FABIANO BRANCO ROCHA

CONTRIBUIÇÕES PARA O CONHECIMENTO DOS FUNGOS FITOPATOGÊNICOS ASSOCIADOS À ESPÉCIE AMEAÇADA *Euterpe edulis* **E PARA O ESCLARECIMENTO DO COMPLEXO** *Trichoderma harzianum*

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

VIÇOSA
MINAS GERAIS - BRASIL
2011

FABIANO BRANCO ROCHA

CONTRIBUIÇÕES PARA O CONHECIMENTO DOS FUNGOS FITOPATOGÊNICOS ASSOCIADOS À ESPÉCIE AMEAÇADA *Euterpe edulis* E PARA O ESCLARECIMENTO DO COMPLEXO *Trichoderma harzianum*

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

APROVADA: 31 de março de 2011.

_____
Prof. João Augusto Alves Meira Neto
(Co-Orientador)

_____
Prof. Maurício Dutra Costa

_____
Prof. Harold Charles Evans

_____
Prof. Gleiber Quintão Furtado

_____
Prof. Olinto Liparini Pereira
(Presidente da banca)

Aos meus pais, dedico...

AGRADECIMENTOS

BIOGRAFIA

FABIANO BRANCO ROCHA, filho de Floriano Rocha Neto e Eunice Branco Rocha, nasceu na cidade de Aracaju, Sergipe, no dia 23 de agosto de 1980, onde concluiu o ensino básico em Dezembro de 1998.

Em 2000, iniciou o curso de graduação em Agronomia na Universidade Federal de Viçosa, graduando-se em janeiro de 2005.

Ainda em 2005, iniciou o programa de Mestrado em Fitopatologia na Universidade Federal de Viçosa, concentrando seus estudos na área de Micologia (Taxonomia de fungos fitopatogênicos).

Em 2007 concluiu o mestrado e iniciou o doutorado também no programa de Fitopatologia na Universidade Federal de Viçosa.

ÍNDICE

RESUMO

ROCHA, Fabiano Branco, D.Sc., Universidade Federal de Viçosa, março de 2011. **Contribuições para o conhecimento dos fungos fitopatogênicos associados à espécie ameaçada *Euterpe edulis* e para o esclarecimento do complexo *Trichoderma harzianum*.** Orientador: Robert Weingart Barreto. Co-orientadores: Olinto Liparini Pereira e João Augusto Alves Meira Neto.

Três estudos cobrindo áreas distintas da micologia foram desenvolvidos: 1) Um estudo visando explorar a diversidade morfológica de oitenta e um isolados reconhecidos como pertencentes à espécie *Trichoderma harzianum sensu lato*, em busca de descontinuidades em caracteres morfológicos que refletissem os doze diferentes clados reconhecidos pelo estudo filogenético feito previamente e permitissem amparar sua distinção como espécies crípticas compondo o "complexo *T. harzianum*"; 2) Um estudo taxonômico sobre espécies de fungos encontradas como parasitas associados à Arecaceae nativa do Brasil *Euterpe edulis* (palmito-juçara), espécie listada como ameaçada de extinção; 3) Uma tentativa de esclarecimento da gama de hospedeiros de fungos associados às plantas nativas ameaçada de extinção *Cousssapoa floccosa* (Cecropiaceae), descritos em estudo anterior, e *E. edulis*. A elucidação do "complexo *T. harzianum*" é de especial importância para o controle biológico de doenças de plantas, área em que isolados pertencentes a este táxon encontram grande aplicação. Desde sua primeira descrição, *T. harzianum* é considerado um fungo morfologicamente diverso e de ampla distribuição geográfica capaz de se estabelecer em diferentes nichos, por esse motivo esse fungo foi alvo de estudos filogenéticos que indicam a existência de um complexo de espécies. Não foi encontrada base morfológica que justifique a distinção dos doze clados reconhecidos com base em estudos moleculares e concluiu-se que, embora exista amparo parcial para distinção baseado em nichos ecológicos e habitats ocupados e distribuição geográfica para os clados. No presente estado da arte para este táxon, conclui-se como mais recomendável a continuidade do tratamento das populações que compõe o "complexo *T. harzianum*" como uma única espécie até que evidências mais conclusivas sejam obtidas ou clados mais bem definidos sejam reconhecidos permitindo uma distinção mais precisa de espécies crípticas ou de taxa infra-específicos que existam sob este "rótulo". Quatro espécies de fungos

anamórficos foram descritos associados à *Euterpe edulis*: *Bipolaris cynodontis*, *Distocercospora* sp., *Melanographium* sp. e *Passalora eitenii*. A gama de hospedeiro desses fungos, assim como a dos fungos associados à *Cousssapoa floccosa*, foi estudada através de teste de especificidade, busca por hospedeiros alternativos no campo e uso de plantas-sentinela no campo. Nenhum fungo originalmente associado a *C. floccosa* foi encontrado associado a outros hospedeiros da ordem Rosales no campo e entre os fungos encontrados originalmente associados a *E. edulis*, *Melanographium* sp. desmonstrou ser patógeno específico para o gênero *Euterpe* e *Distocercospora* sp. foi apenas capaz de parasitar *E. edulis*.

ABSTRACT

ROCHA, Fabiano Branco, D.Sc., Universidade Federal de Viçosa, March of 2011. **Contributions towards the knowledge of plant pathogenic fungi associated with the endangered species *Euterpe edulis* and the elucidation of the *Trichoderma harzianum* species-complex.** Adviser: Robert Weingart Barreto. Co-advisers: Olinto Liparini Pereira and João Augusto Alves Meira Neto.

Three studies covering different area of mycology were developed: 1) A study aiming at exploring the morphological diversity of *Trichoderma harzianum sensu latu* represented by eighty-one isolates recognized as belonging to this species-complex, seeking for disjunction of morphological characters matching/reflecting the twelve different clades recognized by a previous molecular-phylogeny study in support for the distinction and recognition of these taxa as separate cryptic species within the "*T. harzianum* complex"; 2) A taxonomic study about the species of fungi found as parasites associated with an Arecaceae native from Brazil *Euterpe edulsi* (palmito-juçara), and listed as endangered of extinction; 3) An attempt to clarify the host range of the fungi found associated with two species of Brazilian plants endangered of extinction - *Coussapoa floccosa* (Cecropiaceae) and *E. edulis*. The elucidation of the "*T. harzianum* complex" has special relevance for biological control of plant diseases, since isolates belonging to this taxon are widely investigated and used for that purpose. Since its first description, *T. harzianum* was recognized as a fungal species that has a high degree of morphologically diversity, a broad geographical distribution and the ability to exploit a wide range of niches. More recently molecular phylogenetic studies have further indicated that this species may be a species-complex including several cryptic species. The search for key morphological differentiation that would support the distinction of the twelve clades unraveled by the molecular studies did not yield any consistent character to be used with that purpose. Nevertheless, it appears that there is partial support for clade distinction based on niches and habitats occupied by distinct groups of isolates and based on geographic distribution of clade-members. At the present state of the art for this taxon, it appears more logical to continue to treat the populations that fit into the "*T. harzianumi* complex" under the old name until more substantial evidence and consistent characters or well defined clades are found allowing for a precise

distinction of the cryptic species (or infra-specific taxa) that are included. Four species of anamorphic fungi were described in association with *Euterpe edulis*: *Bipolaris cynodontis*, *Distocercospora* sp., *Melanographium* sp. and *Passalora eitenii*. The host range of these fungi, as well as of the fungi associated with *Coussapoa floccosa*, were studied through inoculations under controlled conditions (host-specificity tests), by seeking for possible alternative hosts in the field and by means of using sentinel-plants. None of the fungi originally associated with *C. floccosa* were found in association with other host of Rosales in the field and among the fungi associated originally with *E. edulis*, *Melanographium* sp. showed to be specific to the genus *Euterpe* whereas *Distocercospora* sp. was only capable to parasite *E. edulis*.

INTRODUÇÃO GERAL

A descrição e o relato de espécies de fungos, assim como o esclarecimento da biologia desses organismos, com ênfase no conhecimento dos nichos que estes ocorrem, servem como base para os estudos que envolvam tais organismos nas mais diferentes áreas da pesquisa científica (Rossmann et al. 1998). A estimativa de 1,5 milhões de espécies de fungos existentes no planeta Terra (Hawksworth 2001) é amplamente citada e é a mais aceita no meio científico, porém muito se discute sobre a real estimativa de diversidade de fungos no planeta (Blackwell 2011). A dificuldade em se estimar a diversidade de fungos se deve, em parte ao fato de que inúmeras espécies de fungos foram descritas como sendo uma única espécie, com base em antigos conceitos de espécie baseados unicamente em aspectos morfológicos, porem podendo representar na verdade um conjunto de espécies de morfologia indistinta, formando os denominado complexos de espécies (Kirk et al. 2008). Além disso, a escassez de estudos micológicos em países detentores de megabiodiversidade como o Brasil (Myers et al. 2000) leva a um possível sub-dimensionamento da real diversidade micológica existente.

Espécies do gênero *Trichoderma* (e.g. *Trichoderma brevicompactum* G.F. Kraus, C.P. Kubicek & W. Gams) já foram relatadas como complexo de espécies anteriormente (Degenkolb et al. 2008). Dentre as espécies de *Trichoderma* consideradas com representando um complexo de espécies que necessitam de esclarecimento destaca-se *Trichoderma harzianum* Rifai (Chaverri et al. 2003; Druzhinina et al. 2010; Jaklitsch 2009). *Trichoderma harzianum* foi descrito pela primeira vez por Rifai (1969), que reconheceu em seu trabalho o fato de este fungo apresentar uma grande variação em suas características morfológicas. Além disso, a ampla distribuição geográfica em diferentes nichos ecológicos juntamente com análises filogenéticas (Druzhinina et al. 2010), sugerem a presença de um conjunto de espécies crípticas sob o "rótulo" *Trichoderma harzianum*.

Fungos fitopatogênicos são comumente lembrados como sendo organismos exclusivamente maléficos aos interesses humanos, mas exercem o papel fundamental como bioreguladores (Dighton 2003), contribuindo para a co-existência de espécies de plantas em ecossistemas naturais sem que ocorra a predominância de uma ou

poucas em detrimento de outras. Embora esses organismos possam ser vistos, em uma análise superficial, como uma ameaça para espécies vegetais ameaçadas de extinção, tais fungos podem estar também ameaçados devido a um evento denominado coextinção (Dunn et al. 2009) caso estes estejam associados especificamente com espécies hospedeiras em vias de extinção. Relatos de coextinção de simbiontes associados a animais (Koh et al 2004) corroboram com a hipótese de que fungos parasitas específicos e dependentes de plantas especificas ficam em risco de extinção caso seus hospedeiros se tornem raros ou passem a condição de iminente extinção. Mundialmente, os esforços em reconhecer quais espécies de fungos estão realmente ameaçados de extinção estão basicamente restritos a macrofungos e liquens (Balmford et al, 2000; Dahlberg et. al, 2010). Poucos trabalhos foram dedicados à documentação de espécies de microfungos associados a espécies vegetais raras (Dulymamode et al. 2001, Rocha et. al 2010, Siboe et. al, 2000). Há uma clara necessidade de se levantar e documentar espécies de fungos em risco de extinção e melhor caracterizar deste status.

O presente trabalho teve como objetivo descrever a morfologia de doze clados filogenéticos pertencentes ao complexo *Trichoderma harzianum*. Além disso, descrever e relatar quatro espécies de fungos anamórficos associadas à espécie vegetal *Euterpe edulis* Mart., que é incluída na lista oficial de espécies da flora brasileira ameaçada de extinção do Ministério do Meio Ambiente (2008). Esse trabalho ainda visou determinar a gama de hospedeiros de fungos previamente relatados associados a *Coussapoa floccosa* Akkermans & C.C. Berg (Rocha et al 2010), espécie da Mata Atlântica listada como ameaçada de extinção na IUCN (2006) e assim contribuir para testar, e talvez demonstrar, experimentalmente a condição de cada espécie como estando ameaçada, justificando sua inclusão em programas de conservação

REFERÊNCIA BIBLIOGRÁFICA

Balmford A, Lyon AJE, Lang RM, 2000. Testing the higher-taxon approach to conservation planning in a megadiverse group: the macrofungi. *Biological Conservation*. 93, 209-217.

Blackwell M, 2011. The Fungi: 1, 2, 3 … 5.1 million species? *American Journal of Botany* 98, 426-438.

Chaverri P, Samuels GJ, 2003. *Hypocrea/Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): species with green ascospores. *Studies in mycology*. 48, 1-116.

Dahlberg A, Genney DR, Heilmann-Clausen J, 2010. Developing a comprehensive strategy for fungal conservation in Europe: current status and future needs. *Fungal Ecology*. 3, 50-64.

Degenkolb T, Dieckmann R, Nielsen KF, Gräfenhan T, Theis C, Zafari D, Chaverri P, Ismaiel A, Brückner H, von Döhren H, Thrane U, Petrini O, Samuels GJ, 2008. The *Trichoderma brevicompactum* clade: a separate lineage with new species, new peptaibioticsm, and mycotoxins. *Mycological Progress* 7, 177-219.

Dighton J, 2003. Fungi in ecosystem processes. Vol. 17. CRC Press, Mycology series. USA.

Druzhinina IS, Kubicek CP, Komon-Zelazowska M, Mulaw TB, Bissett J, 2010. The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. BMC *Evolitionary Biology*. 10, 94.

Dulymamode R, Cannon PF, Peerally A, 2001. Fungi on endemic plants of Mauritius. *Mycological Research*. 105, 1472-1479.

Dunn RR, Harris NC, Colwell RK, Koh LP, Sodhi NS, 2009. The sixth mass coextinction: are most endangered species parasites and mutualists? *Proceedings of the Royal Society B*. 276, 3037-3045.

Hawksworth DL, 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycological Research* 105, 1422-1432.

IUCN, 2006. 2006 IUCN Red List of Threatened Species. www.iucnredlist.org. Acesso em 22 de Setembro de 2010.

Jaklitsch WM, 2009. European species of *Hypocrea* Part I. The green-spored species. *Studies in Mycology*. 63, 1–91.

Kirk PM, Cannon PF, Minter DW, Stalpers JA. 2008. Dictionary of the Fungi. Oxon. UK. CAB International. 771p.

Koh LP, Dunn RR, Sodhi NS, Colwell RK, Proctor HC, Smith VS, 2004. Species coextinctions and the biodiversity crisis. *Science*. 305, 1632-1634.

Ministério do Meio Ambiente, 2008. Lista oficial das species da flora brasileira ameaçadas de extinção. www.mma.gov.br. (accessed October 2010).

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J, 2000. Biodiversity hotspots for conservation priorities. *Nature* 403, 853-858.

Rifai MA, 1969. A revision of the genus *Trichoderma. Mycologica Papers.* 116, 1-56.

Rocha FB, Barreto RW, Bezerra JL, Meira Neto JAA, 2010. Foliage mycobiota of *Coussapoa floccosa*, a highly threatened tree of the Brazilian Atlantic Forest. *Mycologia*. 102, 1240-1252.

Rossmann AY, Tulloss RE, O'Dell TE, Thorn RG. Protocols for an all taxa biodiversity inventory of fungi in a Costa Rican conservation area. Parkway Publishers, Inc. Boone, North Carolina. 195pp.

Siboe GM, Kirk PM, David JC, Cannon PF, 2000. Necrotophic fungi from Kenyan endemic and rare plants. *Sydowia*. 52, 286-304.

1. CHAPTER ONE

***Trichoderma harzianum*: a multi-species complex or a morphologically diverse species?**

Fabiano B. Rocha, Priscila Chaverri, Gary J. Samuels, Robert W. Barreto

Abstract

Fungal isolates having a morphology fitting into the traditional concept accepted for *Trichoderma harzianum* were studied. Twelve clades were identified by phylogenetic analyses using gene regions of actin, calmodulin, elongation factor and ITS. Morphological analyses were performed based on clades generated by the coalescent phylogenetic tree and considering general culture aspect and biometrics features of reproductive structures. Among those clades, only two represented single ascospore isolates. All ten other clades represented monoconidial cultures. Some clades represented a continentally restricted distribution whereas others were restricted to some particular climates and others appeared to be host/substrate specialized. Results of this study clearly indicated that morphology alone does not provide sufficient information to allow discrimination between the various clades. It is questionable if the clades recognized within the so-called *T. harzianum* complex deserve recognition at the species level, as originally expected. Until better evidence is generated, perhaps through the investigation of additional genome regions, it appears more appropriate to continue using *T. harzianum sensu lato* for the range of forms having a morphology equivalent to *T. harzianum* instead of erecting formal taxa for each clade.

1. Introduction

Trichoderma harzianum Rifai is well known among biocontrol researchers as a fungus that is widely used as a biological control agent against numerous crop pathogens. Several authors regard *T. harzianum* as a species complex containing numerous cryptic species (Chaverri et al. 2003; Druzhinina et al. 2010; Jaklitsch 2009). A cryptic species complex is a group of closely related species that have indistinguishable morphological features making their distinction impossible (through traditional morphological methods) (Kirk et al. 2008). The search for

precision in taxonomy requires the elucidation of such species complexes and the naming of the cryptic species as, although sharing a common morphology, these taxa may be reproductively isolated entities and may have very distinct features of pratical relevance such as, production of different metabolites, having distinct growth requirements, having distinct pathogenic status or host-ranges, having different geographical distributions, etc. Misidentification of a cryptic taxon by the use of a collective name may have far-reaching negative consequences for strategic matters in industry, plant quarantine and other fields and their clarification is a challenge that has only recently started to be tackled by mycologists using characters other than morphology (physiological features, mating type studies, proteome…). The development and wider use of molecular tools are presently allowing the elucidation of some important species complexes with the discrimination of cryptic–species hiding behind such "umbrella-taxa". Well known examples are *Fusarium graminearum* Schwabe (O'Donnell et al. 2004) and *Colletotrichum gloeosporioides* (Penz.) Sacc. (Phoulivong et al. 2010). Numerous novel species are being described through the genealogical concordance phylogenetic species recognition (GCPSR, Taylor et al. 2000).

Trichoderma Pers:Fr (anamorphic Ascomycota, Sordariomycetes, Hypocreales, Hypocreaceae) is a diverse fungal genus that presently contains 102 named species (Kirk et al. 2008). Although *Trichoderma* is a morphologically diverse genus, there is a consensus among the mycologists dealing with this genus, that in many cases morphology alone does not mirror the real genetic diversity generated by evolution. Groups of populations likely to be genetically separated from others with a highly similar morphology and deserving taxonomic recognition are often kept in hide under a broad name, rendering *Trichoderma*'s taxonomy controversial and imperfect.

Taxonomic treatment of *Trichoderma* was plagued with misidentification problems since it was first described by Persoon (1794). He proposed the genus and included four species in *Trichoderma*, but only the type species, *Trichoderma viride* Pers, really belonged to this genus (Samuels 2006). Nowadays the key characters for *Trichoderma* are well defined, and it is relatively easy to recognize a fungal species as belonging to *Trichoderma*. The situation within the genus is nevertheless different. Morphological characters used for species identification sometimes overlap making

distinction between similar species difficult or even impossible by classical methods. In these cases molecular tools are a mandatory solution for species delimitation and identification. A recent example is provided by Degenkolb et al. (2008) who described four new species discriminated from a species complex formerly treated as *Trichoderma brevicompactum* G.F. Kraus, C.P. Kubicek & W. Gams. In this publication the authors distinguished the species using phylogenetic analyses and protein patterns additionally to morphological analyses and hence were capable of determining the species limit between the components of the *T. brevicompactum* species complex.

