetos encontrados, em sua maioria, são potenciais candidatos a fungos micorrízicos de orquídeas. Ao passo que, as taxa ascomicetos identificados incluem fungos potencialmente endofíticos dessas orquídeas.

As comunidades fúngicas das três espécies de orquídeas analisadas apresentaram alta diversidade, indicando que essas plantas podem apresentar baixa especificidade em relação a certos clados fúngicos.

As orquídeas analisadas apresentaram-se generalistas em suas associações micorrízicas com fungos rizoctonióides sebacinóides e tulasnelóides, sendo a diversidade dessa associação influenciada por fatores inerentes aos locais que essas plantas habitam.

A alta diversidade das comunidades fúngicas associadas a essas plantas oferece uma maior flexibilidade para se adaptarem a mudanças nas condições ambientais, sendo uma grande vantagem para programas de reintrodução dessas espécies em outros habitats.

5. ANEXOS

Supplementary material relating to the article Endophytic and mycorrhizal communities associated with roots of endangered native orchids from the Brazilian Atlantic Forest

Table S1 BLASTn results for the 442 OTUs

ITS rDNA sequences of fungal clones obtained by the clone libraries[a]					
Clones	**Close relative from GenBank (accession nº)**	**Identity (%)**	**Clones**	**Close relative fromGenBank (accession nº)**	**Identity (%)**
HCB1.1	*Sebacina vermifera* (EU625994.1)	89%	HCB1.49	Uncultured Helotiales (FN565262.1)	95%
HCB1.2	*Sebacina vermifera* (EU625994.1)	89%	HCB1.50	Uncultured Helotiales (FN565262.1)	95%
HCB1.3	*Sebacina vermifera* (EU625994.1)	88%	HCB1.51	Uncultured Helotiales(FN565262.1)	95%
HCB1.4	*Sebacina vermifera* (EU625994.1)	88%	HCB1.52	Uncultured Helotiales (FN565262.1)	95%
HCB1.5	Uncultured Sebacinales (EU625950.1)	87%	HCB1.53	Uncultured Helotiales (FN565262.1)	95%
HCB1.6	Uncultured Sebacinales (EU625951.1)	89%	HCB1.54	Uncultured *Meliniomyces* sp. (FN565286.1)	95%
HCB1.7	*Sebacina vermifera* (FN663143.1)	88%	HCB1.55	Uncultured Hyaloscyphaceae (FJ440904.1)	93%
HCB1.8	Uncultured soil fungus (GU083263.1)	91%	HCB1.56	Uncultured Helotiales (FN565262.1)	95%
HCB1.9	*Sebacina vermifera* (EU625994.1)	88%	HCB1.57	Uncultured *Meliniomyces* sp. (FN565286.1)	89%
HCB1.10	*Sebacina vermifera* (EU625994.1)	88%	HCB1.58	Uncultured Sebacinales (HM451822.1)	96%
HCB1.11	*Sebacina vermifera* (EU625994.1)	88%	HCB1.59	Uncultured Sebacinales (HM451822.1)	96%
HCB1.12	*Sebacina vermifera* (EU625994.1)	88%	HCB1.60	Uncultured *Sebacina* sp. (EU909227.1)	100%
HCB1.13	*Sebacina vermifera* (EU625994.1)	89%	HCB1.61	Uncultured fungus (AM260798.11)	82%
HCB1.14	*Sebacina vermifera* (EU625994.1)	88%	HCB1.62	Uncultured fungus (AM260798.1)	80%
HCB1.15	*Sebacina vermifera* (EU625994.1)	88%	HCB1.63	Uncultured fungus (JF300835.1)	86%
HCB1.16	*Sebacina vermifera* (EU625994.1)	88%	HCB1.64	Uncultured *Sebacina* sp. (EU909227.1)	100%
HCB1.17	*Sebacina vermifera* (EU625994.1)	88%	HCB1.65	Uncultured *Sebacina* sp. (EU909227.1)	100%
HCB1.18	*Sebacina vermifera* (EU625994.1)	88%	HCB1.66	Uncultured *Sebacina* sp. (EU909227.1)	100%
HCB1.19	*Stereaceae* sp. (FR846481.1)	91%	HCB1.67	Uncultured *Sebacina* sp. (EU909227.1)	100%
HCB1.20	*Sebacina vermifera* (EU625994.1)	89%	HCB1.68	Uncultured *Sebacina* sp. (EU909227.1))	100%