One of the most important *Trichoderma* species in terms of practical applications in biological control of plant pathogens, is *Trichoderma harzianum* (Vinale et al. 2008; Wells et al. 1972). This species is a typical inhabitant of soil and organic matter, but can also be found as an endophyte (Bailey et al. 2008, Bailey et al. 2006, Evans et al 2003, Samuels 2006). *T. harzianum* is also considered a cosmopolitan species that is adapted to both temperate and tropical regions. *Trichoderma harzianum* was described in the first taxonomic review of *Trichoderma* (Rifai 1969), where nine 'aggregate' species of *Trichoderma* were recognized. Only recently *Hypocrea lixii* Pat. was recognized as the known teleomorph of *T. harzianum sensu latu* (Chaverri et al. 2003). Rifai (1969) recognized in his work, that *T. harzianum* has a considerable morphological variation, particularly in the macroscopic appearance of colonies, suggesting that *T. harzianum* is in fact a species-complex, this hypothesis has been further reinforced in phylogenetic analysis published recently (Chaverri et al. 2003; Druzhinina et al. 2010). Although such studies acknowledged that *Trichoderma harzianum* is, in fact, a species-complex, this still remains a pending matter. The scope of the present work is describing the morphology characters of the clades recognized by phylogenetic analyses previously performed involving numerous isolates belonging to this group.

2. Material and methods

The cultures were obtained from the following culture collections: Systematic Mycology and Microbiology Laboratory of United States Departament of Agriculture (USDA), Centraalbureau voor Schimmelcultures (CBS) and International Mycological Institute (IMI) and were originally obtained from collected

from various countries in the Americas, Africa and Europe and isolated from soil, bark, decorticated wood, and as endophytes from different host-plants (Table 1). All isolates included in the study were preliminarily identified as belonging to the *Trichoderma harzianum* species–complex based on Rifai's description (Rifai 1969). Special considerations were given to geographic origin of isolates and ecology (habitats/niches) – particularly on whether they were isolated as endophytes (in which case host-identity was given special importance) or from soil/decomposing plants.

2.1. Morphological analysis

All strains were grown on cornmeal-dextrose agar (CMD, cornmeal agar Difco + 2% dextrose) for three days. Culture plugs (0.5 cm diam) were collected from the edge of the colonies and transferred to around 1.0 cm from the edge of 9 cm diam individual plates containing each of two media: PDA, potato dextrose agar Difco and synthetic low nutrient agar (SNA: Nirenberg 1976). The cultures were incubated at five different temperatures (15, 20, 25, 30 and 35 °C) in the dark and at 25°C or under a 12h light regime (light provided from 10 to 30 cm above plates). The colonies radii were taken 24, 48, 72 and 96 h after incubation started based on the examination of the cultures incubated in the dark. The experiment was performed three times for each strain. The period taken since incubation onset to the moment of appearance of the typical green sporulation as well as from incubation onset to the appearance of the yellow pigment in the medium were recorded for all cultures. Additionally, strains were also evaluated for the production of characteristic scent that resembles coconut (after 96 h for cultures growing on PDA and incubated on dark at 30 °C). All cultures were described after 96h. Colony texture, development and number of the sporulation rings, radiate aspect of the colony on PDA, sporulation aggregated in small mass of conidia denominated pustules (e.g. Figure 3c) on SNA and yellow pigment tone (pale or dark reddish) were recorded.

The strains were grown on SNA at 25 °C and slides were prepared from fungal structures produced in white to pale green areas of colonies. These were mounted in 3% KOH. Characteres considered relevant to identify *T. harzianum* s.l. were observed and recorded such as length and width of phialides at the widest point, length/width ratio of phialides, width of phialides at the base, width of the cell

subjacent to the phialides, phialide shape, conidial length and width , length/width ratio for conidia, conidial shape, conidial colour, conidial wall surface (warted or smooth), presence of sterile or fertile hairs. The direction of adjacent phialides (phialides adjacent to the tip of conidiophores) was observed. The sexual state, when present, was also examined. Free hand sections of fruit bodies were prepared and the structures were described after examination under the light microscope. Presence of chlamydospores was verified by observing the reverse of the plates under the light microscope (objective 20x). Presence of hairs was verified in colonies of different ages under a stereomicroscope and bright field microscopy as well as how the strains sporulate, if spread on media surface or aggregated in small pustules. Images were captured and biometric data was recorded with Scion Image for Windows[TM] (www.scioncorp.com). Basic descriptive statistics (minimum, maximum, mean, standard deviation and 95% confidence interval) were determined for morphological characters (Table 2 and 3) using Systat 10 (Systat Software, San José, California).

2.2. Phylogenetic analysis.

The phylogenetic analyses were performed by Dr. Priscila Chaverri's mycology lab at the University of Maryland. Each isolate was grown in plates containing potato dextrose broth Difco[TM] and incubated in 25 °C during four days for the production of the mycelia mass for DNA extraction. DNA extraction was performed with a cell/tissue lysis kit (ArchivePure DNA, 5 Prime[TM]). One hundred and forty-one cultures recognized as belonging to *Trichoderma harzianum* complex were used in phylogenetic analysis and two cultures of *Trichoderma aggressivum* Samuels & W. Gams were used as the outgroup.

Four different genome regions were studied, these regions include partial sequence of genes Actin (*act*), Calmodulin (*cal1*), Internal Transcribed Spacers (ITS) and Translation Elongation Factor 1α (*tef1*). Each reaction of PCR amplification was performed in a solution containing 10 µl of Taq 2x Master Mix (New England Biolab[TM]), 1 µl of 25 mM MgCl$_2$, 0.5 µl of forward primer, 0.5 µl of reverse primer and 7.5 µl of nuclease-free water. The primers for *act* were ACT-512F (50-ATGTGCAAGGCCGGTTTCGC-30) and AC T-783R (50-TACGAGTCCTTCTGGCCCAT-30) (Carbone and Kohn, 1999), for *cal* were CAL-228F (50-GAGTTCAAGGAGGCCTTCTCCC-30) and CAL-737R (50-

CATCTTTCTGGCCATCATGG-30), for ITS were ITS 1 (50-TCCGTAGGTGAACCTGCGG-30) and ITS 4 (50-TCCTCCGCTTATTGATATGC-30) (White et al., 1990), and for *tef1* were EF1-728F (50-CATCGAGAAGTTCGAGAAGG-30) and EF1-986R (50-TACTTGAAGGAACCCTTACC-30). The reactions were placed in a PTC-200 MJ Research termocycler (Waltham, Mass). The PCR final product were purified with ExoSAP-it kit (USB[TM]) using manufacture's protocol and sequenced using BigDye Terminator v3.1 in a DNA sequencer (ABI 3100; Applied Biosystems[TM]). Sequences were edited and assembled using Sequencher 4.1 (Gene Codes[TM]). Clustal X 1.81 (Thompson et al., 1997) was used to align the sequences and McClade version 3.06 software to manually adjust the alignment. To determine the relationship between species in *Trichoderma harzianum* species complex in a phylogenetic tree, the sequence alignment of four genes was analysed with maximum likelihood (ML) (Swofford, 2002).

3. Results

The decision about clade limits was based on the coalescence tree represented in Figure 1 where each clade is indicated. This tree was the result of a detailed analyses performed by P. Chaverri (unpub. results) and is being presented here as a complement to the present work as an attempt to match the observation of characters of the isolates in culture with the groupings yielded in the molecular study. Eighty-one isolates were included in this study, which were combined in ten treatments with three repetitions generating a total of 2430 plates which were individually examined for each character under evaluation.

Even for those morphological characters values that appeared to be most informative for separating the different species in *T. harzianum* complex, a significant overlap among different "potential cryptic species" appeared compromising their diagnostic value (Table 2 and 3). Some morphological characters were not consistent even for strains belonging to the same clade. Characters like conidia size, shape and colour were ignored because they were regarded as useless for the purpose of clade distinction. Some isolates did not fit into any clade, and seemed to belong to some other group that was poorly represented in the list of isolates included in this study. These were hence regarded as not identified at this

stage of the work for the evaluation of a possible proposition of new taxa as these would be based on just single strains which would be unwise. The morphological characters of each clade are described below:

Clade 1   (Figure 2)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Fast-growing, $5.5 - 6.0$ cm diam at 25 °C, and $4.0 - 4.5$ cm diam at 35 °C on PDA, $5.0 - 6.0$ cm diam at 25 °C and $3.0 - 4.0$ cm diam at 35 °C on SNA, after 72h. Under twelve hours photoperiod and after 96h at 25 °C aerial mycelium cottonous, sometimes sparse, radiate, sporulating in a concentric halus on PDA; translucent mycelium with scarce aerial hypha, sporulation scarce forming few pustules in the center on SNA. In the dark, sporulation appearing between 48h and 72h on PDA at 25 °C forming one halus surrounding a central sporulating area, a spreading sporulation appearing after 48h on PDA at 35 °C; sporulating between 72h and 96h at 25 °C and after 96h at 35 °C on SNA, two hali at 35 °C after 96h, sporulation appearing after 72h on SNA, forming one halus at 25 °C or few pustules at 35 °C after 96h. *Hidrosoluble pigment* produced after 48h at 35 °C in the dark, brown on PDA; absent on SNA. Sweetish smell sometimes produced on PDA at 30 °C. *Conidiophores* pyramidal, with opposite branches. *Phialides* lageniform, obpyriform or obclavate, and sometimes conical mainly terminal on conidiophores, up to 4 at each conidiophores tip, $4.0 - 8.5 \times 2.0 - 4.0$ μm, (mean 5.9×3.1 μm), base $1.0 - 3.0$ μm, (mean 1.9 μm), supporting cells $2.0 - 4.0$ μm wide (mean 2.8 μm), ratio phialide length/width $1.0 - 3.0$ μm, (mean 1.9 μm), ratio phialide length/width of supporting cell $1.5 - 3.0$ μm, (mean 2.1 μm), ratio phialide width/width of supporting cell $1.0 - 1.5$ μm, (mean 1.1 μm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* globose to subglobose, $2.0 - 3.5 \times 2.0 - 3.5$ μm, ratio length/width $1.0 - 1.5$ μm, hyaline when young becoming green to dark green, rarely yellowish green with age, smooth.

Specimens examined: Culture Dis 377a endophytic in stems of *Cola verticillata* (Thonn.) Stapf ex A. Chev., Korup National Park, Cameroon, H.C. Evans, oct-31-2003; culture Dis 314f endophytic in stems of *Cola altissima*, Korup National Park, Cameroon, H.C. Evans & K.A.Holmes, jul-06-2001; culture GJS 06-

98 from soil, Cameroon, P. Janje, 2005; Culture GJS 99-227 (other number IMI 393967), from soil, Central Province, Cameroon, collector unknown, 1999.

Clade 2   (Figure 3)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Growing quickly, 5.0 – 6.5 cm diam at 25 °C, and 3.5 – 5.0 cm diam at 35 °C on PDA, 4.0 – 6.5 cm diam at 25 °C and 2.0 – 4.5 cm diam at 35 °C on SNA, after 72h. Under twelve hours photoperiod and after 96h at 25 °C aerial mycelium cottonous, irradiating, mostly sporulating in the center and in two concentric hali on PDA, rarely no sporulation or sporulating only centrally; translucent mycelium usually with sparse aerial hypha, sporulation rarely spread and mostly forming pustules spread on one or two hali on SNA. In the dark, sporulation appearing between 48h and 72h forming two hali besides a central halus at 25 °C, and appearing after 48h forming mostly two hali besides a center sporulation or just two hali at 35 °C on PDA; sporulation appearing between 72h and 96h, sometimes no sporulation at 25 °C and 35 °C, when sporulating sometimes forming two hali at 25 °C, but mostly forming evenly spread sporulation after 96h at 25 °C and 35 °C on SNA. Hidrosoluble pigment produced mosty after 48h at 35 °C on the dark, pale yellow on PDA; absent on SNA. Sweetish smell mostly absent on PDA at 30 °C. Conidiophores pyramidal, opposite branches. Phialides lageniform, obpyriform or obclavate, up to 5 at the the conidiophores tips, $3.5 – 9.5 \times 2.0 – 4.5$ µm, (mean 5.4×3.3 µm) , base 1.0 – 3.5 µm wide, (mean 2.0 µm), supporting cell 2.0 – 5.0 µm wide (mean 3.1 µm), ratio phialide length/width 1.0 – 3.0 µm, (mean 1.6 µm), ratio phialide length/width of supporting cell 1.0 – 3.5 µm, (mean 1.8 µm) , ratio phialide width/width of supporting cell 0.5 – 1.5 µm, (mean 1.1 µm). Adjacent phialides inclined and sometimes curved towards the apex. Conidia globose, subglobose to ovoid, $2.0 – 3.5 \times 2.0 – 3.5$ µm, ratio length/width 1.0 – 1.5 µm, hyaline when young becoming green to dark green with age, smooth.

Specimens examined: Cultures Dis 217a, Dis 217h, Dis 217o, Dis 218f, Dis 220j, Dis 220k, Dis 221d isolated as an endophyte from stem of *Theobroma gileri* Cuatrec., Esmeraldas Province, Ecuador, H.C. Evans and K.A. Holmes, may-05-2000; culture GJS 11-184 (other number: LA 10) isolated as an endophyte from *Hevea guianensis* Aubl., Los Amigos Biological station, Manu, Madre de Dios, Peru,

R. Gazis, may-2008; culture GJS 11-186 (other number: T 38) isolated as an endophyte from *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg**.**, Picaflor Biological station, Tambopata, Peru, R. Gazis and P. Chaverri, jun-2007.

Clade 3   (Figures 4)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Growing quickly, 5.5 – 6.5 cm diam at 25 °C, and 3.0 – 4.5 cm diam at 35 °C on PDA, 6.0 – 6.5 cm diam at 25 °C and 1.5 – 5.0 cm diam at 35 °C on SNA, after 72h. Under twelve hours photoperiod and after 96h at 25 °C aerial mycelium cottonous, irradiating, sporulating in the center and in two concentric halui, just on halus or with no sporulation on PDA; translucent mycelium with sparse aerial hypha and sparse spread sporulation on SNA. In the dark, sporulation appearing after 48h on PDA, forming one halus or two hali, and sometimes two hali besides a central sporulation at 25 °C and spread or sometimes two hali besides a central sporulation at 35 °C after 96h, sporulation appearing after 72h on SNA at 25 °C and after 48h at 35 °C, and forming evenly spread sporulation at 25 °C and 35 °C after 96hm some strains not sporulating at 35 °C. *Hidrosoluble pigment* produced after 48h or after 96h at 35 °C on the dark, pale yellow on PDA; absent on SNA. Some strains producing sweetish smell on PDA at 30 °C. *Conidiophores* pyramidal with opposite branches. *Phialides* lageniform, obpyriform or obclavate, and some strains developing conical phialides mainly in the end of the conidiophores, up to 5 at the end of the conidiophores, 4.0 – 9.0 × 2.5 – 4.5 μm (mean 6.0 × 3.5 μm), base 1.5 – 3.0 μm wide (mean 2.0 μm), supporting cell 2.0 – 4.0 μm wide (mean 2.9 μm), ratio phialide length/width 1.0 – 3.0 μm, 1.5 μm mean, ratio phialide length/width of supporting cell 1.5 – 3.5 μm, (mean 2.1 μm), ratio phialide width/width of supporting cell 1.0 – 1.5 μm, (mean 1.2 μm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* globose, subglobose to ovoid, 2.0 – 3.5 × 2.0 – 3.0 μm, ratio length/width 1.0 – 1.5 μm, hyaline when young becoming green to dark green, rarely yellowish green with age, smooth.

Specimens examined: Culture Dis 269c endophytic in *Herrania* sp., Mocache-Vinces, Los Rios Province, Ecuador, H.C. Evans, feb-11-2001; culture Dis 337f endophytic in stem of *Theobroma gileri*, Pichincha Province, Ecuador, H.C. Evans

and R. Reeder, apr-13-2002; culture Dis 355b endophytic in stems of *T. gileri*, Ecuador, collector unknown, date unknown.

Clade 4   (Figure 5)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Growing quickly, 4.3 – 5.9 cm diam at 25 °C, and 3.1 – 3.5 cm diam at 35 °C on PDA, 4.6 – 5.4 cm diam at 25 °C and 2.7 – 3.0 cm diam at 35 °C on SNA, after 72h. Under twelve hours photoperiod and after 96h at 25 °C aerial mycelium cottonous, some strains producing sparse aerial mycelium and forming a milky inner medium mycelium, , some strains sporulating densely in the middle of the colony, others forming two hali besides a small central sporulation, and some strains sterile on PDA; translucent mycelium with some aerial hypha, sporulation on pustules in one or two hali besides a central sporulation and some strains sterile on SNA. In the dark, sporulation appearing after 48h on PDA and forming one halus besides a central sporulation, and some strains sterile at 25 °C and 35 °C after 96h, sporulation appear after 72h on SNA, some strains do not produce spores at 35 °C, forming two hali at 25 °C and when sporulating some strains form one halus and producing even sporulation at 35 °C after 96h. *Hidrosoluble pigment* produced after 48h, pale yellow at 35 °C on PDA in the dark; absent on SNA. Sweetish smell absent on PDA at 30 °C. *Conidiophores* pyramidal, opposite branches. *Phialides* obpyriform or obclavate, some strains developing few conical shape phialides, mainly at the end of the conidiophore, up to 5 at the end of the conidiophore, 3.5 – 12.5 × 2.5 – 5.0 µm, (mean 5.8 × 3.6 µm) base 1.5 – 3.0 µm wide (mean 2.2 µm), supporting cell 2.0 – 4.0 µm wide (mean 3.0 µm), ratio phialide length/width 1.0 – 4.0 µm (mean 1.7 µm), ratio phialide length/width of supporting cell 1.5 – 4.0 µm (mean 2.0 µm), ratio phialide width/width of supporting cell 1.0 – 1.5 µm (mean 1.2 µm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* globose to subglobose, sometimes ovoid, 2.5 – 3.5 × 2.0 – 3.5 µm, ratio length/width 1.0 – 1.5µm, hyaline when young becoming green to dark green with age, smooth.

Specimens examined: Culture Dis 219f endophytic in stems of *Theobroma gileri*, Esmeraldas Province, Ecuador, H.C. Evans & K.A. Holmes, may-05-2000; culture GJS 11-185 (other number: LA 11) endophytic in stems of *Hevea guianensis*, Los Amigos Biological station, Manu, Madre de Dios, Peru, R. Gazis, may-2008;

culture GJS 11-187 (other number: T 51) from stem of *Hevea brasiliensis*, Picaflor Biological station, Tambopata, Peru, R. Gazis and P. Chaverri, june-2007.

Clade 5 - *Trichoderma harzianum* sensu strictu   (Figure 6)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Growing quickly, 5.0 – 6.5 cm diam at 25 °C, and 4.0 – 5.5 cm diam at 35 °C on PDA, 4.0 – 5.5 cm diam at 25 °C and 3.5 – 4.5 cm diam at 35 °C on SNA, after 72h. Under twelve hours photoperiod and after 96h at 25 °C aerial mycelium cottonous, irradiating, sporulating mostly in two hali besides a central sporulation, sometimes sporulation spread in the center of the colony but with one recognizable halus at the edge of the sporulation, and rarely no sporulation on PDA; translucent mycelium with some aerial hypha on the edge, sporulation spread on the colony, sometimes with two recognizable hali, producing pustules mainly on the hali on SNA. In the dark, sporulation appearing between 48h and 72h on PDA forming a central sporulating zone besides one or two hali, rarely with evenly spread sporulation at 25 °C and sporulation appearing after 48h forming three hali at 35 °C. Sporulation appearing after 72h on SNA, and forming two hali at 25 °C and 35 °C after 96h. *Hidrosoluble pigment* produced after 48h at 35 °C on the dark, pale yellow on PDA some strains not producing any pigment; absent on SNA. Sweetish smell mostly absent on PDA at 30 °C, few strains producing such smell. *Conidiophores* pyramidal, opposite branches. *Phialides* lageniform, obpyriform or obclavate, and usually terminal phialides conical, up to 5 at the end of the conidiophores, 4.5 – 16.0 × 2.5 – 4.5 μm (mean 6.9× 3.5 μm), base 1.0 – 3.0 μm wide (mean 2.0 μm), supporting cell 2.0 – 4.0 μm wide (mean 3.0 μm), ratio phialide length/width 1.0 – 5.0 μm (mean 2.0 μm), ratio phialide length/width of supporting cell 1.5 – 5.0 μm, (mean 2.3 μm), ratio phialide width/width of supporting cell 0.5 – 2.0 μm (mean 1.2 μm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* subglobose to ovoid, 2.5 – 4.0 × 2.0 – 4.0 μm, ratio length/width 1.0 – 1.5 μm, hyaline when young becoming green to dark green with age, smooth.