Clones	Close relative from GenBank (accession nº)	Identity (%)	Clones	Close relative from GenBank (accession nº)	Identity (%)
HCB1.21	*Sebacina vermifera* (EU625994.1)	88%	HCB1.69	Uncultured *Sebacina* sp. (EU909227.1)	100%
HCB1.22	*Sebacina vermifera* (EU625994.1)	88%	HCB1.70	Uncultured *Sebacina* sp. (EU909227.1)	100%
HCB1.23	*Sebacina vermifera* (EU625994.1)	88%	HCB1.71	Uncultured *Sebacina* sp. (EU909227.1)	100%
HCB1.24	*Sebacina vermifera* (EU625994.1)	88%	HCB1.72	Uncultured Fungus (JF691222.1)	86%
HCB1.25	*Sebacina vermifera* (EU625994.1)	89%	HCB1.73	Uncultured *Sebacina* sp.(EU909227.1)	100%
HCB1.26	*Sebacina vermifera* (EU625994.1)	89%	HCB1.74	Uncultured Sebacinales (JF691222.1)	94%
HCB1.27	*Sebacina vermifera* (EU625994.1)	88%	HCB1.75	Uncultured Sebacinales (JF691222.1)	94%
HCB1.28	Uncultured soil fungus (GU083242.1)	90%	HCB1.76	Uncultured Sebacinales (HM451822.1)	95%
HCB1.29	Uncultured Letiomycetes(HM230871.1)	95%	HCB1.77	Uncultured Sebacinales (HM451822.1)	96%
HCB1.30	Uncultured Letiomycetes (FN565262.1)	95%	HCB1.78	Uncultured fungus (AM260798.1)	80%
HCB1.31	Uncultured Helotiaceae (EU668920.1)	96%	HCB1.79	Uncultured fungus (JF300835.1)	86%
HCB1.32	Uncultured Helotiales (FN565262.1)	95%	HCB1.80	Uncultured fungus (JF300835.1)	86%
HCB1.33	Uncultured Helotiales (FN565262.1)	95%	HCB1.81	Uncultured Sebacinales (HM451822.1)	95%
HCB1.34	Uncultured Helotiales (FN565262.1)	95%	HCB1.82	Uncultured fungus (AM260798.1)	79%
HCB1.35	Uncultured Helotiales (FN565262.1)	95%	HCB1.83	Uncultured *Sebacina* sp.(EU909227.1)	100%
HCB1.36	Uncultured Helotiales (FN565262.1)	95%	HCB1.84	Uncultured Sebacinales (HM451799.1)	95%
HCB1.37	Uncultured Helotiales (FN565262.1)	95%	HCB1.85	Uncultured Sebacinales (FN663742.1)	89%
HCB1.38	Uncultured Helotiales (FN565262.1)	94%	HCB1.86	Uncultured Sebacinaceae (EU625984.1)	99%
HCB1.39	Uncultured Helotiales (FN565262.1)	95%	HCB1.87	Uncultured Sebacinales (FN663742.1)	89%
HCB1.40	Uncultured Helotiales (FN565262.1)	95%	HCB1.88	Uncultured Sebacinales (FN663742.1)	87%
HCB1.41	Uncultured *Meliniomyces* sp. (FN565286.1)	91%	HCB1.89	Uncultured fungus (AM260798.1)	80%
HCB1.42	Uncultured Helotiales (FN565262.1)	95%	HCB1.90	Uncultured Sebacinales (FN663742.1)	88%
HCB1.43	Uncultured Helotiales (FN565262.1)	95%	HCB1.91	Uncultured Sebacinales (FN663742.1)	88%
HCB1.44	Uncultured fungus (GU721579.1)	98%	HCB1.92	Uncultured Sebacinales (FN663742.1)	89%
HCB1.45	Uncultured Helotiales (FN565262.1)	95%	HCB1.93	Uncultured Sebacinales (FN663742.1)	89%

Clones	Close relative from GenBank (accession nº)	Identity (%)	Clones	Close relative from GenBank (accession nº)	Identity (%)
HCB1.46	Uncultured Helotiales (FN565262.1)	95%	HCB1.94	Uncultured Sebacinales (FN663696.1)	88%
HCB1.47	Uncultured Helotiales (FN565262.1)	95%	HCB1.95	Uncultured Sebacinales (FN663696.1)	88%
HCB1.48	Uncultured *Meliniomyces* sp.	91%	HCB1.96	Uncultured fungus (EU625986.1)	80%
HCB1.97	Uncultured Sebacinales (EU625986.1)	88%	HCB2.35	*Piriformospora indica* (AF019636.1)	99%
HCB1.98	Uncultured fungus (AM260798.1)	78%	HCB2.36	Uncultured Leotiomycetes (HM230888.1)	97%
HCB1.99	Uncultured fungus (AM260798.1)	78%	HCB2.37	Uncultured Leotiomycetes (HM230888.1)	97%
HCB1.100	Uncultured Sebacinales (FN663696.1)	88%	HCB2.38	Uncultured Leotiomycetes (HM230888.1)	96%
HCB1.101	Uncultured Sebacinales (EU625985.1)	87%	HCB2.39	Uncultured Leotiomycetes (HM230888.1)	96%
HCB1.102	Uncultured Sebacinales (EU625985.1)	87%	HCB2.40	Uncultured Leotiomycetes (HM230888.1)	97%
HCB1.103	Uncultured Sebacinales (FN663742.1)	88%	HCB2.41	Uncultured Leotiomycetes (HM230888.1)	96%
HCB1.104	Uncultured Sebacinales (FN663742.1)	88%	HCB2.42	Uncultured Leotiomycetes (HM230888.1)	96%
HCB1.105	Uncultured fungus (AM260798.1)	80%	HCB2.43	Uncultured Leotiomycetes (HM230888.1)	97%
HCB1.106	Uncultured fungus (AM260798.1)	79%	HCB2.44	Uncultured Leotiomycetes (HM230888.1)	97%
HCB1.107	Uncultured Sebacinales(FN663742.1)	88%	HCB2.45	Uncultured Leotiomycetes (HM230888.1)	97%
HCB1.108	Uncultured Sebacinales (FN663742.1)	88%	HCB2.46	Uncultured Leotiomycetes (HM230888.1)	97%
HCB1.109	Uncultured Sebacinales (FN663742.1)	88%	HCB2.47	Uncultured Leotiomycetes (HM230888.1)	97%
HCB1.110	Uncultured Sebacinales (FN663742.1)	89%	HCB2.48	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.1	*Chaetomium aureum* (HQ607894.1)	99%	HCB2.49	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.2	Uncultured fungus (GU174309.1)	94%	HCB2.50	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.3	Uncultured Helotiales(FN565262.1)	95%	HCB2.51	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.4	Uncultured fungus (GU174309.1)	95%	HCB2.52	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.5	*Chaetomium aureum* (HQ607894.1)	99%	HCB2.53	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.6	Uncultured fungus (GU370758.1)	99%	HCB2.54	Uncultured fungus (HQ625462.1)	98%
HCB2.7	*Pisolithus* sp. (HQ896485.1)	99%	HCB2.55	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.8	Uncultured basidiomycete (AM901966.1)	86%	HCB2.56	Uncultured Leotiomycetes (HM230888.1)	97%

Clones	Close relative from GenBank (accession nº)	Identity (%)	Clones	Close relative from GenBank (accession nº)	Identity (%)
HCB2.9	Uncultured fungus (GQ851818.1)	90%	HCB2.57	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.10	Uncultured fungus (GU174309.1)	94%	HCB2.58	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.11	Uncultured fungus (GU174309.1)	94%	HCB2.59	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.12	Uncultured fungus (GU174309.1)	94%	HCB2.60	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.13	Uncultured fungus (GU370758.1)	99%	HCB2.61	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.14	Uncultured fungus (GU566296.1)	95%	HCB2.62	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.15	Uncultured fungus (GU174309.1)	94%	HCB2.63	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.16	Uncultured Basidiomycota (GU328562.1)	93%	HCB2.64	Uncultured Leotiomycetes (HM230888.1)	97%
HCB2.17	Uncultured fungus (AM229065.1)	87%	HCB2.65	Teratosphaeria microspora (EU343297.1)	85%
HCB2.18	Uncultured Hyaloscyphaceae (FJ440904.1)	94	HCB2.66	Uncultured fungus (FJ857927.1)	99%
HCB2.19	Uncultured fungus (JF497142.1)	99%	HCB2.67	Uncultured fungus (FJ857927.1)	99%
HCB2.20	*Piriformospora indica* (AF019636.1)	99%	HCB2.68	Uncultured fungus (FJ857927.1)	99%
HCB2.21	*Piriformospora indica* (AF019636.1)	99%	HCB2.69	Uncultured fungus (FJ857927.1)	99%
HCB2.22	*Phacellium paspali* (GU214669.1)	88%	HCB2.70	Uncultured fungus (FJ857927.1)	99%
HCB2.23	*Piriformospora indica* (AF019636.1)	99%	HCB2.71	Uncultured Helotiales (FN565262.1)	95%
HCB2.24	*Piriformospora indica* (AF019636.1)	99%	HCB2.72	Uncultured fungus (FJ857927.1)	99%
HCB2.25	Uncultured Sebacinales (FN663677.1)	88%	HCB2.73	Uncultured fungus (FJ857927.1)	99%
HCB2.26	*Piriformospora indica* (AF019636.1)	95%	HCB2.74	Uncultured fungus (FJ857927.1)	92%
HCB2.27	*Piriformospora indica* (AF019636.1)	99%	HCB2.75	Uncultured fungus (FJ857927.1)	99%
HCB2.28	*Sebacina vermifera* (EU625994.1)	100%	HCB2.76	Uncultured fungus (JN890484.1)	99%
HCB2.29	*Teratosphaeria microspora* (EU343297.1)	85%	HCB2.77	Uncultured fungus (FJ857927.1)	99%
HCB2.30	*Colletotrichum* sp. (FJ042520.1)	99%	HCB2.78	Uncultured fungus (FJ857927.1)	99%
HCB2.31	*Piriformospora indica* (AF019636.1)	99%	HCB2.79	Uncultured fungus (FJ857927.1)	99%
HCB2.32	*Piriformospora indica* (AF019636.1)	99%	HCB2.80	Uncultured Basidiomycota (GU328570.1)	96%
HCB2.33	*Colletotrichum gloeosporioides* (JN399218.1)	92%	HCB2.81	Uncultured Basidiomycota (GU328570.1)	96%