Specimens examined: culture GJS 04-71 endophytic in twig of *Castanea sativa* Mill., Massa-Carrara province, Italy, G. Vannacci, 1998; culture CBS 227.95 from soil, Sheffield Botanical Garden, England, A. Lyon, may-1994; culture CBS 226.95 from soil, Sheffield Botanical Garden, England, J.L. Kinderlerer, jan-1994

(NEOTYPE: CBS 226.95, designated in Gams and Meyer, 1998); culture GJS 05-107 endophytic in stems of *Ricunus communis* L., Italy, G. Vannacci, 2001; culture GJS 99-5 from soil, USA, collector unknown, date unknown; culture IMI 359823 from mushroom compost, Ireland, collector unknown, date unknown.

Clade 6   (Figures 7 and 8)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Fast-growing, 6.0 – 6.5 cm diam at 25 °C and 4.0 – 5.0 cm diam at 35 °C on PDA, 4.5 – 6.0 cm diam at 25 °C and 3.0 – 3.5 cm diam at 35 °C on SNA, after 72h. Under 12-hour light regime after 96h at 25 °C aerial mycelium cottony, irradiating, sporulating in the center and in a concentric halus on PDA; translucent mycelium with very sparse aerial hypha at the edge of colonies, sporulation forming pustules in the center, sometimes with a sparse sporulation past the halus, and rarely without sporulation on SNA. In the dark, sporulation appearing after 48h on PDA and forming one halus plus a central halus at 25 °C and two hali at 35 °C after 96h, sporulation appearing after 72h on SNA, and forming a central halus, sometimes plus an additional halus at 25 °C and three hali, rarely only with central sporulation at 35 °C after 96h. *Hidrosoluble pigment* produced after 48h at 35 °C in the dark, mostly brown but sometimes pale yellow on PDA; absent on SNA. Sweetish smell absent on PDA at 30 °C. *Conidiophores* pyramidal, branches opposite. *Phialides* lageniform, obpyriform or obclavate, and rarely conical, up to 5 at the tip of the conidiophores, 4.0 – 9.0 × 2.5 – 4.0 μm, (mean 6.2 × 3.3 μm), base 1.0 – 3.0 μm, (mean 2.0 μm), supporting cells 2.0 – 4.0 μm wide (mean 2.8 μm) , ratio phialide length/width 1.0 – 3.5 μm, (mean 1.9 μm), ratio phialide length/width of supporting cell 1.5 – 4.0 μm, (mean 2.3 μm), ratio phialide width/width of supporting cell 0.5 – 1.5 μm (mean 1.2 μm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* subglobose to ovoid, 2.5 – 3.5 × 2.0 – 3.5 μm, ratio length/width 1.0 – 1.5 μm, hyaline when young becoming green to dark green, rarely yellowish green with age, smooth. *Stromata* solitary, pulvinate when seen from above, 0.04 – 0.12 mm height, 1.0 – 3.0 mm diam., glabrous, dark brown or black, pseudoparenchymatous, angular cells, cells at surface pigmented (dark brown or black), and internal cells ranging from hyaline to pale brown. *Perithecia* immersed in stroma, forming dense amorphous aggregates, sometimes scattered, distorted when densely packed, globose to subglobose when scattered, 152.5 – 275.0 × 93.0 – 182.0 μm, wall formed of

compact pseudoparenchyma, 5.0 – 25.0 µm thick, ostiole one per perithecium, central, 43.5 – 83.5 µm diam. *Asci* cylindrical, 61.5 – 113.0 × 3.5 – 6.5 µm, ascospores uniseriate. *Ascospores* splitting to form 16 spores per asci, dimorphic, distal spores subglobose, oval or suboval with truncate base and rounded apex, 3.0 – 5.5 × 3.0 – 5.0 µm, brown in KOH, warted, proximal spores subcylindrical sometimes with rounded base and truncate apex, 3.5 – 6.0 × 3.0 – 4.5 µm, brown in KOH, warted.

Specimens examined: Culture GJS 90-127 isolated from teleomorph (BPI 1109390, also examined) on bark, Blue Valley, North Carolina, USA, Y. Doi, A.Y Rossmann and G.J. Samuels, oct-16-1990; culture GJS 94-53 isolated from teleomorph (BPI 749348, also examined) on decorticated wood, Shawnee National Forest, Illinois, USA, G.J. Samuels and W. Sundberg sep-30-1994; culture GJS 90-22 isolated from teleomorph (BPI 802600, also examined) on decorticated wood, Aldo Leopold Reserve, Wisconsin, USA, G.J. Samuels, jun-23-1990; culture GJS 91-138 isolated from teleomorph (BPI 1112907, also examined) on bark, Prince George County, Maryland, USA, G.J. Samuels, S.E. Rehner, A.Y. Rossmann and F.A Uecker, oct-11-1991.

Clade 7 (Figure 9)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Fast-growing at 25 °C but slower at 35 °C, 4.5 – 6.0 cm diam at 25 °C, and 2.5 – 3.5 cm diam at 35 °C on PDA, 4.5– 5.5 cm diam at 25 °C and 2.0 – 3.0 cm diam at 35 °C on SNA, after 72h. Under 12-hours light regime and after 96h at 25 °C aerial mycelium cottonous, irradiating, sporulating densely in the center with some sparse sporulation around the center on PDA; translucent mycelium with very sparse aerial mycelium spread over the colony, sparse and evenly-spread sporulation sometimes forming pockets in the center or close to the border of plates, sometimes without sporulation on SNA. In the dark, sporulation appearing between 48h and 72h on PDA and forming mostly two hali and sometimes a evenly-spread sporulation at 25 °C and two hali or sometimes evenly-spread sporulation at 35 °C after 96h; sporulation sparce between 72h and 96h at 25°C and 35 °C on SNA, sometimes sterile. Hidrosoluble pale yellow pigment starting to appear between 72h and 96h at 35 °C in the dark on PDA; absent on SNA. Sweetish smell rarely present on PDA at

30 °C. *Conidiophores* pyramidal, opposite branches. *Phialides* lageniform, obpyriform or obclavate, and sometimes conical, mainly terminal on conidiophores, up to 5 at the end of each conidiophores tip, 4.0 – 10.5 × 2.0 – 4.0 μm, (mean 6.3 × 3.3 μm), base 1.0 – 3.0 μm wide (mean 2.0 μm), supporting cell 2.0 – 4.0 μm wide (mean 2.8 μm), ratio phialide length/width 1.5 – 4.0 μm, (mean 2.0 μm), ratio phialide length/width of supporting cell 1.5 – 4.5 μm, (mean 2.3 μm) , ratio phialide width/width of supporting cell 1.0 – 2.0 μm, (mean 1.2 μm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* globose, subglobose to ovoid, 2.0 – 4.0 × 2.0 – 3.5 μm, ratio length/width 1.0 – 1.5 μm, hyaline when young becoming green to dark green, rarely yellowish green with age, smooth.

Specimens examined: Cultures Dis 314d, Dis 314i and Dis 314b endophytic in stem of *Cola altissima* Engl., Korup National Park, Cameroon, H.C. Evans and K.A. Holmes, jul-06-2001; culture Dis 375g endophytic in stem of *Cola lateritia* K. Schum., Korup National Park, Cameroon, H.C. Evans, oct-31-2003; culture isolated Dis 382aii endophytic in stem of *Cola rostrata* K. Schum., Cameroon, H.C. Evans, nov-01-2003; culture Dis 386fi endophytic in stem of *Ancistrocladus korupensis* D.W. Thomas & Gereau, Korup National Park, Cameroon, H.C. Evans, nov-02-2003.

Clade 8 (Figure 10)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Growing quickly, 5.0 – 6.5 cm diam at 25 °C, and 3.5 – 5.0 cm diam at 35 °C on PDA, 5.0 –6.0 cm diam at 25 °C and 3.5 – 5.0 cm diam at 35 °C on SNA, after 72h. Under twelve hours photoperiod and after 96h at 25 °C some isolates presnt aerial mycelium cottonous with an irradiating aspect, and some isolates do not present a radiate aspect or even without aerial mycelium but presenting a radiate aspect. When sporulating forming two hali and sometimes with a central sporulating zone on PDA; translucent mycelium with sparce aerial mycelium, sporulation in pustules spread on 2/3 of the colony, usually one visible halus, sometimes aerial mycelium more dense without sporulation on SNA. In the dark, sporulation appearing between 48h and 72h on PDA and forming a diverse range of sporulation layouts, some strains with evenly-spread sporulation, others with two or three hali and sometimes with two hali besides a central sporulating area at 25 °C and mostly

spread and some strains sterile at 35 °C after 96h, sporulation appearing between 72h and 96h on SNA at 25 °C, forming one or two hali, sporulation appearing bwtween 48h and 72h at 35 °C, forming one halus after 96h, some strains sterile at 35 °C. *Hidrosoluble pigment* produced between 48h and 72h at 35 °C on the dark, pale yellow and some strains producing a redish yellow pigment on PDA; absent on SNA. Some strains producing sweetish smell and others strains with no smell on PDA at 30 °C. *Conidiophores* pyramidal, opposite branches *Phialides* lageniform, obpyriform or obclavate, and rarely conical, up to 5 at the end of the conidiophores, $4.5 - 17.5 \times 2.0 - 4.5$ μm, (mean 8.5×3.1 μm), base $1.5 - 3.0$ μm wide, (mean 1.9 μm), supporting cell $2.0 - 4.0$ μm wide (mean 2.6 μm), ratio phialide length/width $1.5 - 7.0$ μm, (mean 2.8 μm), ratio phialide length/width of supporting cell $1.5 - 7.0$ μm, (mean 3.3 μm), ratio phialide width/width of supporting cell $1.0 - 2.0$ μm, (mean 1.2 μm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* subglobose to ovoid, $2.0 - 4.5 \times 2.0 - 4.0$ μm, ratio length/width $1.0 - 1.5$ μm, hyaline when young becoming green to dark green with age, smooth.

Specimens examined: culture GJS 00-24 isolated from soil, Cerro de Ortega, Mexico, A.M. Aceves, apr-03-1998; culture GJS 05-113 isolated as an endophyte from seed of *Triticum aestivum* L., Italy, G. Vannacci, 2002; culture GJS 04-186 isolated from *Moniliophthora roreri* (Cif.) H.C. Evans, Stalpers, Samson & Benny, Junin, Peru, W. Soberanis, date unknown; culture GJS 04-197, isolated from *Moniliophthora roreri* (Cif.) H.C. Evans, Stalpers, Samson & Benny, Cusco, Peru, W. Soberanis, date unknown; culture GJS 04-02, missing data, USA, D. Lokken, date unknown; culture GJS 97-268 isolated from soil, Okinawa, Japan, T. Okuda, 1988; culture GJS 99-225 isolated from soil, Central Province, Cameroon, collector unknown, 1999.

Clade 9   (Figure 11 and 12)

Characteristics in culture: Optimum temperature for growth on PDA and SNA around 30 °C. Growing quickly at 25 °C and slower at 35 °C, $3.5 - 5.5$ cm diam at 25 °C, and $0.5 - 2.0$ cm diam at 35 °C on PDA, $3.5 - 5.5$ cm diam at 25 °C and $1.5 - 2.5$ cm diam at 35 °C on SNA, after 72h. Under twelve hours photoperiod and after 96h at 25 °C aerial mycelium cottonous, irradiating, sporulating in the center and in one or two concentric halus on PDA, sometimes sterile; translucent

mycelium , just few strains produce a poor aerial mycelium, sporulation sometimes forming pustules n one halus, and sometimes producing sparse sporulation on SNA. In the dark, sporulation appear between 48h and 72h on PDA and rarely sporulating after 96h at 35 °C, forming evenly-spread sporulation and rarely with one halus at 25 °C and when fertile evenly-spread sporulating at 35 °C after 96h; sporulation evenly-spread and sometimes with one halus after 96h at 25°C and infertile at 35 °C on SNA. *Hidrosoluble pigment* produced between 48h and 96h at 35 °C on the dark, pale yellow and sometimes the pigment is absent on PDA; absent on SNA. Sweetish smell mostly present on PDA at 30 °C. *Conidiophores* pyramidal, opposite branches *Phialides* lageniform, obpyriform or obclavate, sometimes conical at the end, up to four philaides at the end of each conidiophore, 4.5 − 13.0 × 2.5 − 4.5 µm, (mean 7.0 µm ×3.4 µm) , base 1.5 − 3.0 µm wide (mean 2.1 µm), supporting cell 2.0 − 4.0 µm wide (mean 2.9 µm), ratio phialide length/width 1.0 − 4.5 µm (mean 2.1 µm) , ratio phialide length/width of supporting cell 1.5 − 5.0 µm (mean 2.5 µm), ratio phialide width/width of supporting cell 1.0 − 2.0 µm, (mean 1.2 µm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* subglobose to ovoid, 2.5 − 3.5 × 2.0 − 3.5 µm, ratio length/width 1.0 − 1.5 µm, hyaline when young becoming green to dark green with age, smooth. *Stromata* solitary or aggregated, pulvinate (when seen from above), 0.04 − 0.08 mm height, 0.9 − 2.5 mm diam., glabrous, dark brown or black, pseudoparenchymatous, angular cells, cells at surface pigmented dark brown or black, and internal cells hyaline to pale brown or black. *Perithecia* cryptic, immersed in stroma, closely aggregated, sometimes scattered, distorted when crowded, and globose to subglobose when scattered, 180.5 − 322.0 × 91.0 − 228.0 µm, wall formed of compact pseudoparenchyma, 6.5 − 16.0 µm thick, ostiole one per perithecium, central, 6.0 − 101.0 µm diam. *Asci* cylindrical, 53.0 − 102.0 × 3.0 − 5.5 µm, ascospores uniseriate. *Ascospores* splitting to form16 spores per asci, dimorphic, distal spores subglobose, oval or suboval with truncate base and rounded apex, 3.0 − 5.5 × 3.0 − 5.0 µm, brown in KOH, warted, proximal spores subcylindrical sometimes with rounded base and truncate apex, 3.5 − 5.5 × 2.5 − 4.0 µm, brown in KOH, warted.

Specimens examined: Culture GJS 04-67 isolated from soil, Pisa, Italy, G. Vanacci, 1983; cultures GJS 05-101 and GJS 05-106 isolated from soil, Salina Natural Park, Italy, G. Vanacci, 1983; culture GJS 98-183 isolated from teleomorph

(BPI 841387, also examined) on *Ulmus* sp., Austria, W.J. Jaklitsch, oct-30-1998; culture GJS 90-254 from teleomorph (BPI 1109306, also examined) on *Pinus sylvestris* L., Weimar, Germany, G. Arnald, aug-10-1990; culture GJS 92-110 (other number: CBS 549.92) isolated from teleomorph (BPI 802854, also examined) on *Fagus* sp., France, C.M.F. Candassau, sep-13-1992.

Clade 10   (Figure 13)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Growing quickly, 5.0 – 7.5 cm diam at 25 °C, and 1.5 – 5.5 cm diam at 35 °C on PDA, 4.5 – 6.5 cm diam at 25 °C and 1.5 – 4.5 cm diam at 35 °C on SNA, after 72h. Under twelve hours photoperiod and after 96h at 25 °C aerial mycelium cottonous, irradiating, mostly sporulating sparsely on the surface of the mycelium, sometimes with a dense sporulation on the center, some strains with a small and central sporulation and others with a wide central zone on PDA; translucent mycelium with some aerial hypha, sporulation evenly-spread on the colony, some strains forming pustules in one or two hali on SNA. In the dark, sporulation appearing between 48h and 72h on PDA and forming mostly a central sporulating area or one halus besides a central sporulation, but some strains having a different layout (evenly-spread, two or more sporulating hali…) at 25 °C, at 35 °C sporulation evenly-spread or forming two hali besides a central sporulation zone after 96h, sporulation appearing after 72h on SNA, in some strains appearing after 48h at 35 °C, forming one or two hali, some other strains forming two hali besides a central sporulation zone at 25 °C, either evenly-spread sporulation or sporulating centrally at 35 °C after 96h. *Hidrosoluble pigment* mostly produced after 48h at 35 °C on the dark, some strains producing the pigment after 24h, 72h or 96h, some strains not producing pigment, mostly pale yellow on PDA, but some strains producing a redish or brownish pigment; pigmentation absent on SNA. Some strains producing a sweetish smell and others not producint it on PDA at 30 °C. *Conidiophores* pyramidal, opposite branches *Phialides* lageniform, obpyriform or obclavate, some strains developing conical phialides mainly at the end o the condidiophores, up to 5 at the end of each conidiophore, 3.0 – 12.5 × 2.5 – 5.0 μm, (mean 5.5 × 3.6 μm), base 1.0 – 3.5 μm wide, (mean 2.1 μm), supporting cell 2.0 – 5.0 μm wide (mean 3.2 μm), ratio phialide length/width 1.0 – 3.5 μm, (mean 1.5 μm), ratio phialide length/width of supporting cell 1.0 – 4.5 μm, (mean 1.8 μm), ratio phialide

width/width of supporting cell 0.5 – 2.0 μm, (mean 1.2 μm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* globose to subglobose, or subglobose to ovoid, 2.0 – 4.0 × 2.0 – 3.5 μm, ratio length/width 1.0 – 1.5 μm, hyaline when young becoming green to dark green with age, some strains developing a yellowish green conidia, smooth surface.

Specimens examined: Cultures Dis 55f and Dis 55j isolated as an endophyte from stem of *T. cacao*, Tafo, Akim, Ghana, H.C. Evans, mar-07-1999; culture Dis 67b isolated as an endophyte from stem of *Theobroma bicolor* Bonpl., Nueva Primavera, Napo Province, Ecuador, H.C. Evans & K.A. Holmes, mac-24-1999; culture Dis 93d isolated as an endophyte from stem of *T. cacao*, Quebrada de Sabalillo, Rio Maranon, Peru, H.C. Evans & D.H. Djeddour, may-03-1999; culture Dis 94d isolated as an endophyte from stem of *Theobroma* sp., Quebrada Payarote, Rio Maranon, Peru, H.C. Evans & D.H. Djeddour, may-03-1999; culture Dis 168a isolated as an endophyte from stem of *Theobroma cacao* L., Itabuna, Bahia, Brazil, H.C. Evans and K.A. Holmes, feb-02-2000; culture Dis 169c isolated as an endophyte from *Theobroma speciosum* Willd. ex Spreng., H.C.Evans & K.A. Holmes, feb-25-2000; culture Dis 167c isolated as an endophyte from stem of *T. cacao*, Itabuna, Bahia, Brazil, H.C. Evans & K.A. Holmes, feb-29-2000; cultures Dis 173d and Dis 173f isolated as an endophyte from stem of *Theobroma* sp., Belem, Pará, Brazil, feb-29-2000; culture Dis 218e isolated as an endophyte from stem of *Theobroma gileri*, Esmeraldas province, Ecuador, H.C. Evans & K.A.Holmes, may-05-2000; cultures Dis 110a and Dis 233g isolated as an endophyte from stem of *T. cacao*, Rio Napo, Napo Province, Ecuador, H.C. Evans & K.A.Holmes, may-12-2000; cultures Dis 246e, Dis 246j and Dis 246k isolated as an endophyte from stem of a single individual of *T. cacao*, Archidona, Ecuador; cultures Dis 253b and Dis 264u isolated as an endophyte from stem of *T. cacao*, Los Rios Province, Ecuador, H.C. Evans & K.A. Holmes, may-23-2000; culture GJS 05-394 isolated from soil, Bokito, Cameroon, P. Tondge, apr-2005; cultures GJS 11-183 (other number: LA 228), GJS 11-181 (other number: LA 25), GJS 11-188 (other number: LA 251), GJS 11-182 (other number: LA 30), GJS 11-189 (other number: LA 35) isolated as an endophyte from stem of *Hevea guianensis*, Los Amigos Biological station, Manu, Madre de Dios, Peru, R. Gazis, may-2008; cultures GJS 11-190 (other number: T 33)

and GJS 11-180 (other number: T 42) isolated from stem of *Hevea brasiliensis*, Picaflor Biological station, Tambopata, Peru, R. Gazis and P. Chaverri, june-2007.

Clade 11   (Figure 14)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Growing quickly, 5.5 – 7.0 cm diam at 25 °C, and 1.5 – 4.5 cm diam at 35 °C on PDA, 5.0 – 6.5 cm diam at 25 °C and 1.0 – 4.0 cm diam at 35 °C on SNA, after 72h. Under twelve hours photoperiod and after 96h at 25 °C aerial mycelium cottonous, irradiating, sporulating sparsely on mycelium, some strains forming one halus besides a central sporulation on PDA; translucent mycelium with some sparse aerial hypha, sporulation forming pustules and two hali, sometimes with a sparse sporulation besides the halus on SNA. In the dark, sporulation appearing after 48h on PDA and forming two halus besides a central halus at 25 °C and 35 °C after 96h, sporulation appearing after 72h at 25 °C and after 48h at 35 °C on SNA, forming two hali or evenly-spread sporulation at 25 °C and 35 °C after 96h. *Hidrosoluble pigment* produced after 48h at 35 °C on the dark, pale yellow on PDA; absent on SNA. Some strains producing a sweetish smell on PDA at 30 °C, smell absent in some strains. *Conidiophores* pyramidal, opposite branches *Phialides* lageniform, obpyriform or obclavate, and some strains developing few conical phialides, mainly at the end of conidiophores, up to 5 at the end of each conidiophore, 3.5 – 9.5 × 2.5 – 4.5 μm, (mean 5.3× 3.3 μm), base 1.0 – 3.0 μm (mean 2.0 μm), supporting cell 2.0 – 4.5 μm wide (mean 3.1 μm), ratio phialide length/width 1.0 – 3.5 μm, (mean 1.6 μm), ratio phialide length/width of supporting cell 1.0 – 5.5 μm, (mean 1.8 μm), ratio phialide width/width of supporting cell 1.0 – 2.0 μm, (mean 1.1 μm). *Adjacent phialides* inclined and sometimes curved towards the apex, with lower side swollen and the upper part straight. *Conidia* subglobose to ovoid some strains subglobose to ovoid, 2.0 – 4.0 × 2.0– 3.0 μm, ratio length/width 2.0 – 1.5 μm, hyaline when young becoming green to dark green, some strains also developing some yellowish green conidia, smooth.

Specimens examined: Culture Dis 64a isolated as an endophyte from stem of *Theobroma cacao*, Imbabura Province, Ecuador, H.C. Evans & S.E. Thomas, mar-21-1999; culture Dis 218h isolated as an endophyte from stem of *Theobroma gileri*, Esmeraldas Province, Ecuador, H.C. Evans & K.A. Holmes, may-05-2000; culture

Dis 264v isolated as an endophyte from stem of *T. cacao*, Los Rios Province, Ecuador, H.C. Evans & K.A. Holmes, may-23-2000; culture Dis 325ai isolated as an endophyte from stem of *T. cacao*, Esmeraldas Province, Ecuador, H.C. Evans & R. Reeder, nov-05-2001; culture Dis 354a isolated as an endophyte from stem of *T. gileri*, Ecuador, collector unknown, date unknown.

Clade 12   (Figure 15)

Characteristics in culture: Optimum temperature for growth on PDA and SNA 30 °C. Growing quickly, 6.0 – 6.5 cm diam at 25 °C, and 4.0 – 5.0 cm diam at 35 °C on PDA, 6.0 – 7.0 cm diam at 25 °C and 3.5 – 4.0 cm diam at 35 °C on SNA, after 72h Under twelve hours photoperiod and after 96h at 25 °C aerial mycelium cottonous, irradiating, sporulating sparsely and evenly-spread on mycelium, two hali and a central sporulation can be distinguished on PDA; translucent mycelium with some aerial hypha and sporulation evenly-spread, some strains sporulating on pustules in the center of the colony, two hali are distinguishable in those strains on SNA. In the dark, sporulation appearing after 48h on PDA and forming one halus besides a central halus or evenly-spread sporulation at 25 °C and 35 °C after 96h, sporulation appearing after 72h on SNA, forming two hali besides a central sporulating zone at 25 °C and one halus at 35 °C after 96h. *Hidrosoluble pigment* produced between 48h and 72h, pale yellow at 35 °C on PDA in the dark; absent on SNA. Sweetish smell present in some strains and absent in others on PDA at 30 °C. *Conidiophores* pyramidal, opposite branches *Phialides* lageniform, obpyriform or obclavate, and forming some conical phialides mainly at the end of the condidiophore, up to 5 at the end of the conidiophores, $4.0 - 8.5 \times 3.0 - 4.5$ μm, (mean 5.7×3.6 μm) , base 1.5 – 3.0 μm wide (mean 2.0 μm), supporting cell 2.0 – 6.5 μm wide (mean 2.9 μm), ratio phialide length/width 1.0 – 2.5 μm, (mean 1.6 μm), ratio phialide length/width of supporting cell 1.0 – 3.0 μm, (mean 2.0 μm), ratio phialide width/width of supporting cell 0.5 – 1.5 μm, (mean 1.3 μm). *Adjacent phialides* inclined and sometimes curved towards the apex. *Conidia* subglobose to ovoid, $2.5 - 3.5 \times 2.5 - 3.0$ μm, ratio length/width 1.0 – 1.5 μm, hyaline when young becoming green to dark green with age, smooth.

Specimens examined: cultures GJS 00-06 and GJS 00-08 from soil, Cerro de Ortega, Mexico, A.M. Aceves, apr-03-1998.

4. Discussion

Comparing the confidence interval values (95%), of character values that were evaluated, appeared to be the best alternative to generate a reliable way for identifying morphologically the clades/taxa within the 'harzianum' complex species. This was attempted in this work as an attempt to combine morphology, gene data, ecology (habitat) and geographic origin as a form of discriminating the possible cryptic species hidden within *T. harzianum sensu lato*. The boxplot graphics prepared with the present results on SYSTAT were not informative and it was useless to include it in the results.

The phylogenetic analysis has indicated that at least twelve clades could be distinguished within *T. harzianum* complex. The results obtained in phylogenetic analyses is corroborate by Druzhinina et al. (2010). Additional collections are needed for a decision on the species status of some strains for with there is insufficient data. One of these isolates is the GJS 97-96, the ex-type of *Hypocrea lixii* (Chaverri and Samuels 2002). This isolate appeared close to clade 3 in the phylogenetic tree but it is clearly not related with this species. No description of the anamorph of this species was provided, but our analysis has shown that *H. lixii* is not the teleomorph of *Trichoderma harzianum* s.st. (clade 5) and this clade has an unknown teleomorph. Among the clades described in this work, only clade 6 and clade 9 have been isolated from the sexual stages, but these two *Hypocrea* states are morphologically indistinguishable.

This study indicates that the trend towards speciation within the *T. harzianum* complex occurred under the influence of geographic isolation followed, secondarily, by their niche/habitat. Among these clades only four were obtained from temperate regions, namely: clade 6 (collected in the USA), and clade 8 (which is a cosmopolitan species collected both in temperate regions and in the tropics), clade 9 (collected in Europe), *T. harzianum* s.st. (collected in Europe and USA). All other clades in the *T. harzianum* complex were obtained from tropical sites. These also appear to have remained continentally isolated. Examples are clade 1 and clade 7 both collected only from Cameroon; clade 2, clade 3, clade 4, clade 11, clade 12 from tropical Latin America. The only intercontinental distribution for tropical clades in the *T. harzianum* complex is clade 10 collected from Africa and South America.

Perhaps future studies involving searches for additional isolates of *T. harzianum* from poorly collected tropical areas will reveal a pantropical distribution for some clades of *T. harzianum*, but, at present, these appear to be geographically restricted in distribution. It is acknowledged that the range of isolates included in this work is not sufficiently broad for a complete discussion on geographical distribution as no isolate from Asia and Oceania were included. Among the clades described herein five appear to be strictly endophytic (clade 2, clade 3, clade 4, clade 7 and clade 11) and some seem to be rather versatile and capable of also existing as saprophytes on plant material or in the soil namely: clade 6, clade 9 and clade 12. The latter was, at least once also found to occur as an endophyte.

Since Rifai's review of *Trichoderma*, more than forty years ago, when *Trichoderma harzianum* was described, this species has been treated as a species complex by mycologists. Nevertheless, practical users of isolates of fungi belonging to this taxon as biological control agents have taken little consideration to the fact that *T. harzianum* s.l. is likely to be a species complex. The recognition and description of twelve clades for the taxa hidden under the *T. harzianum* label finally paves the way towards clarifying this difficult taxon. Morphological similarities hiding cryptic taxa are likely to be behind some of the heterogeneous results obtained by researchers involving in the application of *T. harzianum* s.l. in biological control. Results of this study clearly indicated that morphology alone does not provide sufficient information to allow discrimination between the various clades. It is questionable if the clades recognized within the so-called *T. harzianum* complex deserve recognition at the species level, as originally expected. Until better evidence is generated, perhaps through the investigation of additional genome regions, it appears more appropriate to continue using *T. harzianum sensu lato* for the range of forms having a morphology equivalent to *T. harzianum* instead of erecting formal taxa for each clade. Information on ecology and origin of isolates may prove in the future to be of relevance for taxonomic distinction and identification but additional work, inclusive on the grounds of molecular phylogeny are still needed.

6. Bibliography

Bailey BA, Bae H, Strem MD, Crozier J, Thomas SE, Samuels GJ, Vinyard BT, Holmes KA, 2008. Antibiosis, mycoparasitism, and colonization success for endophytic Trichoderma isolates with biological control potential in Theobroma cacao. *Biological Control* 46, 24-35.

Bailey BA, Bae H, Strem MD, Roberts DP, Thomas SE, Crozier J, Samuels GJ, Choi IY, Holmes KA, 2006. Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four Trichoderma species. *Planta* 224, 1449-1464.

Carbone I, Kohn LM, 1999. A method for designing primer sets for speciation studies in filamentous Ascomycetes. *Mycologia* 91, 553– 556.

Chaverri P, Castlebury LA, Samuels GJ, Geiser DM, 2003. Multilocus phylogenetic structure within the Trichoderma harzianum/Hypocrea lixii complex. *Molecular Phylogenetics and Evolution* 27, 302-313.

Chaverri P, Samuels GJ, 2002. *Hypocrea lixii*, the teleomorph oh *Trichoderma harzianum. Mycological Progress* 1, 283–286.

Chaverri P, Samuels GJ, 2003. *Hypocrea/Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): species with green ascospores. *Studies in mycology*. 48, 1-116.

Degenkolb T, Dieckmann R, Nielsen KF, Gräfenhan T, Theis C, Zafari D, Chaverri P, Ismaiel A, Brückner H, von Döhren H, Thrane U, Petrini O, Samuels GJ, 2008. The *Trichoderma brevicompactum* clade: a separate lineage with new species, new peptaibioticsm, and mycotoxins. *Mycological Progress* 7, 177-219.

Druzhinina IS, Kubicek CP, Komon-Zelazowska M, Mulaw TB, Bissett J, 2010. The Trichoderma harzianum demon: complex speciation history resulting in

coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. *Bmc Evolutionary Biology* 10, 94-108.

Evans HC, Holmes KA, Thomas SE, 2003. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and preliminary assessment of their potencial as biological control agents if cacao diseases. *Mycological Progress*. 2, 149-160.

Gams W, Meyer W, 1998. What Exactly Is Trichoderma harzianum? *Mycologia*. 90, 904-915.

Jaklitsch WM, 2009. European species of *Hypocrea* Part I. The green-spored species. *Studies in Mycology*. 63, 1–91.

Kirk PM, Cannon PF, Minter DW, Stalpers JA. 2008. Dictionary of the Fungi. Oxon. UK. CAB International. 771p.

Nirenberg H, 1976. Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion Liseola. *Mitt Biol Bundesant Land-Forstwirtsch Berlin-Dahlem*. 169: 1-117.

O'Donnell K, Ward TJ, Geiser DM, Kistler HG, Aoki T. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology* 41, 600-623.

Persoon CH, 1794. Neuer Versuch Einer Systematischen Eintheilung der Schwamme. Neues Magazin für die Botanik. *Römer*. 1, 63-80.

Phoulivong S, Cai L, Chen H, McKenzie EHC, Abdelsalam K, Chukeatirote E, Hyde KD. 2010. *Colletotrichum gloeosporioides* is a commom pathogen on tropical fruits. *Fungal Diversity* 44, 33-43.

Rifai MA, 1969. A revision of the genus *Trichoderma. Mycologica Papers.* 116, 1-56.

Samuels GJ, 2006. *Trichoderma*: Systematics, the sexual state, and ecology. *Phytopathology* 96, 195-206.

Taylor JW, Jacobson DJ, Kronken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. 2000. Phylogenetic species recognition and species concepts in Fungi. *Fungal Genetics and Biology*. 31, 21-31.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24, 4876–4882.

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M, 2008. Trichoderma-plant-pathogen interactions. *Soil Biology & Biochemistry* 40, 1-10.

Wells HD, Bell DK, Jaworski CA, 1972. Efficacy of trichoderma-harzianum as a biocontrol for sclerotium-rolfsii. *Phytopathology* 62, 442-&.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A. et al. (Eds.), PCR Protocols: A guide to Methods and Applications. Academic Press, San Diego, pp. 315– 322.

7. Figures and Tables
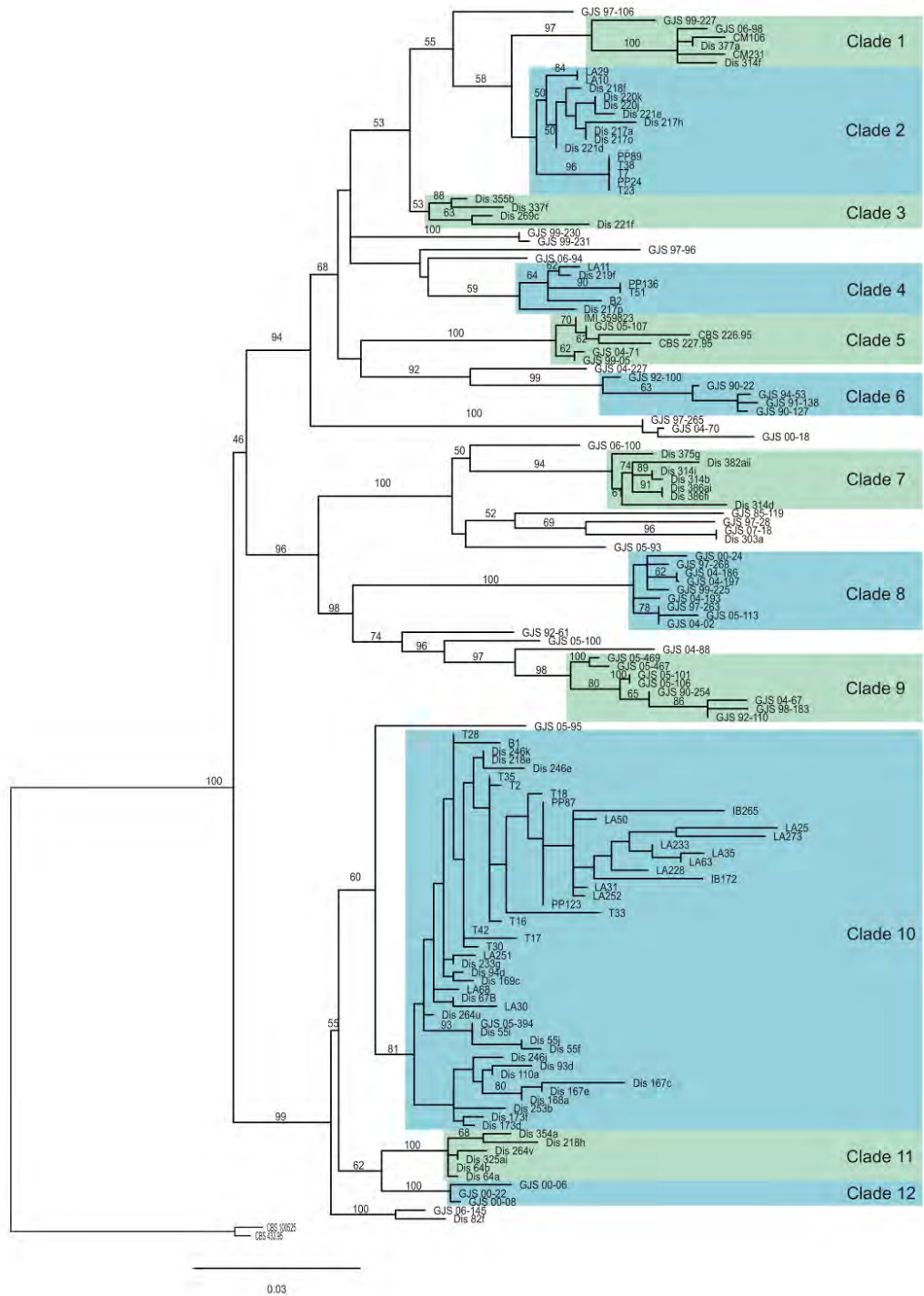
Figure 1. *Trichoderma harzianum* species complex. Multilocus philogenetic tree of obtained by Maximum Likelihood analysis of *Act*, *Cal*, *ITS* and *Tef* sequence data. Species limit proposed are highlighted and named as numbered clades.

.

Figure 2. Ilustrations of clade 1. Cultures grown at 25 °C on PDA (a) and SNA (b) under 12h light regime; pustules (c) developed on SNA; Phialides arising from the apex of conidiophores (d; e); Conidia (f) mostly subglobose. Bars: a= 1.5 mm; b= 1.5 mm; c= 35 µm; d=5.0 µm; e= 2.5 µm; f= 5.0 µm.

Figure 3. Ilustrations of clade 2. Different aspects of cultures grown at 25 °C on PDA (a; f; i) and SNA (b; g; j) under 12h light regime; pustule (c) and chlamydospore (d) developed on SNA; Pyramidal conidiophores (e); phialides arising from the apex of conidiophores (h); conidia subglobose to ovoid (k). Bars: a=1.5 mm; b=1.5 mm; c= 50 µm; d= 10 µm; e= 25 µm; f= 1.5 mm; g= 1.5 mm; h= 10 µm ; i= 1.5 mm;j= 1.5 mm; k= 5.0 µm.

Figure 4. Ilustrations of clade 3. Different aspects of cultures grown at 25 °C on PDA (a; e) and SNA (b; f) under 12h light regime; chlamydospore (c) developed on SNA; pyramidal conidiophore (d); phialides arising from the apex of conidiophores (g); conidia subglobose to ovoid (h). Bars: a= 1.5 mm; b= 1.5 mm; c= 5.0 µm; d= 25 µm; e= 1.5 mm; f= 1.5 mm; g= 5.0 µm; h= 5.0 µm.

Figure 5. Ilustrations of clade 4. Different aspects of cultures grown at 25 °C on PDA (a; f; i) and SNA (b; g; j) under 12h light regime; pustule (c) and chlamydospore (d) developed on SNA; pyramidal conidiophore (e); phialides arising from the apex of conidiophores (h); conidia subglobose to ovoid (k). Bars: a= 1.5 mm; b= 1.5 mm; c= 50 µm ; d= 5.0 µm ; e= 25 µm ; f= 1.5 mm; g= 1.5 mm; h= 10 µm ; i= 1.5 mm ; j= 1.5 mm; k= 5.0 µm.

Figure 6. Ilustrations of clade 5 - *Trichoderma harzianum* sensu strictu. Cultures grown at 25 °C on PDA (a) and SNA (b) under 12h light regime; pustule (c) developed on SNA; pyramidal conidiophores (d); phialides arising from the apex of conidiophores (e); conidia subglobose to ovoid (f). Bars: a= 1,5 mm; b= 1,5 mm; c= 50 µm; d= 25.0 µm; e= 5.0 µm; f= 5.0 µm.