Clones	Close relative from GenBank (accession nº)	Identity (%)	Clones	Close relative from GenBank (accession nº)	Identity (%)
HCB2.34	*Piriformospora indica* (AF019636.1)	99%	HCB2.82	Uncultured Basidiomycota (GU328570.1)	96%
HCB2.83	Uncultured Basidiomycota (GU328570.1)	96%	HC8	*Sebacina vermifera* (EU625992.1)	88%
HCB2.84	*Rhizoctonia solani* (FJ746968.1)	99%	HC9	*Sebacina vermifera* (EU625992.1)	87%
HCB2.85	Uncultured Basidiomycota (GU328570.1)	96%	HC10	*Sebacina vermifera* (EU625992.1)	88%
HCB2.86	*Rhizoctonia solani* (FJ746968.1)	99%	HC11	*Sebacina vermifera* (EU625992.1)	87%
HCB2.87	*Rhizoctonia solani* (FJ746969.1)	99%	HC12	Uncultured sebacinales EU625948.1)	86%
HCB2.88	Uncultured Basidiomycota (GU328570.1)	96%	HC13	*Sebacina vermifera* (EU625992.1)	88%
HCB2.89	*Rhizoctonia solani* (FJ746968.1)	99%	HC14	*Sebacina vermifera* (EU625992.1)	88%
HCB2.90	Uncultured Basidiomycota (GU328570.1)	96%	HC15	Uncultured Sebacinales (JF691462.1)	93%
HCB2.91	Uncultured Basidiomycota (GU328570.1)	96%	HC16	*Sebacina vermifera* (EU625992.1)	88%
HCB2.92	Uncultured Basidiomycota (GU328570.1)	96%	HC17	*Sebacina vermifera* (EU625994.1)	87%
HCB2.93	Uncultured Basidiomycota (GU328570.1)	96%	HC18	Uncultured fungus (GU370759.1)	99%
HCB2.94	Uncultured Sebacinales (EU625967.1)	87%	HC19	*Sebacina vermifera* (EU625992.1)	88%
HCB2.95	Uncultured Sebacinales (EU625967.1)	87%	HC20	*Sebacina vermifera* (EU625992.1)	88%
HCB2.96	Uncultured Sebacinales (EU625967.1)	87%	HC21	*Sebacina vermifera* (EU625992.1)	88%
HCB2.97	Uncultured Sebacinales (EU625967.1)	87%	HC22	Uncultured Hyaloscyphaceae (FJ440904.1)	96%
HCB2.98	Uncultured Sebacinales (EU625967.1)	87%	HC23	Uncultured fungus (JF926980.1)	99%
HCB2.99	Uncultured Sebacinales (EU625967.1)	87%	HC24	Uncultured fungus (FJ524314.1)	87%
HCB2.100	Uncultured Sebacinales (EU625967.1)	87%	HC25	Ascomycota sp. (HM208732.1)	83%
HCB2.101	Uncultured Sebacinales (EU625967.1)	87%	HC26	*Trichoderma gamsii* (HM176560.1)	99%
HCB2.102	Uncultured Sebacinales (HM451820.1)	97%	HC27	Uncultured Ascomycota (EU490074.1)	80%
HCB2.103	Uncultured Sebacinales (EU625967.1)	87%	HC28	*Chaetosphaeria* sp. (HQ630994.1)	97%
HCB2.104	Uncultured Sebacinales (EU625967.1)	87%	HC29	*Chaetosphaeria* sp. (HQ630994.1)	97%
HCB2.105	Uncultured Sebacinales (FN663742.1)	88%	HC30	Uncultured fungus (EU144657.1)	83%
HCB2.106	Uncultured Sebacinales (EU625967.1)	87%	HC31	Uncultured fungus (GU174272.1)	85%