Figure 7. Ilustrations of clade 6. Cultures grown at 25 °C on PDA (a) and SNA (b) under 12h light regime; pustule (c) and chlamydospore (d) developed on SNA; phialides arising from the apex of pyramidal conidiophores (e; f); conidia subglobose to ovoid (g). Bars: a= 1.5 mm; b= 1.5 mm; c= 50.0 μm; d= 10.0 μm; e= 10.0 μm; f= 25.0 μm; g= 2.5 μm.
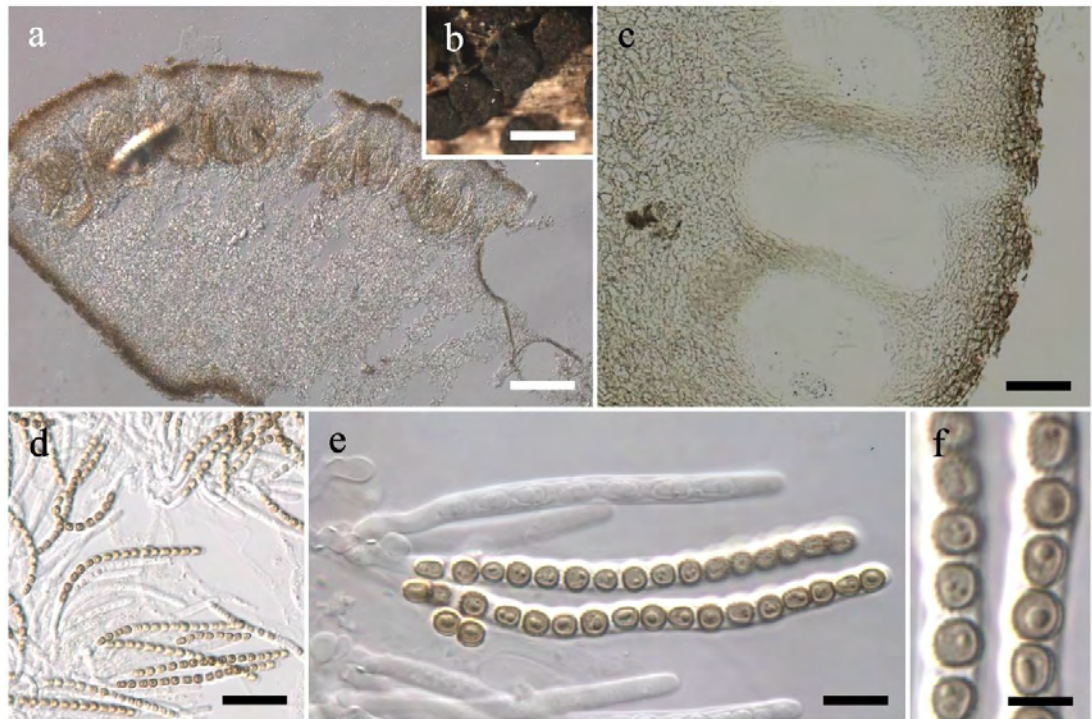
Figure 8. Ilustrations of sexual state (*Hypocrea*) of clade 6. Solitary stromata (a; b) with perithecia (c) on the upper side; uniseriate asci (d; e) with 16 ascospores; warted ascospores (f). Bars: a= 100.0 µm; b= 2.0 µm; c= 50.0 µm; d= 25.0 µm ; e= 10.0 µm; f= 5.0 µm.
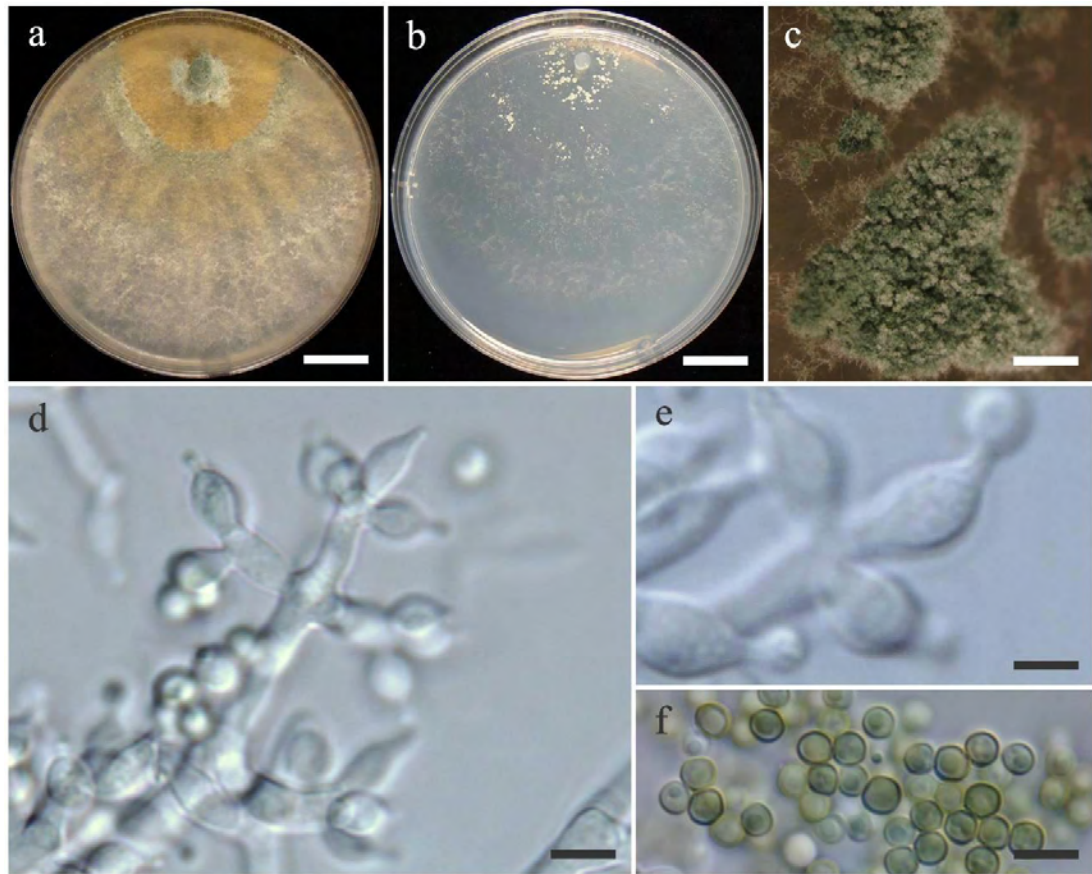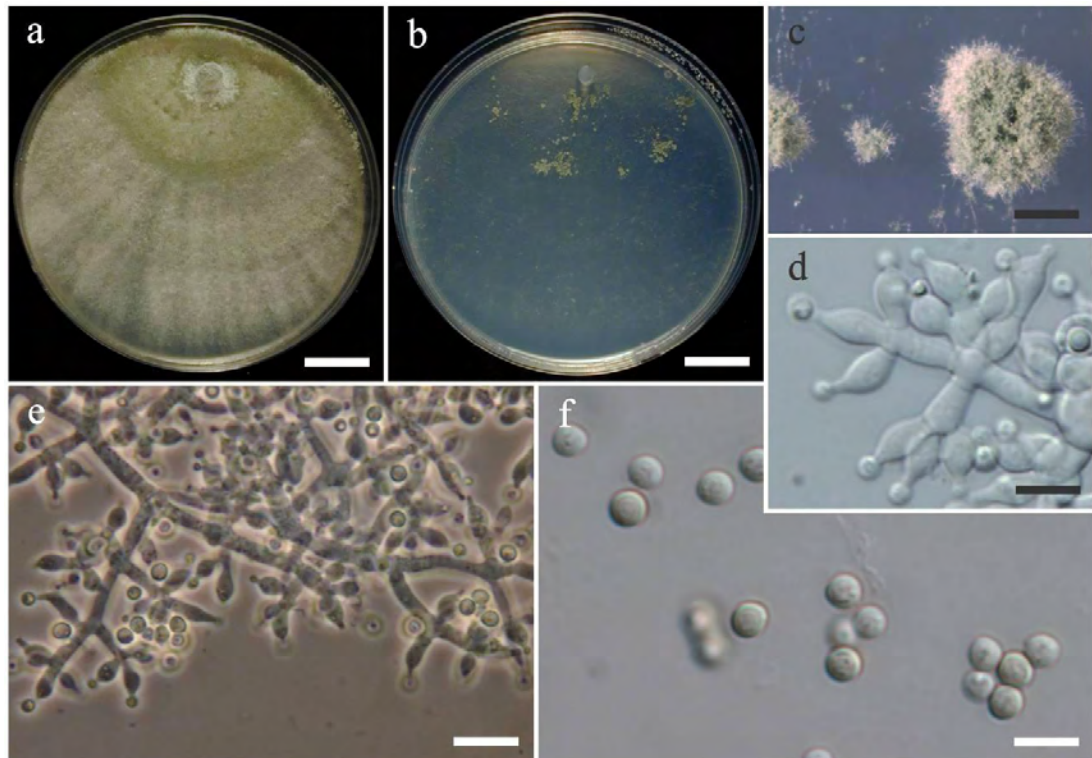
Figure 9. Ilustrations of clade 7. Cultures grown at 25 °C on PDA (a) and SNA (b) under 12h light regime; pustule (c) developed on SNA; phialides arising from the apex of pyramidal conidiophores (d; e); conidia subglobose to ovoid (f). Bars: a= 1.5 mm; b= 1.5 mm; c= 50.0 µm; d= 5.0 µm; e= 10.0 µm; f= 5.0 µm.
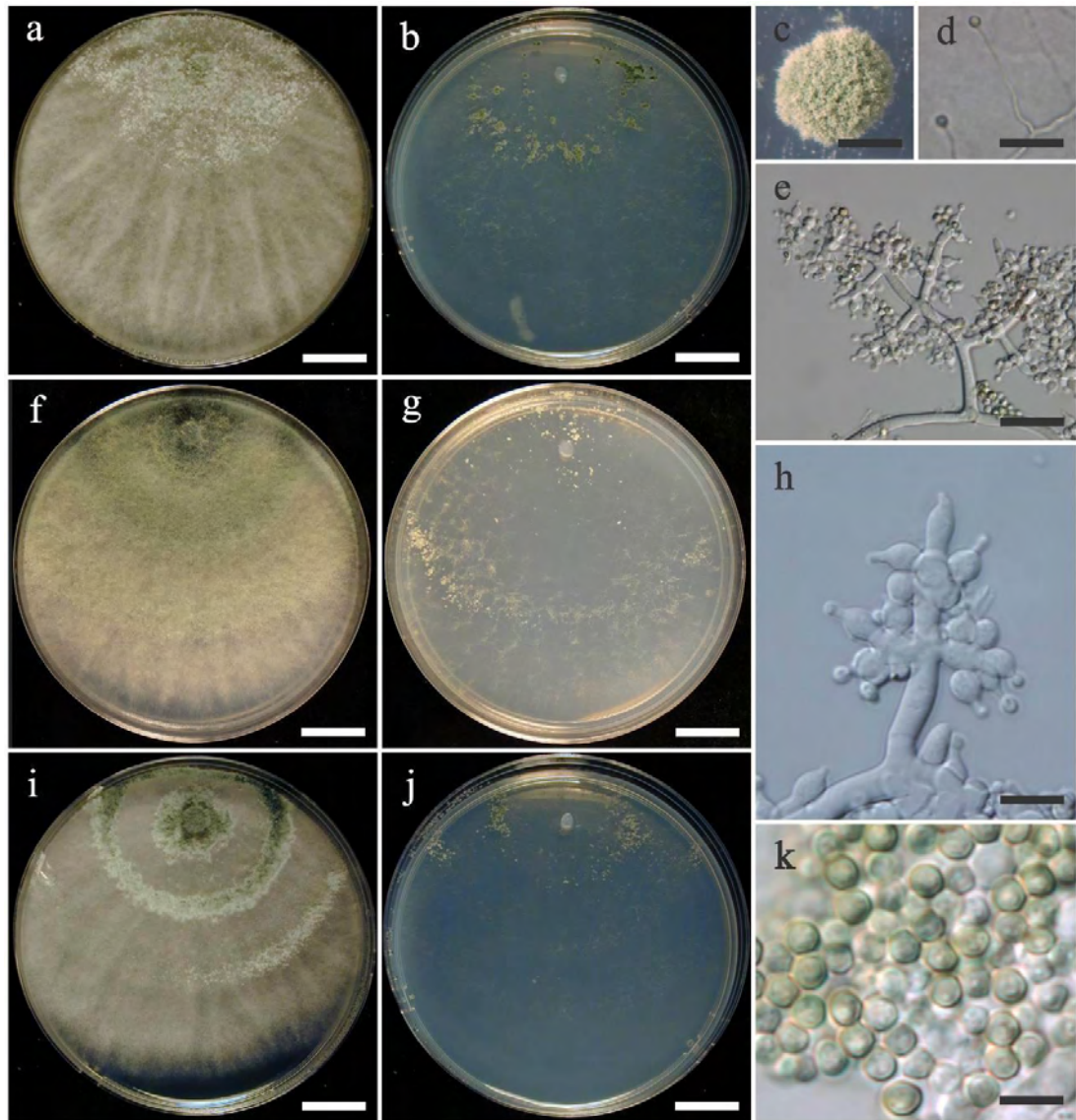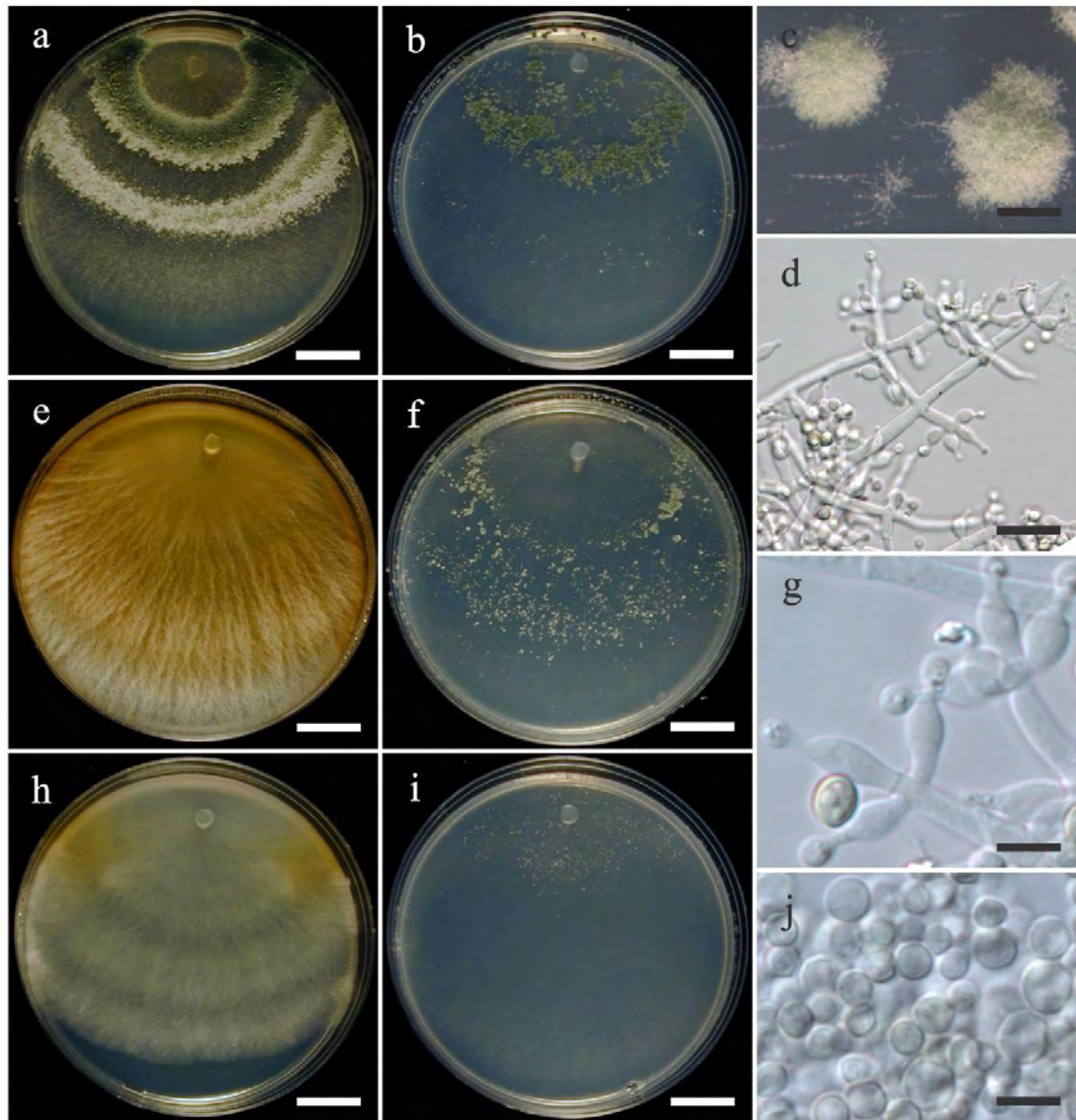
Figure 10. Ilustrations of clade 8. Different aspects of cultures grown at 25 °C on PDA (a; e; h) and SNA (b; f; i) under 12h light regime; pustule (c) developed on SNA; pyramidal conidiophore (d); phialides arising from the apex of conidiophores (g); conidia subglobose to ovoid (j). Bars: a= 1.5 mm; b= 1.5 mm; c= 50.0 μm; d= 25.0 μm; e= 1.5 mm; f= 1.5 mm; g= 10.0 μm ; h= 1.5 mm; i= 1.5 mm; j= 5.0 μm.
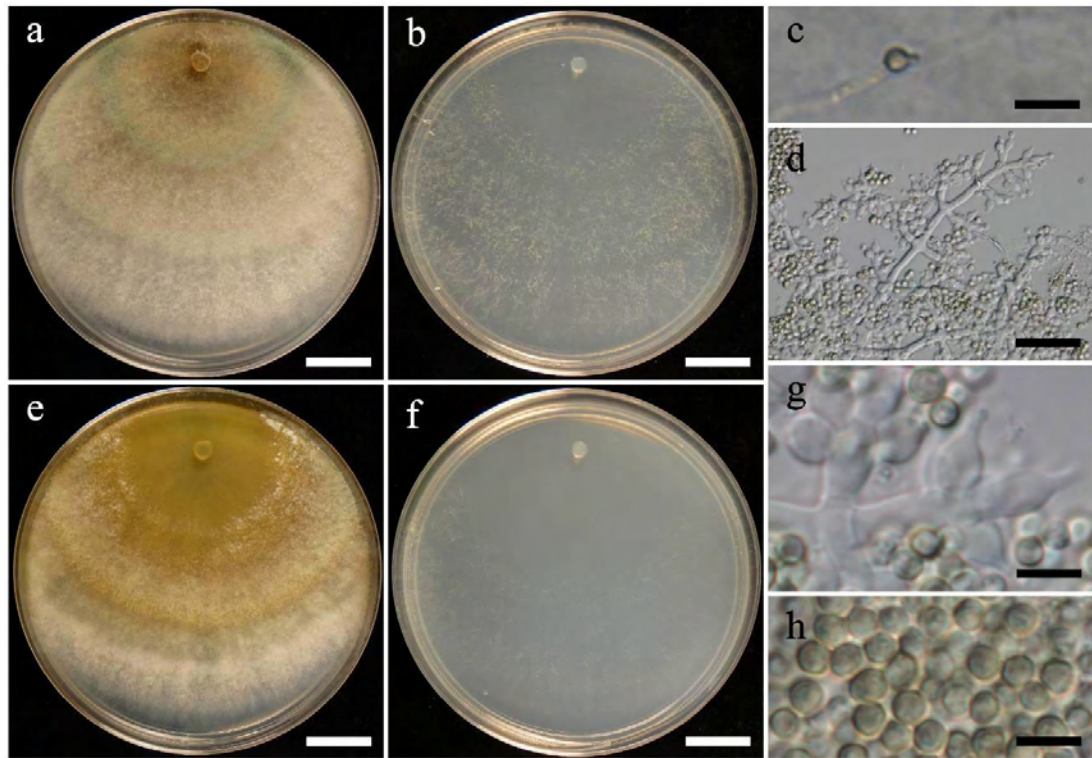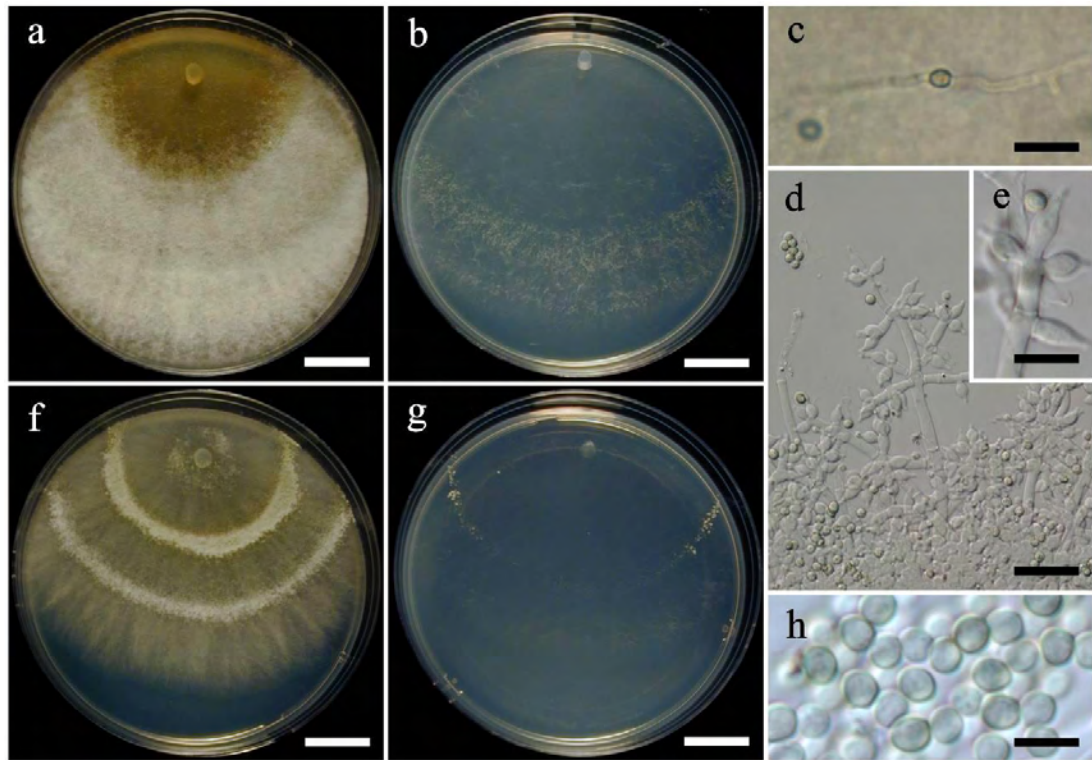
Figure 11. Ilustrations of clade 9. Different aspects of cultures grown at 25 °C on PDA (a; f) and SNA (b; g) under 12h light regime; chlamydospores (c) developed on SNA; pyramidal conidiophore (d); phialides arising from the apex of conidiophores (e); conidia subglobose to ovoid (h). Bars: a= 1.5 mm; b= 1.5 mm; c= 10.0 μm; d= 25.0 μm; e= 10.0 μm; f= 1.5 mm; g= 1.5 mm; h= 5.0 μm.
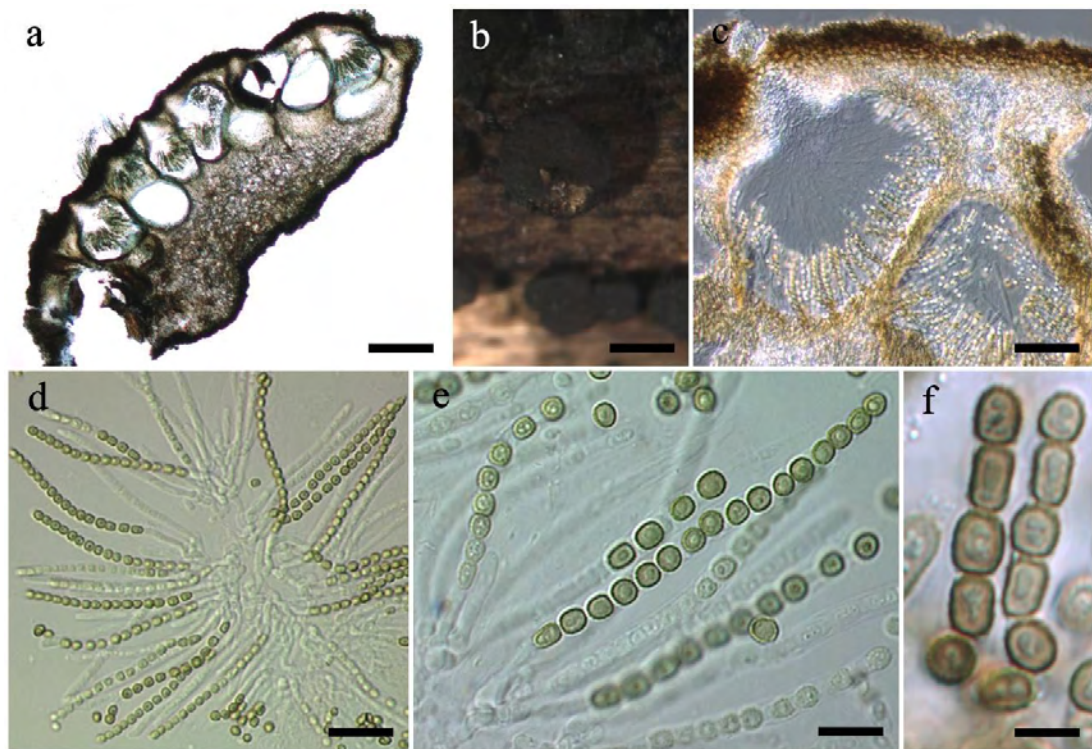
Figure 12. Ilustrations of sexual state (*Hypocrea*) of clade 9. Solitary stromata (a; b) with perithecia (c) on the upper side; uniseriate asci (d; e) with 16 ascospores; warted ascospores (f). Bars: a= 200.0 μm; b= 1.0 μ; c= 50.0 μm; d= 25.0 μm; e= 10.0 μm; f= 5.0 μm.
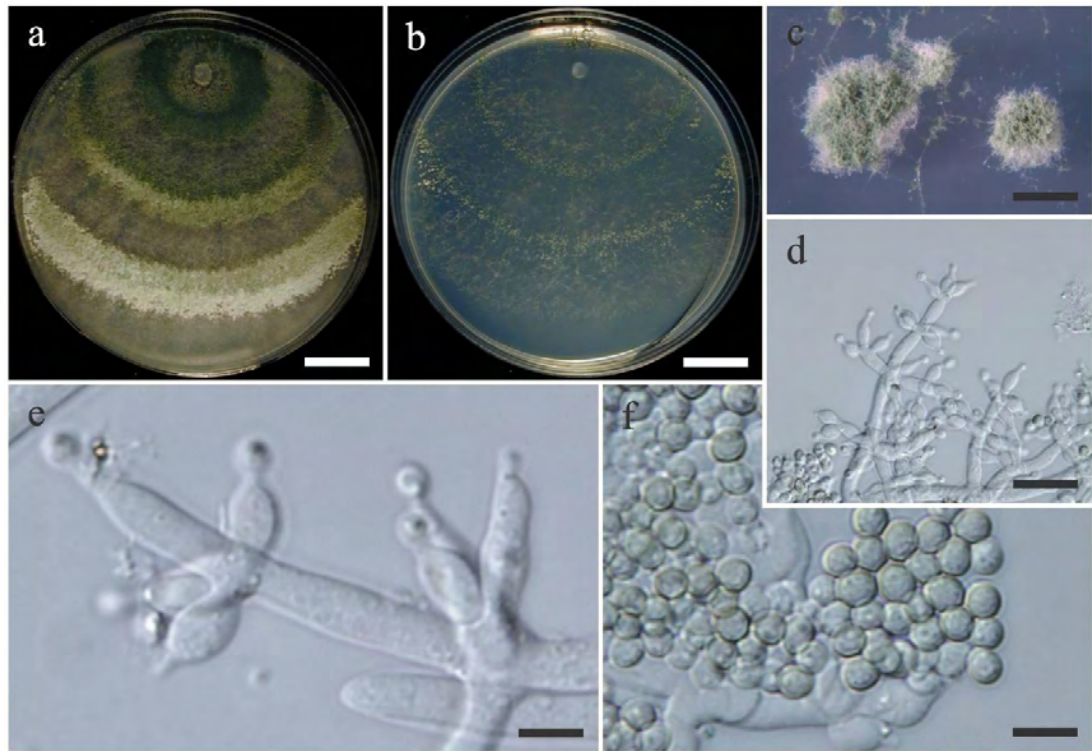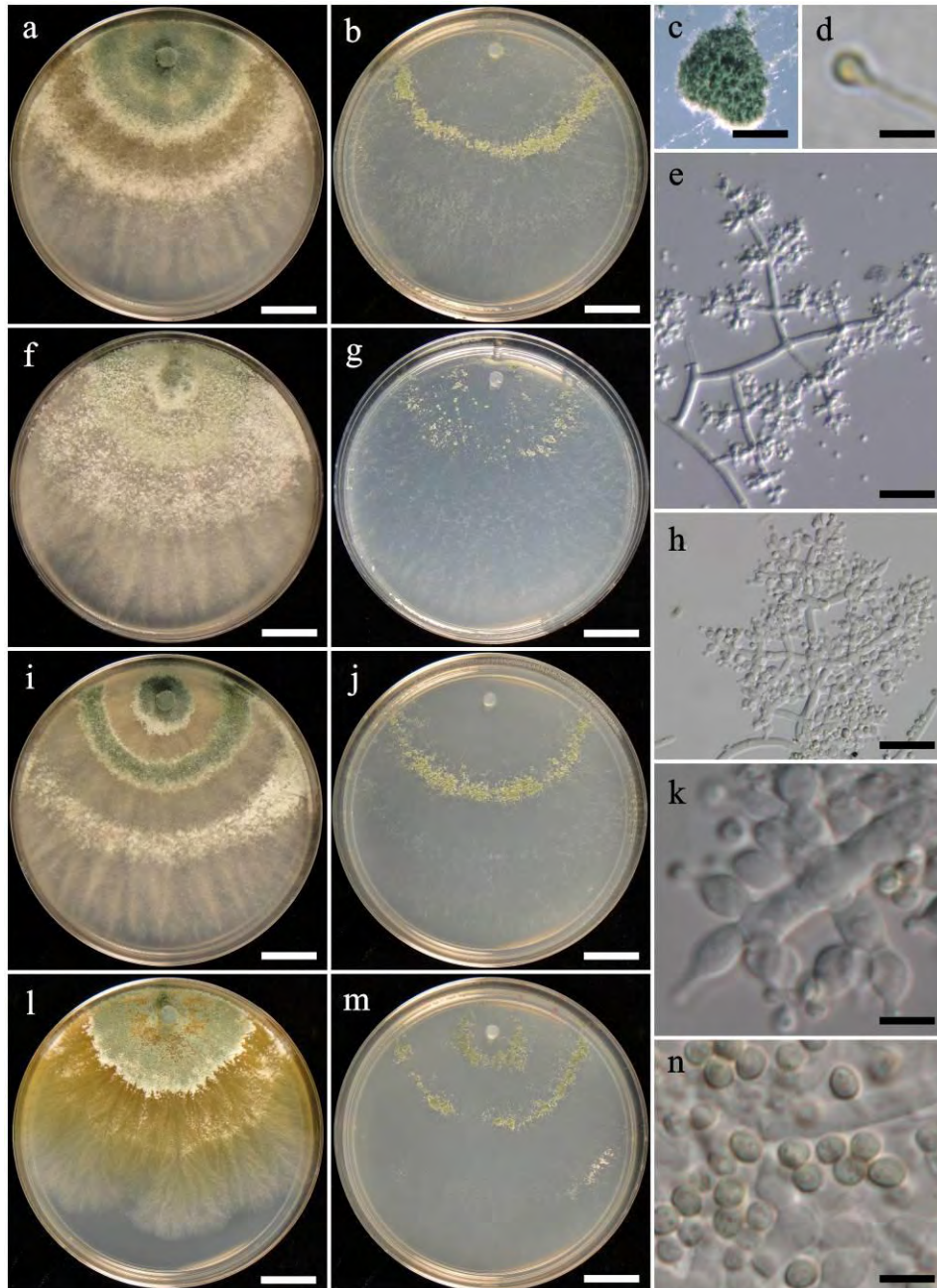
Figure 13. Ilustrations of clade 10. Different aspects of cultures grown at 25 °C on PDA (a; f; i; l) and SNA (b; g; j; m) under 12h light regime; pustule (c) and chlamydospore (d) developed on SNA; pyramidal conidiophore (e; h); phialides arising from the apex of conidiophores (k); conidia subglobose to ovoid (n). Bars: a= 1.5 mm; b= 1.5 mm; c= 100 µm; d= 5.0 µm; e= 25.0 µm; f= 1.5 mm; g= 1.5 mm; h= 25.0 µm; i= 1.5 mm; j= 1.5 mm; k= ; 5.0 µm; l= 1.5 mm; m= 1.5 mm; n= 5.0 µm.
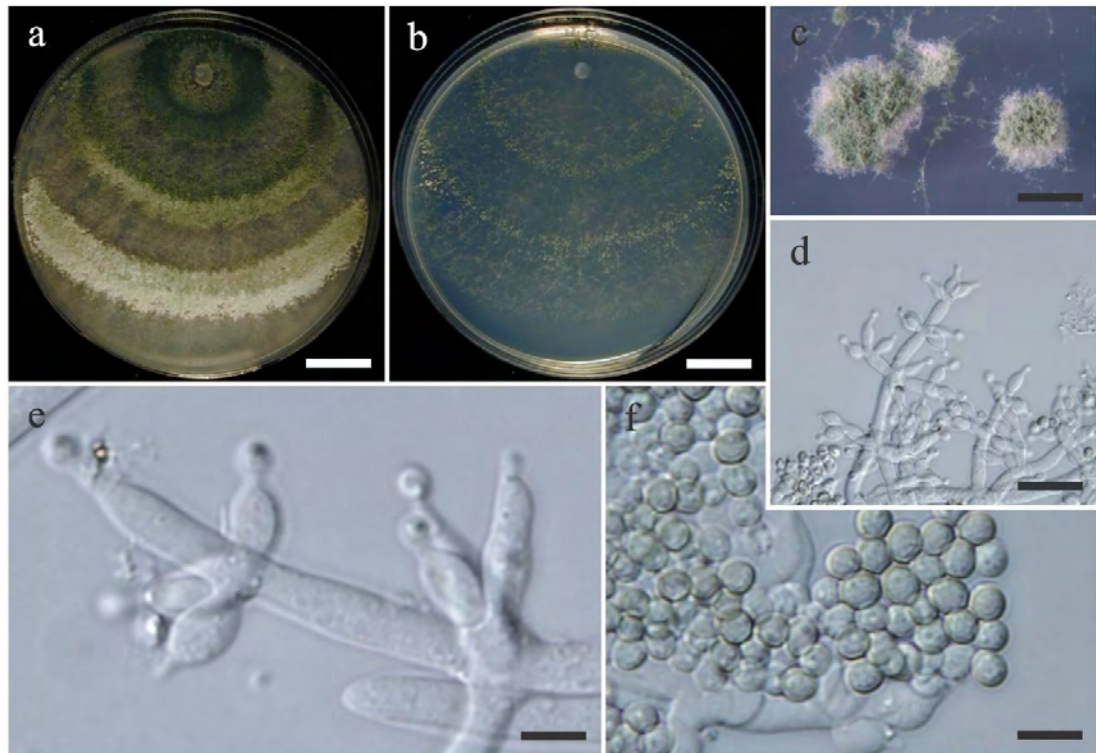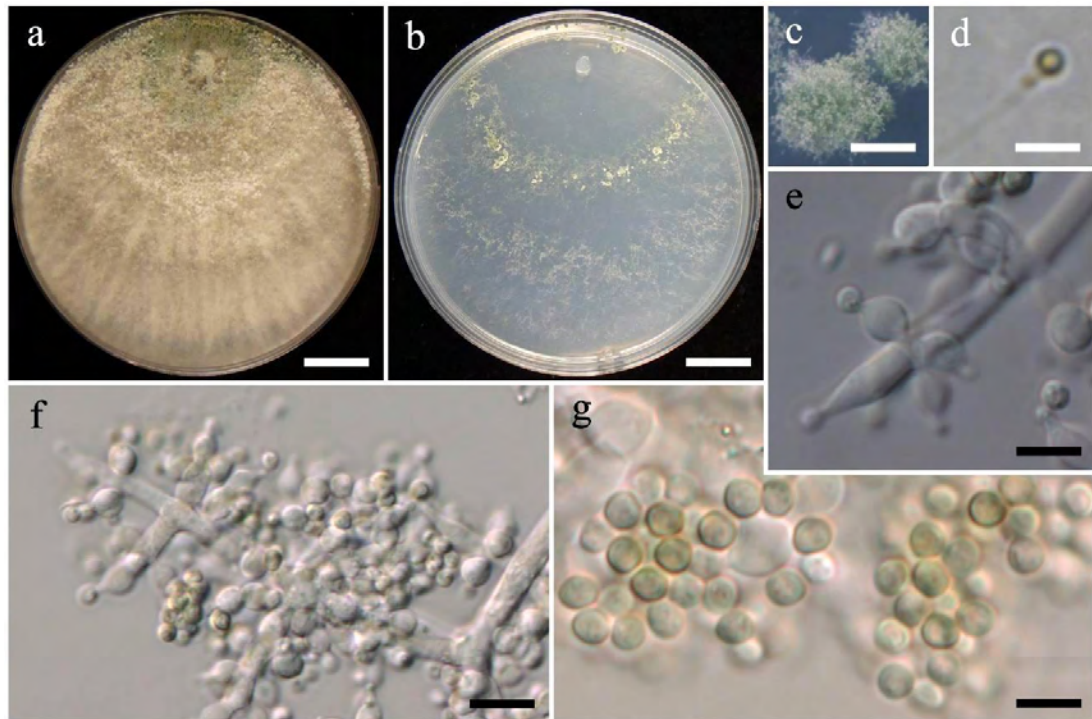
Figure 14. Ilustrations of clade 11. Cultures grown at 25 °C on PDA (a) and SNA (b) under 12h light regime; pustule (c) and chlamydospore (d) developed on SNA; phialides arising from the apex of pyramidal conidiophores (e; f); conidia subglobose to ovoid (g). Bars: a= 1.5 mm; b= 1.5 mm; c= 50.0 μm; d= 5.0 μm; e= 5.0 μm; f= 10.0 μm; g= 5.0 μm.
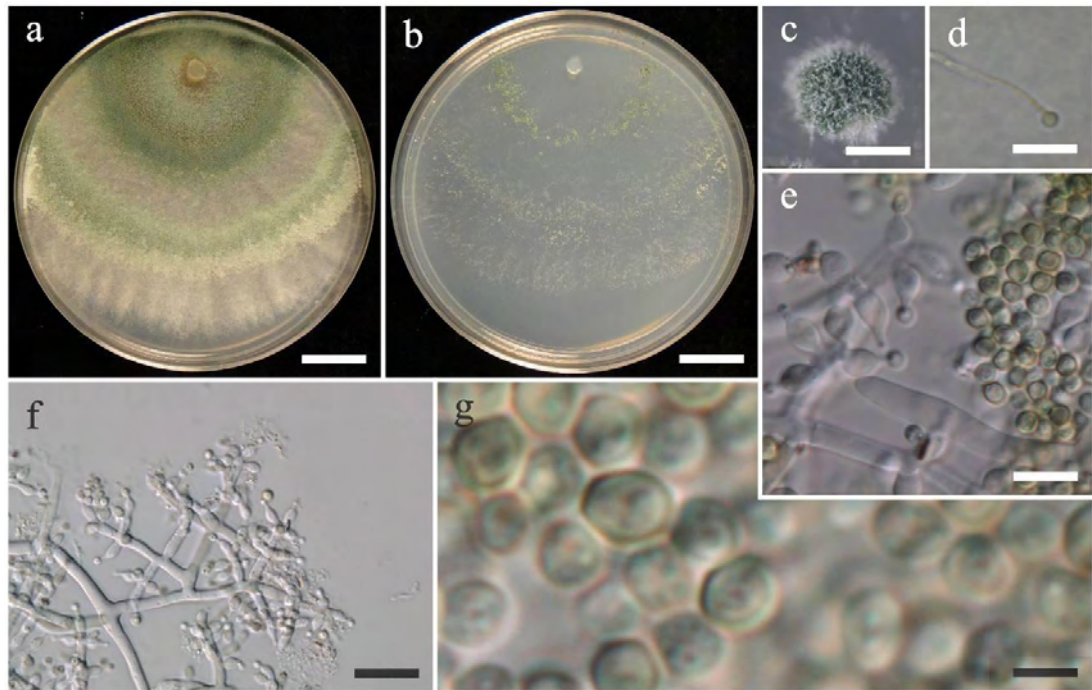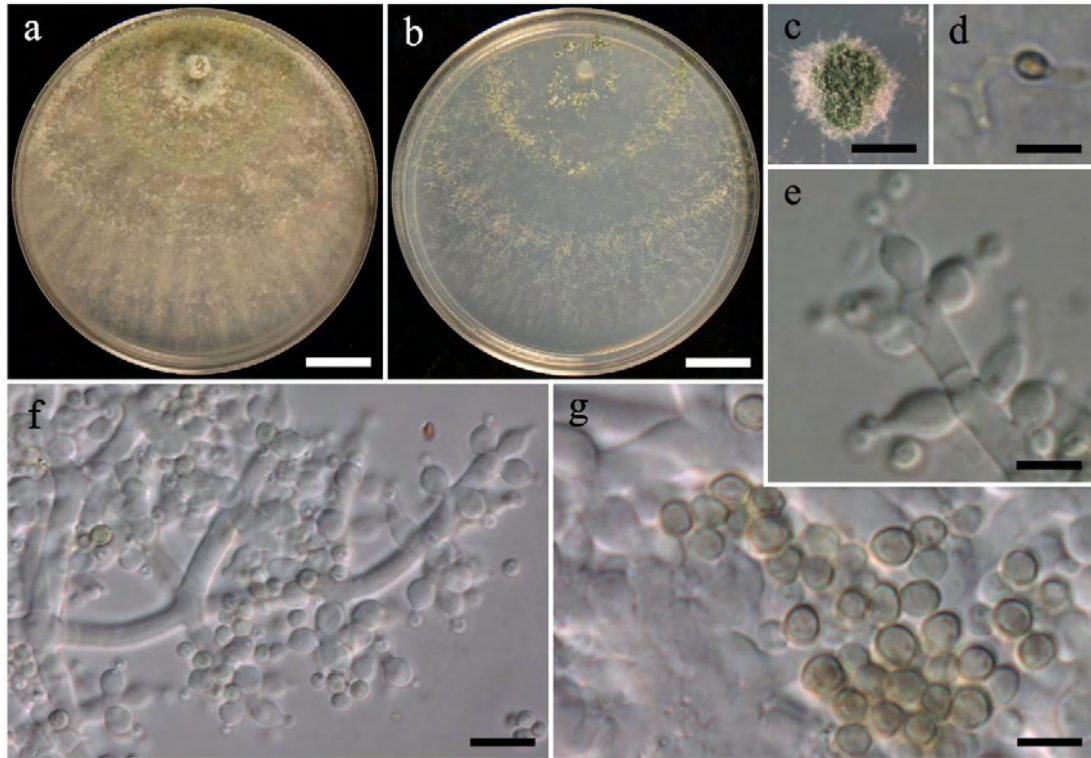
Figure 15. Ilustrations of clade 12. Cultures grown at 25 °C on PDA (a) and SNA (b) under 12h light regime; pustule (c) and chlamydospore (d) developed on SNA; phialides arising from the apex of pyramidal conidiophores (e; f); conidia subglobose to ovoid (g). Bars: a= 1.5 mm; b= 1.5 mm; c= 100.0 μm; d= 5.0 μm; e= 5.0 μm; f= 10.0 μm; g= 5.0 μm.