Clones	Close relative from GenBank (accession nº)	Identity (%)	Clones	Close relative from GenBank (accession nº)	Identity (%)
HCB2.107	Uncultured Sebacinales (EU625967.1)	87%	HC32	Ascomycota sp. (HM208732.1)	83%
HCB2.108	Uncultured Sebacinales (EU909227.1)	100%	HC33	Uncultured soil fungus (HM037670.1)	93%
HCB2.109	Uncultured Sebacinales (JF691222.1)	100%	HC34	Uncultured fungus (GU174272.1)	85%
HCB2.110	Uncultured Sebacinales (FN663742.1)	89%	HC35	Uncultured Ascomycete (EU490094.1)	80%
HCB2.111	Uncultured Sebacinales (EU625967.1)	87%	HC36	Uncultured soil fungus (EU479762.1)	82%
HCB2.112	Uncultured Sebacinales (EU625967.1)	87%	HC37	Ascomycota sp. (HM208732.1)	83%
HCB2.113	Uncultured Sebacinales (FN663666.1)	87%	HC38	Uncultured fungus (FN397287.1)	84%
HCB2.114	Uncultured Sebacinales (EU625967.1)	86%	HC39	Uncultured Leotiomycetes (HQ211516.1)	85%
HCB2.115	Uncultured Sebacinales (EU625967.1)	87%	HC40	Uncultured fungus (GU174272.1)	85%
HCB2.116	Uncultured Sebacinales (EU625967.1)	87%	HC41	Uncultured fungus (JF497148.1)	79%
HCB2.117	Uncultured Sebacinales (EU625967.1)	87%	HC42	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HCB2.118	Uncultured Sebacinales (HM451822.1)	96%	HC43	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HCB2.119	Uncultured Sebacinales (EU625967.1)	87%	HC44	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HCB2.120	Uncultured Sebacinales (EU625967.1)	87%	HC45	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HCB2.121	Uncultured Sebacinales (EU625987.1)	100%	HC46	Uncultured *Rhizoctonia* sp.DQ061931.1)	95%
HCB2.122	Uncultured Sebacinales (EU909227.1)	100%	HC47	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HCB2.123	Uncultured Sebacinales (EU909227.1)	100%	HC48	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HC1	*Sebacina vermifera* (EU625992.1)	88%	HC49	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HC2	*Sebacina vermifera* (EU625992.1)	88%	HC50	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HC3	Uncultured *Phoma* sp. (JX010732.1)	99%	HC51	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HC4	Uncultured Ascomycota (FJ475785.1)	87%	HC52	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HC5	Uncultured Sebacinales (FN663657.1)	82%	HC53	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HC6	Uncultured Ascomycota (HM162104.1)	88%	HC54	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HC7	Uncultured *Phoma* sp. (JX010732.1)	89%	HC55	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%
HC56	Uncultured *Rhizoctonia* sp.(DQ061931.1)	95%	HC104	*Chloridium* sp. (HQ630978.1)	99%

Clones	Close relative from GenBank (accession nº)	Identity (%)	Clones	Close relative from GenBank (accession nº)	Identity (%)
HC57	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%	HC105	*Penicillium* sp. (GU566211.1)	99%
HC58	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%	HC106	Fungal endophyte sp. (HM537063.1)	99%
HC59	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%	HC107	*Chloridium* sp. (HQ630978.1)	99%
HC60	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%	HJ1	Uncultured Ascomycota (HM146819.1)	99%
HC61	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%	HJ2	Uncultured Ascomycota (HM146819.1)	99%
HC62	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%	HJ3	Uncultured fungus (GU366722.1)	99%
HC63	Uncultured *Rhizoctonia* sp. (DQ061931.1)	95%	HJ4	Uncultured fungus (AM260821.1)	99%
HC64	*Chloridium* sp. (HQ630978.1)	88%	HJ5	Uncultured fungus (GU366722.1)	99%
HC65	Uncultured soil fungus (JQ666320.1)	99%	HJ6	Uncultured fungus(GU366722.1)	99%
HC66	Uncultured soil fungus (JQ666320.1)	99%	HJ7	Uncultured fungus(GU366717.1)	99%
HC67	Uncultured endophytic fungus (FJ524301.1)	99%	HJ8	Uncultured fungus (GU366722.1)	99%
HC68	Uncultured fungus (GU174309.1)	95%	HJ9	Uncultured fungus (GU366722.1)	99%
HC69	Uncultured fungus (EF090491.1)	92%	HJ10	Uncultured fungus (GU366717.1)	99%
HC70	*Sebacina vermifera* (EU625992.1)	88%	HJ11	*Cladosporium tenuissimum* (AJ300331.1)	99%
HC71	Uncultured soil fungus (HM037670.1)	99%	HJ12	Uncultured Hyaloscyphaceae (FJ440904.1)	93%
HC72	*Sebacina vermifera* (EU625992.1)	88%	HJ13	Uncultured fungus (GU366722.1)	99%
HC73	*Sebacina vermifera* (EU625992.1)	88%	HJ14	Uncultured fungus GU366722.1	99%
HC74	Uncultured *Rhizoctonia* sp.(DQ061931.1)	95%	HJ15	Uncultured fungus (GU366717.1)	99%
HC75	*Sebacina vermifera* (EU625992.1)	88%	HJ16	Uncultured fungus (JF300835.1)	84%
HC76	*Sebacina vermifera* (EU625992.1)	91%	HJ17	*Sebacina vermifera* (EU625994.1)	100%
HC77	*Sebacina vermifera* (EU625992.1)	81%	HJ18	Uncultured fungus (JF300835.1)	84%
HC78	Helotiaceae (EU668920.1)	97%	HJ19	Uncultured fungus (JF300835.1)	85%
HC79	Uncultured Helotiales (FN565262.1)	95%	HJ20	*Sebacina vermifera* (EU625994.1)	100%
HC80	*Sebacina vermifera* (EU625992.1)	88%	HJ21	Uncultured fungus (JF300835.1)	84%
HC81	*Sebacina vermifera* (EU625992.1)	88%	HJ22	Uncultured Sebacinales (EU625994.1)	100%