Table 1. Isolates characterized morphologicaly, their species name, substratum and collecting place.

Strain	Species Hypothesis	Substratum	Geographic location
Dis 377a	Clade 1	Endophytic in *Cola sp.*	Cameroon
Dis 314f	Clade 1	Endophytic in *Cola altissima*	Cameroon
GJS 06-98	Clade 1	Soil	Cameroon
GJS 99-227	Clade 1	Soil	Cameroon
Dis 218f	Clade 2	Endophytic in *Theobroma gileri*	Ecuador
Dis 220j	Clade 2	Endophytic in *Theobroma gileri*	Ecuador
Dis 217a	Clade 2	Endophytic in *Theobroma gileri*	Ecuador
Dis 217h	Clade 2	Endophytic in *Theobroma gileri*	Ecuador
Dis 217o	Clade 2	Endophytic in *Theobroma gileri*	Ecuador
Dis 221d	Clade 2	Endophytic in *Theobroma gileri*	Ecuador
GJS 11-184	Clade 2	Endophytic in *Hevea guianensis*	Peru
GJS 11-186	Clade 2	Endophytic in *Hevea brasiliensis*	Peru
Dis 220k	Clade 2	Endophytic in *Theobroma gileri*	Ecuador
Dis 355b	Clade 3	Endophytic in *Theobroma gileri*	Ecuador
Dis 337f	Clade 3	Endophytic in *Theobroma cacao*	Panama
Dis 269c	Clade 3	Endophytic in *Herrania sp.*	Ecuador
Dis 219f	Clade 4	Endophytic in *Theobroma gileri*	Ecuador
GJS 11-185	Clade 4	Endophytic in *Hevea guianensis*	Peru
GJS 11-187	Clade 4	Endophytic in *Hevea brasiliensis*	Peru
GJS 04-71	Clade 5	Endophytic in *Castanea sativa*	Italy
CBS 227.95	Clade 5	Soil	England
CBS 226.95	Clade 5	Soil	England
GJS 05-107	Clade 5	Endophytic in *Ricunus communis*	Italy
GJS 99-5	Clade 5	Soil	USA

Table 1. Isolates characterized morphologicaly, their species name, substratum and collecting place. (cont.).

Strain	Species Hypothesis	Substratum	Geographic location
IMI 359823	Clade 5	On mushroom compost	Ireland
GJS 90-127	Clade 6	Bark	USA
GJS 94-53	Clade 6	Decorticated wood	USA
GJS 90-22	Clade 6	Decorticated wood	USA
GJS 91-138	Clade 6	Bark	USA
Dis 314d	Clade 7	Endophytic in *Cola altissima*	Cameroon
Dis 314i	Clade 7	Endophytic in *Cola altissima*	Cameroon
Dis 314b	Clade 7	Endophytic in *Cola altissima*	Cameroon
Dis 375g	Clade 7	Endophytic in *Cola lateritia*	Cameroon
Dis 382aii	Clade 7	Endophytic in *Cola rostrata*	Cameroon
Dis 386fi	Clade 7	Endophytic in *Cola sp.*	Cameroon
GJS 00-24	Clade 8	Soil	Mexico
GJS 05-113	Clade 8	Endophytic in *Triticum aestivum*	Italy
GJS 04-186	Clade 8	On *Moniliophthora roreri*	Peru
GJS 04-197	Clade 8	On *Moniliophthora roreri*	Peru
GJS 04-02	Clade 8	unknown	USA
GJS 97-268	Clade 8	Soil	Japan
GJS 99-225	Clade 8	Soil	Cameroon
GJS 04-67	Clade 9	Soil	Italy
GJS 05-101	Clade 9	Soil	Italy
GJS 98-183	Clade 9	Teleomorph on *Ulmus* sp.	Austria
GJS 05-106	Clade 9	Soil	Italy
GJS 90-254	Clade 9	Teleomorph on *Pinus sylvestris*	Germany
GJS 92-110	Clade 9	Teleomorph on *Fagus* sp.	France

Table 1. Isolates characterized morphologicaly, their species name, substratum and collecting place (cont.).

Strain	Species Hypothesis	Substratum	Geographic location
Dis 110a	Clade 10	Endophytic in *Theobroma cacao*	Ecuador
Dis 167c	Clade 10	Endophytic in *Theobroma sp.*	Brazil
GJS 05-394	Clade 10	Soil	Camaroon
Dis 168a	Clade 10	Endophytic in *Theobroma cacao*	Brazil
Dis 169c	Clade 10	Endophytic in *Theobroma speciosum*	Brazil
Dis 173d	Clade 10	Endophytic in *Theobroma sp.*	Brazil
Dis 173f	Clade 10	Endophytic in *Theobroma sp.*	Brazil
Dis 218e	Clade 10	Endophytic in *Theobroma gileri*	Ecuador
Dis 233g	Clade 10	Endophytic in *Theobroma cacao*	Ecuador
Dis 246e	Clade 10	Endophytic in *Theobroma cacao*	Ecuador
Dis 246j	Clade 10	Endophytic in *Theobroma cacao*	Ecuador
Dis 246k	Clade 10	Endophytic in *Theobroma cacao*	Ecuador
Dis 253b	Clade 10	Endophytic in *Theobroma cacao*	Ecuador
Dis 264u	Clade 10	Endophytic in *Theobroma cacao*	Ecuador
Dis 67b	Clade 10	Endophytic in *Theobroma bicolor*	Ecuador
Dis 93d	Clade 10	Endophytic in *Theobroma cacao*	Peru
Dis 94d	Clade 10	Endophytic in *Theobroma sp.*	Peru
GJS 11-183	Clade 10	Endophytic in *Hevea guianensis*	Peru

Table 1. Isolates characterized morphologicaly, their species name, substratum and collecting place (cont.).

Strain	Species Hypothesis	Substratum	Geographic location
GJS 11-181	Clade 10	Endophytic in *Hevea guianensis*	Peru
GJS 11-188	Clade 10	Endophytic in *Hevea guianensis*	Peru
GJS 11-182	Clade 10	Endophytic in *Hevea guianensis*	Peru
GJS 11-189	Clade 10	Endophytic in *Hevea guianensis*	Peru
GJS 11-190	Clade 10	Endophytic in *Hevea brasiliensis*	Peru
GJS 11-180	Clade 10	Endophytic in *Hevea brasiliensis*	Peru
Dis 55f	Clade 10	Endophytic in *Theobroma cacao*	Ghana
Dis 55j	Clade 10	Endophytic in *Theobroma cacao*	Ghana
Dis 218h	Clade 11	Endophytic *in Theobroma gileri*	Ecuador
Dis 264v	Clade 11	Endophytic in *Theobroma cacao*	Ecuador
Dis 325ai	Clade 11	Endophytic in *Theobroma cacao*	Ecuador
Dis 354a	Clade 11	Endophytic in *Theobroma gileri*	Ecuador
Dis 64a	Clade 11	Endophytic in *Theobroma cacao*	Ecuador
GJS 00-06	Clade 12	Soil	Mexico
GJS 00-08	Clade 12	Soil	Mexico

Table 2. Morphological characteristics and growth rate of 'harzianum' complex species hypothesis.

Character		Clade 1	Clade 2	Clade 3	Clade 4.	Clade 5	Clade 6
	Species Hypothesis						
Colony radius PDA 72 H (mm)							
15 °C	Range	15-17	15-22	15-18	14-18	13-19	15-18
	Mean	16	18	17	16	17	18
	SD	1	3	1	2	2	2
	95% CI	13-18	16-21	14-19	10-21	14-19	15-20
	n	3	8	3	3	6	4
20 °C	Range	30-32	28-37	32-33	28-29	23-35	28-33
	Mean	31	33	33	29	30	31
	SD	1	4	0	1	5	2
	95% CI	29-33	30-36	32-34	27-30	25-35	27-34
	n	3	8	3	3	6	4
25 °C	Range	56-61	51-66	55-67	43-59	48-65	60-67
	Mean	59	59	63	53	57	64
	SD	2	5	7	8	6	3
	95% CI	53-64	55-63	46-79	32-73	51-64	59-68
	n	3	8	3	3	6	4
30 °C	Range	66-69	61-73	60-72	49-68	56-72	70-71
	Mean	68	68	68	59	67	71
	SD	2	4	7	10	6	1
	95% CI	64-73	65-71	51-86	35-84	61-74	69-72
	n	3	8	3	3	6	4
35 °C	Range	38-44	37-48	32-43	31-35	41-57	41-52
	Mean	41	41	39	32	51	48
	SD	4	4	6	2	6	4
	95% CI	33-50	38-45	23-54	26-38	45-57	40-55
	n	3	8	3	3	6	4

Table 2. Morphological characteristics and growth rate of 'harzianum' complex species hypothesis (Cont.).

Character		Species Hypothesis					
		Clade 1	Clade 2	Clade 3	Clade 4.	Clade 5	Clade 6
Colony radius SNA 72 H (mm)							
15 °C	Range	11-16	5-23	17-19	12-12	11-17	14-17
	Mean	12	14	18	12	14	16
	SD	3	6	1	0	2	1
	95% CI	6-19	9-19	15-20	12-12	12-16	14-18
	n	3	8	3	3	6	4
20 °C	Range	27-31	23-38	34-36	28-29	24-29	28-32
	Mean	29	29	35	29	27	29
	SD	2	6	1	1	2	2
	95% CI	24-34	24-34	32-39	26-31	25-29	26-33
	n	3	8	3	3	6	4
25 °C	Range	50-60	41-65	61-65	46-54	39-54	45-58
	Mean	55	53	63	51	49	51
	SD	5	8	2	4	6	5
	95% CI	44-67	46-59	58-68	40-62	43-56	43-60
	n	3	8	3	3	6	4
30 °C	Range	61-65	47-70	60-69	44-58	45-67	50-67
	Mean	62	58	64	52	56	57
	SD	2	8	4	7	7	7
	95% CI	57-67	51-64	53-75	33-71	49-64	46-68
	n	3	8	3	3	6	4
35 °C	Range	31-41	20-43	28-42	27-30	37-44	32-36
	Mean	35	34	35	28	40	34
	SD	5	6	7	1	3	2
	95% CI	23-48	29-39	17-52	25-32	37-43	31-36
	n	3	8	3	3	6	4
Phialides length (µm)	Range	4.1-8.7	3.5-9.5	4.2-9	3.6-12.3	4.7-16.2	4.1-9.1
	Mean	5.9	5.4	6	5.8	6.9	6.2
	95% CI	5.7-6.1	5.3-5.5	5.7-6.2	5.6-6.1	6.7-7.2	6-6.4
	SD	1	0.9	1.1	1.3	1.6	1.1
	n	90	241	90	90	180	120

Table 2. Morphological characteristics and growth rate of 'harzianum' complex species hypothesis (Cont.).

Character		Species Hypothesis					
		Clade 1	Clade 2	Clade 3	Clade 4.	Clade 5	Clade 6
Max. Width (µm)	Range	2.2-4	2.1-4.3	2.7-4.4	2.6-4.8	2.7-4.4	2.5-4.1
	Mean	3.1	3.3	3.5	3.6	3.5	3.3
	95% CI	3.1-3.2	3.3-3.4	3.4-3.6	3.5-3.7	3.5-3.6	3.2-3.4
	SD	0.4	0.4	0.4	0.5	0.4	0.4
	n	90	241	90	90	180	120
Base (µm)	Range	1.1-2.9	1-3.4	1.4-3	1.4-3.1	1.2-3	1.1-2.9
	Mean	1.9	2	2	2.2	2	2
	95% CI	1.8-2	2-2.1	1.9-2.1	2.1-2.2	1.9-2	1.9-2.1
	SD	0.4	0.4	0.4	0.3	0.4	0.3
	n	90	241	90	90	180	120
Width of supporting cell (µm)	Range	1.8-3.9	2-5.1	2-4.3	2.2-4.2	1.9-4.2	1.9-3.9
	Mean	2.8	3.1	2.9	3	3	2.8
	95% CI	2.8-2.9	3-3.2	2.8-3	2.9-3.1	3-3.1	2.7-2.8
	SD	0.4	0.5	0.4	0.5	0.4	0.4
	n	90	241	90	90	180	120
Ratio phialide length/max. width (µm)	Range	1.2-2.9	0.9-2.9	1.1-3.1	1.1-4.1	1.2-5	1.2-3.3
	Mean	1.9	1.6	1.7	1.7	2	1.9
	95% CI	1.8-2	1.6-1.7	1.7-1.8	1.6-1.8	1.9-2.1	1.8-2
	SD	0.4	0.3	0.4	0.5	0.5	0.5
	n	90	241	90	90	180	120
Ratio phialide length/width of supporting cell (µm)	Range	1.3-3	0.9-3.3	1.3-3.4	1.3-4	1.3-5.2	1.4-4
	Mean	2.1	1.8	2.1	2	2.3	2.3
	95% CI	2-2.2	1.7-1.8	2-2.2	1.9-2.1	2.3-2.4	2.2-2.4
	SD	0.4	0.4	0.4	0.4	0.7	0.5
	n	90	241	90	90	180	120

Table 2. Morphological characteristics and growth rate of 'harzianum' complex species hypothesis (Cont.).

Character		Species Hypothesis					
		Clade 1	Clade 2	Clade 3	Clade 4.	Clade 5	Clade 6
Ratio phialide max. width/width of supporting cell (μm)	Range	0.8-1.6	0.6-1.7	0.8-1.7	0.8-1.7	0.7-1.9	0.7-1.7
	Mean	1.1	1.1	1.2	1.2	1.2	1.2
	95% CI	1.1-1.2	1.1-1.1	1.2-1.3	1.2-1.3	1.2-1.2	1.2-1.3
	SD	0.2	0.2	0.2	0.2	0.2	0.2
	n	90	241	90	90	180	120
Conidia length (μm)	Range	2-3.3	2.2-3.6	2.2-3.4	2.5-3.6	2.3-4.2	2.4-3.7
	Mean	2.7	2.7	2.8	3	3.2	3
	95% CI	2.7-2.8	2.7-2.8	2.8-2.8	2.9-3	3.1-3.2	2.9-3
	SD	0.3	0.2	0.2	0.2	0.4	0.3
	n	90	241	90	90	180	120
Conidia width (μm)	Range	2-3.3	1.8-3.3	2-3.1	2.1-3.3	2-3.8	2.2-3.4
	Mean	2.6	2.5	2.6	2.7	2.8	2.7
	95% CI	2.5-2.6	2.5-2.6	2.6-2.6	2.7-2.8	2.7-2.8	2.6-2.7
	SD	0.3	0.2	0.2	0.2	0.3	0.2
	n	90	241	90	90	180	120
Ratio conidia length/width (μm)	Range	0.8-1.3	0.8-1.4	0.9-1.3	0.9-1.3	0.9-1.6	0.9-1.4
	Mean	1.1	1.1	1.1	1.1	1.1	1.1
	95% CI	1-1.1	1.1-1.1	1.1-1.1	1.1-1.1	1.1-1.2	1.1-1.1
	SD	0.1	0.1	0.1	0.1	0.1	0.1
	n	90	241	90	90	180	120
Condiophore main axis length (μm)	Range	00	22.9-104.4	32.2-103	24.6-104.6	00	16.5-105.9
	Mean	0	59.4	62.1	59.7	0	55.3
	95% CI	00	55.5-63.3	58.6-65.6	54.1-65.3	00	52-58.6
	SD	0	17.8	16.8	21.6	0	17.3
	n	0	83	90	60	0	106

Table 3. Morphological characteristics and growth rate of 'harzianum' complex species hypothesis, other clades.