Clones	Close relative from GenBank (accession nº)	Identity (%)	Clones	Close relative from GenBank (accession nº)	Identity (%)
HC82	Uncultured soil fungus (HM037670.1)	99%	HJ23	Uncultured Sebacinales (HM451825.1)	87%
HC83	*Sebacina vermifera* (EU625992.1)	88%	HJ24	*Sebacina vermifera* (EU625994.1)	100%
HC84	*Sebacina vermifera* (EU625992.1)	88%	HJ25	*Sebacina vermifera* (EU625994.1)	100%
HC85	*Sebacina vermifera* (EU625992.1)	88%	HJ26	*Sebacina vermifera* (EU625994.1)	100%
HC86	*Sebacina vermifera* (EU625992.1)	88%	HJ27	*Sebacina vermifera* (EU625994.1)	100%
HC87	*Sebacina vermifera* (EU625992.1)	88%	HJ28	*Sebacina vermifera* (EU625994.1)	100%
HC88	Uncultured soil fungus (HM037670.1)	99%	HJ29	*Sebacina vermifera* (EU625994.1)	100%
HC89	Uncultured soil fungus (HM037670.1)	99%	HJ30	*Sebacina vermifera* (EU625994.1)	100%
HC90	Uncultured soil fungus (FN397212.1)	72%	HJ31	*Sebacina vermifera* (EU625994.1)	100%
HC91	Uncultured soil fungus (HM037670.1)	99%	HJ32	*Sebacina vermifera* (EU625994.1)	100%
HC92	Uncultured Helotiales (FN565262.1)	95%	HJ33	*Sebacina vermifera* (EU625994.1)	100%
HC93	*Sebacina vermifera* (EU625992.1)	88%	HJ34	*Sebacina vermifera* (EU625994.1)	100%
HC94	*Cladosporium* sp. (HQ631003.1)	100%	HJ35	*Sebacina vermifera* (EU625994.1)	100%
HC95	*Chaetosphaeria* sp. (HQ157859.1)	96%	HJ36	*Sebacina vermifera* (EU625994.1)	100%
HC96	Ascomycota sp. (HM208732.1)	81%	HJ37	Uncultured Sebacinales (HM451811.1)	97%
HC97	*Cladosporium* sp (HQ631003.1)	97%	HJ38	Uncultured fungus (JF300835.1)	85%
HC98	*Chaetosphaeria* sp. (HQ630994.1)	97%	HJ39	Uncultured fungus (JF300835.1)	85%
HC99	*Chaetosphaeria* sp. (HQ630994.1)	97%	HJ40	*Sebacina vermifera* (EU625994.1)	100%
HC100	*Chaetosphaeria* sp (HQ630994.1)	97%	HJ41	*Sebacina vermifera* (EU625994.1)	100%
HC101	*Ochrocladosporium elatum* (EU040233.1)	89%	HJ42	*Sebacina vermifera* (EU625994.1)	100%
HC102	*Chaetosphaeria* sp. (HQ630994.1)	97%	HJ43	*Sebacina vermifera* (EU625994.1)	100%
HC103	Uncultured Ascomycete (EU490102.1)	95%	HJ44	*Sebacina vermifera* (EU625994.1)	100%
HJ45	*Sebacina vermifera* (EU625994.1)	100%	HJ74	Uncultured Sebacinales (EU625988.1)	88%
HJ46	Uncultured fungus (HM030589.1)	100%	HJ75	Uncultured Sebacinales (EU625988.1	88%

Clones	Close relative from GenBank (accession nº)	Identity (%)	Clones	Close relative from GenBank (accession nº)	Identity (%)
HJ47	*Sebacina vermifera* (EU625994.1)	100%	HJ76	Uncultured Sebacinales (EU625988.1)	88%
HJ48	*Sebacina vermifera* (EU625994.1)	100%	HJ77	Uncultured Sebacinales (EU625988.1)	88%
HJ49	*Sebacina vermifera* (EU625994.1)	100%	HJ78	Unculturedss Sebacinales (EU625988.1)	88%
HJ50	*Sebacina vermifera* (EU625994.1)	100%	HJ79	Uncultured Sebacinales (EU625988.1)	88%
HJ51	*Sebacina vermifera* (EU625994.1)	100%	HJ80	Uncultured Sebacinales(EU625988.1)	88%
HJ52	*Sebacina vermifera* (EU625994.1)	100%	HJ81	Uncultured Capnodiales (GU055983.1)	96%
HJ53	*Sebacina vermifera* (EU625994.1)	100%	HJ82	Uncultured Capnodiales (GU055983.1)	99%
HJ54	Uncultured *sebacina* (EU909222.1)	86%	HJ83	*Readeriella guyanensis* (EU707900.1)	89%
HJ55	Uncultured fungus (JF300835.1)	86%	HJ84	Uncultured *Sebacina* (EU909227.1)	100%
HJ56	*Sebacina vermifera* (EU625994.1)	100%	HJ85	Uncultured fungus (FN397328.1)	90%
HJ57	*Sebacina vermifera* (EU625994.1)	100%	HJ86	Uncultured fungus (DQ421060.1)	89%
HJ58	Uncultured fungus (JF300835.1)	85%	HJ87	Uncultured Capnodiales (GU055983.1)	99%
HJ59	Uncultured fungus (JF300835.1)	87%	HJ88	UnculturedCapnodiales (GU055983.1)	99%
HJ60	Uncultured fungus (JF300835.1)	87%	HJ89	Uncultured fungus (HQ260284.1)	83%
HJ61	Uncultured fungus (JF300835.1)	87%	HJ90	Uncultured Capnodiales (GU055983.1)	99%
HJ62	Uncultured fungus (JF300835.1)	87%	HJ91	Uncultured Capnodiales(GU055983.1)	99%
HJ63	Uncultured fungus (JF300835.1)	87%	HJ92	Uncultured fungus (EF521206.1)	97%
HJ64	Uncultured fungus (JF300835.1)	87%	HJ93	Uncultured CapnodialesGU055983.1	100%
HJ65	*Sebacina vermifera* (EU625994.1)	100%	HJ94	*Readeriella guyanensis* (EU707900.1)	98%
HJ66	Uncultured fungus (JF300835.1)	87%	HJ95	Uncultured fungus (DQ421060.1)	89%
HJ67	Uncultured fungus (JF300835.1)	87%	HJ96	Uncultured Capnodiales (GU055983.1)	99%
HJ68	Uncultured fungus (JF300835.1)	87%	HJ97	Uncultured Capnodiales (GU055983.1)	99%
HJ69	*Sebacina vermifera* (EU625994.1)	100%	HJ98	Uncultured Capnodiales (GU055983.1)	98%
HJ70	Uncultured fungus (JF300835.1)	87%	HJ99	Uncultured Capnodiales (GU055983.1)	99%
HJ71	Uncultured fungus (JF300835.1)	87%	HJ100	Uncultured Capnodiales (GU055983.1)	99%

Clones	Close relative from GenBank (accession nº)	Identity (%)	Clones	Close relative from GenBank (accession nº)	Identity (%)
HJ72	*Sebacina vermifera* (EU625994.1)	100%	HJ101	Uncultured Capnodiales (GU055983.1)	100%
HJ73	Uncultured Sebacinales (EU625988.1)	88%	HJ102	Uncultured fungus (DQ421060.1)	90%

(a) The database sequences that exhibited the highest identity, query and score and the lowest e-values relative to the sequences obtained in the present study allowed us to determine that the fungi belong to different taxonomic levels. HCB1 - clones obtained from HCBg; HCB2 - clones from HCBc; HC - clones from HCC; HJ - clones from HJ. HCBg - *Hoffmannseggella cinnabarina* from the Serra do Brigadeiro State Park (granitic inselberg); HCBc - *Hoffmannseggella cinnabarina* from a Ferriferous Formation (*canga*)*;* HCC - *Hoffmannseggella caulescens* from a Ferriferous Formation (*canga*)*;* HJ - *Hadrolaelia jongheana* from the State Park of Serra do Brigadeiro (Semideciduous Seasonal Forest).

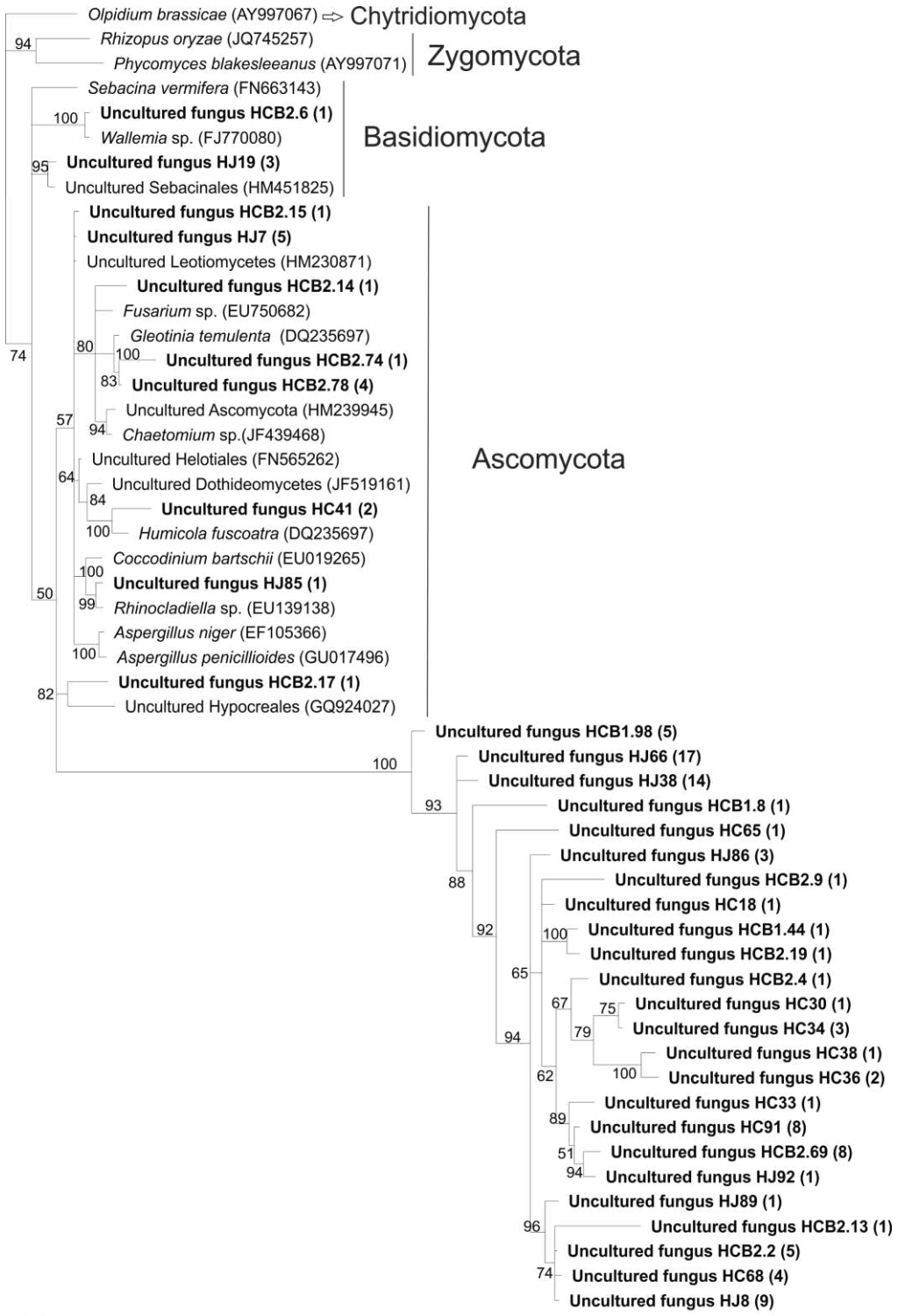

Figure S1 Phylogenetic relationships of uncultured fungi ITS sequences from orchid species roots. The numbers on the branches indicate the *a posteriori* probability obtained by Bayesian likelihood (only values exceeding 50% are shown). The tree was rooted with *Olpidium brassicae*. HCB1 - clones obtained from the HCBg library; HCB2 - clones from HCBc; HC - clones from HCC; HJ - clones from HJ. The numbers in parentheses after the clone names represent the sequence number for this specific clone library. GenBank accession numbers are listed after species names.