Character		Species Hypothesis					
		Clade 7	Clade 8	Clade 9	Clade 10	Clade 11	Clade 12
Colony radius PDA 72 H (mm)							
15 °C	Range	12-16	12-20	17-22	12-20	16-20	16-18
	Mean	14	15	19	16	18	17
	SD	2	2	2	2	2	1
	95% CI	12-17	13-18	16-21	15-17	16-20	7-26
	n	5	7	5	26	5	2
20 °C	Range	24-33	27-36	26-36	24-37	31-34	29-32
	Mean	30	31	30	31	33	30
	SD	4	3	4	4	1	2
	95% CI	26-34	28-34	25-35	29-32	31-34	8-52
	n	5	7	5	26	5	2
25 °C	Range	45-62	49-64	37-55	51-74	54-69	62-63
	Mean	53	59	49	65	63	63
	SD	6	5	7	6	5	1
	95% CI	46-61	54-64	40-58	62-67	57-70	56-69
	n	5	7	5	26	5	2
30 °C	Range	54-69	56-70	35-59	33-74	67-74	70-72
	Mean	63	64	51	69	71	71
	SD	6	5	10	10	3	1
	95% CI	56-70	60-69	39-62	65-73	68-75	58-83
	n	5	7	5	26	5	2
35 °C	Range	25-36	34-51	4-22	15-57	13-46	42-49
	Mean	31	43	13	39	38	45
	SD	5	6	7	11	14	5
	95% CI	25-38	37-48	4-22	35-44	21-55	-2-93
	n	5	7	5	26	5	2

Table 3. Morphological characteristics and growth rate of 'harzianum' complex species hypothesis, other clades (Cont.)

Character		Species Hypothesis					
		Clade 7	Clade 8	Clade 9	Clade 10	Clade 11	Clade 12
Colony radius SNA 72 H (mm)							
15 °C	Range	6-13	12-20	16-20	11-21	18-21	16-17
	Mean	9	15	18	17	19	16
	SD	3	3	1	3	1	0
	95% CI	6-13	12-18	16-20	16-18	18-20	13-19
	n	5	7	5	26	5	2
20 °C	Range	25-34	27-36	27-36	21-38	30-37	30-36
	Mean	29	30	30	32	33	33
	SD	3	3	4	4	2	4
	95% CI	25-33	27-33	26-35	31-34	30-36	-5-71
	n	5	7	5	26	5	2
25 °C	Range	44-55	49-59	35-53	44-65	50-64	62-68
	Mean	51	56	47	59	59	65
	SD	4	4	8	5	5	4
	95% CI	46-56	53-59	37-57	57-61	53-66	26-103
	n	5	7	5	26	5	2
30 °C	Range	52-65	62-65	32-57	45-72	62-71	65-71
	Mean	59	64	48	65	68	68
	SD	4	1	10	5	3	5
	95% CI	53-64	63-65	35-60	62-67	64-72	26-109
	n	5	7	5	26	5	2
35 °C	Range	18-29	34-49	13-26	14-47	10-42	37-40
	Mean	25	43	20	35	33	38
	SD	5	5	6	8	14	2
	95% CI	19-31	38-47	13-27	32-39	16-50	16-60
	n	5	7	5	26	5	2
Phialides length (µm)	Range	4.1-10.7	4.6-17.5	4.6-13	3-12.3	3.4-9.7	4.2-8.3
	Mean	6.3	8.5	7	5.5	5.3	5.7
	95% CI	6.1-6.5	8.1-8.8	6.8-7.3	5.4-5.6	5.1-5.5	5.4-5.9
	SD	1.3	2.4	1.4	1.1	1.1	1
	n	180	180	120	750	120	60

Table 3. Morphological characteristics and growth rate of 'harzianum' complex species hypothesis, other clades (Cont.)

Character		Species Hypothesis					
		Clade 7	Clade 8	Clade 9	Clade 10	Clade 11	Clade 12
Max. Width (µm)	Range	2.2-4.1	2.2-4.4	2.3-4.4	2.4-5.1	2.3-4.4	3.1-4.4
	Mean	3.3	3.1	3.4	3.6	3.3	3.6
	95% CI	3.2-3.3	3.1-3.2	3.4-3.5	3.6-3.6	3.2-3.4	3.5-3.7
	SD	0.4	0.4	0.4	0.4	0.5	0.3
	n	180	180	120	750	120	60
Base (µm)	Range	1.1-3	1.3-2.9	1.3-2.8	1.1-3.5	1-2.9	1.3-3.1
	Mean	2	1.9	2.1	2.1	2	2
	95% CI	1.9-2	1.8-1.9	2-2.2	2-2.1	1.9-2	1.9-2.1
	SD	0.4	0.3	0.3	0.4	0.4	0.4
	n	180	180	120	750	120	60
Width of supporting cell (µm)	Range	1.8-4	1.9-4	2-4.1	1.8-4.8	1.8-4.4	2.2-6.6
	Mean	2.8	2.6	2.9	3.2	3.1	2.9
	95% CI	2.7-2.9	2.6-2.7	2.8-2.9	3.1-3.2	3-3.2	2.8-3.1
	SD	0.4	0.4	0.3	0.5	0.5	0.6
	n	180	180	120	750	120	60
Ratio phialide length/max. width (µm)	Range	1.1-3.8	1.3-7	1.2-4.4	0.9-3.7	1.1-3.3	1.1-2.4
	Mean	2	2.8	2.1	1.5	1.6	1.6
	95% CI	1.9-2	2.6-3	2-2.2	1.5-1.6	1.5-1.7	1.5-1.7
	SD	0.5	1.1	0.5	0.3	0.3	0.3
	n	180	180	120	750	120	60
Ratio phialide length/width of supporting cell (µm)	Range	1.3-4.6	1.4-7	1.3-5.1	0.9-4.5	1-5.3	0.9-2.9
	Mean	2.3	3.3	2.5	1.8	1.8	2
	95% CI	2.2-2.4	3.2-3.5	2.4-2.6	1.7-1.8	1.7-1.9	1.9-2.1
	SD	0.6	1.2	0.6	0.5	0.5	0.4
	n	180	180	120	750	120	60
Ratio phialide max. width/width of supporting cell (µm)	Range	0.8-1.9	0.8-1.9	0.8-1.8	0.6-2.1	0.8-1.9	0.6-1.7
	Mean	1.2	1.2	1.2	1.2	1.1	1.3
	95% CI	1.2-1.2	1.2-1.2	1.2-1.2	1.1-1.2	1.1-1.1	1.2-1.3
	SD	0.2	0.2	0.2	0.2	0.2	0.2
	n	180	180	120	750	120	60

Table 3. Morphological characteristics and growth rate of 'harzianum' complex species hypothesis, other clades (Cont.)

Character		Species Hypothesis					
		Clade 7	Clade 8	Clade 9	Clade 10	Clade 11	Clade 12
Conidia length (µm)	Range	2-4.1	2.1-4.6	2.3-3.7	2.2-4.1	2.1-3.8	2.4-3.5
	Mean	2.8	3.2	3.1	2.8	2.7	2.9
	95% CI	2.8-2.9	3.1-3.2	3-3.1	2.8-2.8	2.7-2.8	2.8-3
	SD	0.3	0.4	0.3	0.3	0.3	0.3
	n	180	180	120	750	120	60
Conidia width (µm)	Range	2-3.5	2.1-4.1	2-3.5	1.9-3.4	1.8-3	2.3-3.2
	Mean	2.7	2.8	2.8	2.6	2.5	2.7
	95% CI	2.6-2.7	2.8-2.9	2.8-2.8	2.6-2.6	2.4-2.5	2.7-2.8
	SD	0.3	0.3	0.3	0.2	0.2	0.2
	n	180	180	120	750	120	60
Ratio conidia length/width (µm)	Range	0.8-1.5	0.9-1.5	0.9-1.4	0.8-1.5	0.8-1.7	0.9-1.3
	Mean	1.1	1.1	1.1	1.1	1.1	1.1
	95% CI	1.1-1.1	1.1-1.1	1.1-1.1	1.1-1.1	1.1-1.1	1-1.1
	SD	0.1	0.1	0.1	0.1	0.1	0.1
	n	180	180	120	750	120	60
Condiophore main axis length (µm)	Range	13.2-92	27-134.5	21-97.1	5.1-138	10-130	28-102.3
	Mean	54.1	66.1	56.5	60.3	61.7	59.8
	95% CI	42.5-65	61.1-71	52.8-60	58-61.9	56.5-67	55-64.4
	SD	24.8	24.6	17.3	21.5	23	18.1
	n	20	98	86	680	77	60

2. CHAPTER TWO

Hyphomycetes associated with *Euterpe edulis* (Arecaceae), an endangered palm tree from the Brazilian Atlantic Forest

Fabiano B. Rocha, Robert W. Barreto, Graciele V. Poleto, Olinto L. Pereira and João A. A. Meira Neto

Abstract

Four anamorphic fungi were found associated with *Euterpe edulis* (Arecaceae) a palm species included in the official list of endangered species in Brazil. Fungi associated with that host plant were collected in three Brazilian states: Espírito Santo, Minas Gerais and Rio de Janeiro. Two of these fungi were recognized as known to science, *Bipolaris cynodontis* and *Passalora eitenii*, whereas two are described herein as new to science: *Distocercospora* sp. nov and *Melanographium* sp. nov. It is possible that both newly described species should be recognized as also being in danger of extinction in case it is demonstrated that they are specialized, host-specific pathogens.

1. Introduction

Reports of fungi associated with endangered plants usually are made without emphasis about their status of being threatned of becoming extinct (Braun and Freire 2004). There are few publications addressing the issue of endangered microfungi on plants (Dulymamode et al. 2001, Rocha et al; 2010, Siboe et al. 2000). Most of the microfungi reported associated with endangered plants are plant pathogenic fungi and hence often viewed solely as noxious organisms even by professional plant pathologists and mycologists. In reality only a minority of such fungal species cause significant impact on their hosts that would justify such negative views. In general, it can be said that the majority of fungal pathogens of plants are organisms associated with wild plants and are not negative to human interests. In fact, they often play important roles in regulation of plant populations contributing to maintaining a high diversity of plants in natural ecosystems (Dighton 2003). Although there are no precise accounts, it is certain that plant pathogenic fungi on wild plants represent a very significant part of the global biodiversity. Unfortunately, most of it remains to

be discovered and named. The loss of such species is probably happening at a fast pace. It is known that each plant species supports a unique mycobiota, often including highly specialized parasites and mutualistic symbionts which would become extinct if their host plant disappears in a process long ago named co-extinction (Dunn et al. 2009).

As a large tropical country including numerous unique ecosystems Brazil is widely recognized as one of the few countries having a megadiversity (Myers et al 2000). One major Brazilian ecosystem which is recognized as a biodiversity hot-spot is Atlantic forest. This, originally occupied 1,100,000 km^2 of the Brazilian territory and covered areas ranging from the extreme south in Rio Grande do Sul to humid parts of the northeast. The destruction of this ecosystem has been immense and it is estimated that only 11.4-16.0 % of the original area of Atlantic forest remains. (Ribeiro et al. 2009). Among the species typical of the primary Atlantic rainforest *Euterpe edulis* Mart. (Arecaceae) (Figure 1a) used to be a prominent and common species broadly spread in Brazil from the extreme south in the state of Rio Grande do Sul to humid areas of the northeast (Lorenzi, 2010). It became increasingly rare because of a combination of habitat destruction and overexploitation as the main source of heart of palm for the canning industry and for fresh consumption (Fig 1b). A study of the mycobiota of *E. edulis* was started as part of an ongoing study that was recently started in Brazil aimed at surveying fungi associated with endangered plant species. Discovering and naming fungal species possibly threatened with co-extinction is the first step towards highlighting their endangered status and the need to preserve them and their hosts both *in situ* - in their natural habitat and *ex situ* - in botanic gardens and culture collections (Moore et al 2001). A preliminary survey of fungi associated with *Euterpe edulis* was performed in different fragments of Atlantic rainforest in the states of Espírito Santo, Minas Gerais and Rio de Janeiro. The objective of this work was to provide a taxonomic assessment of the four anamorphic fungi associated with the endangered of extinction *Euterpe edulis*.

2. Material and Methods

The survey for fungi associated with *Euterpe edulis* was initiated in small forest fragments in areas of the municipalities of Viçosa, (state of Minas Gerais), Venda Nova dos Imigrantes (state of Espirito Santo) and Nova Friburgo (state of Rio

de Janeiro). Additionally a specimen was collected in a commercial nursery in Viçosa.. The focus was plant pathogenic fungi associates to leaves. Fresh leaves were collected using a long poled prunner. Freshly collected *E. edulis* leaves colonized by fungi were examined under a stereomicroscope. Leaves bearing fungal structures were selected for further study. Hand sections containing fungal structures were prepared or fungal material was scraped from colonized tissues and mounted in lactophenol (20 g phenol, 20 ml lactic acid, 40 ml glycerin and 40 ml water). Observations were made and measurements and line drawing were prepared with an Olympus BX 51 light microscope fitted with a drawing tube.

The fungal isolation was performed by directly transferring conidia with a sterile fine-pointed needle from the lesions onto plates containing Vegetable Broth Agar (Pereira et al. 2003). Pure cultures were preserved in silica-gel (as described in Dhingra and Sinclair 1995), and were deposited at the culture collection of the Departamento de Fitopatologia of the Universidade Federal de Viçosa.

Identification was based on observation of distinctive morphological characters and the use of keys for genera (Carmichael et al. 1980, Ellis 1971). Identification to the species level was attempted through comparison with original descriptions of species belongint to each genus.

3. Results

Four anamorphic fungi were found associated with *Euterpe edulis* leaves during the survey. These are described and discussed below.

3.1. Taxonomy

Bipolaris cynodontis (Marignoni) Shoemaker   (Figure 2)

Lesions on leaves, amphigenous, 0.4 – 2.1 cm, necrotic, edge dark brown, center pale brown to grey, initially rounded becoming elliptical following the veins. *Internal mycelium* indistct. *External mycelium* absent. *Stromata* absent. *Conidiophores* amphigenous, solitary, straight to flexuous, subcylindrical, 60.0 – 185.0 × 5.0 – 7.5 μm, 1 – 10 septate, rarely branched, yellowish brown to brown, paler towards the apex, smooth. *Conidiogenous cell* terminal and intercalary, integrated, sympodial, subcylindrical, 7.5 – 40.0 × 4.0 – 7.5 μm, pale brown to brown. *Conidiogenous loci* conspicuous, rounded, 4.0 – 5.0 μm, thickened, black.

Conidia dry, solitary, fusiform, straight to slightly curved, $27.5 - 110.0 \times 10.0 - 22.5$ μm, apex and base rounded, base $4.0 - 7.5$ μm, $2 - 9$ septate, conidial scar externally thickened truncate, black, eguttulate, yellowish pale brown to brown, smooth.

Specimens examined. BRAZIL. Minas Gerais: Viçosa, Viveiro Antuérpia. On living leaves of *Euterpe edulis*, Sept 2009, R.W. Barreto. Herbarium accession number: FBR 100.

Note: There are 73 species in the genus *Bipolaris* (Kirk et al. 2008) but only three are known in association with the Arecaceae: *Bipolaris incurvata* (C. Bernard) Alcorn, *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker and *Bipolaris setariae* Shoemaker (Farr et al. 2006). The fungus collected on *E. edulis* had the morphological features typical of *B. cynodontis* as described in Sivanesan (1987). This is the first time that *B. cynodontis* is reported in a plant species belonging to Arecaceae.

Distocercospora sp.   (Figure 3)

Lesions amphigenous on leaves, beginning as small black dots, becoming ellipsoid following the veins, with grey necrotic center and a black to dark purple edge, 0. $0.7 - 2.0 \times 0.5 - 4.5$ cm. *Internal mycelium* subhyaline, septate, branched and smooth. *External mycelium* absent. *Stroma* limited to a few brown cells in the stomatal cavity. *Conidiophores* hypophyllous, fasciculate, subcylindrical, $52.5 - 130.0 \times 3.75 - 5.0$ μm, $1 - 3$ septate, unbranched, brown, smooth. *Conidiogenous cell* terminal, integrated, sympodial, subcylindrical, $12.5 - 47.5 \times 3.5 - 5.0$ μm, smooth, brown. *Conidiogenous loci* conspicuous, on the tip of the conidiophores apex, round, $2.5 - 4.5$ μm, thickened, black. *Conidia* dry, solitary, obclavate, $26.0 - 79.0 \times 2.5 - 5.0$ μm, $3 - 7$ distoseptate, conidial scar thickened, dark, egutulate, hyaline, smooth.

Specimens examined. BRAZIL. Minas Gerais: Viçosa, Fazenda Bom Sucesso. On living leaves of *Euterpe edulis*, May 2006, F.B. Rocha. Herbarium accession number: FBR 74.

Note: Three species have been described in the genus *Distocercospora* and their main morphological features are given in Table 1 for comparison. Only *Distocercospora livistonae* U. Braun & C.F. Hill was described in association with a

member of the Arecaceae (*Livistona chinensis* (Jacq.) R. Br. ex Mart.) in New Zealand (Braun et al 2006). *Distocercospora africana* Crous & U. Braun (Craus and Braun 1994) and *Distocercospora pachyderma* (Syd. & P. Syd.) N. Pons & B. Sutton (Pons and Sutton 1988) are associated with different species of *Dioscorea*. None of these species were reported in any country of the Americas. *Distocercospora livistonae* differs from *Distocercopora sp.*, by having well developed stromata, larger conidiophores (40.0 − 280.0 × 3.0 − 6.0 μm), thicker conidia (4.0 − 7.0 μm diam) wich are narrower at base (2.0 − 3.0 μm) (Braun et al 2006). The type species of *Distocercospora*, *D. pachyderma*, differs from *Distocercospora sp.* by having branched, larger and smooth to verrucose conidiophores (16.0 − 400.0 × 4.0 − 6.0 μm). It also has strongly incurved conidia that bear a smaller number of septae (1 − 4 septate), are finely verrucose and subhyaline to pale brown (Pons and Sutton 1988). *Distocercospora africana* resembles *Distocercospora* sp. nov., but has shorter (15.0 − 80.0 μm) and thicker (3.0 − 10.0 μm) conidiophores that are sometimes branched arising from a well developed stroma (Crous and Braun 1994). We regard the fungus on *E. edulis* as sufficiently distinct morphologically from other species in the genus to justify its recognition as a new species.

Melanographium sp.   (Figure 4)

Lesions necrotic, 20.0 − 285.0 × 4.0 − 65.0 mm, on the apex of the leaves. *Internal mycelium* branched, septate, pale brown. *External mycelium* absent. *Stroma* brown to dark brown. *Conidiophores* amphigenous, often forming synemata but also appearing as somewhat loose fascicles and in isolation, subcylindrical, straight, 227.5 − 407.5 × 3.75 − 6.25 μm, 7 − 14 septate, unbranched, brown, paler towards the apex, smooth. *Conidiogenous cell* terminal or intercalary, integrated, holoblastic, poliblastic, denticulate, sympodial, subcylindrical, 12.5 − 81.25 × 3.75 − 7.5 μm, pale brown. *Conidiogenous loci* conspicuous, denticulate, 2.0 − 2.5 μm diam. *Conidia* dry, solitary, reniform to ellipsoid, with a longitudinal germ slit, 11.0 − 21.0 × 9.0 − 13.0 μm, 1-celled, conidial scar unthickened, guttulate, brown to dark brown, smooth.

Specimens examined. BRAZIL. Rio de Janeiro: Nova Friburgo, Riograndina. On living leaves of *Euterpe edulis*, Sept 2009, R.W. Barreto. Herbarium accession number: FBR 75.

Note: There are fifteen species described in the genus *Melanographium* (Robert et al. 2005), seven of which are known in association to members of the Areceaceae (Farr and Rossmann 2006), the main morphological characters for each of these species are listed on Table 2. The sole exception was *Melanographium trachycarpi* I. Hino & Katum (Katumoto 1962) for which very little information is available. *Melanographium calami* N. Srivast., A.K. Srivast. & Kamal differs from *Melanographium* sp. nov. by having conidiophores with up to 6 septa, and conidia which are very strongly curved giving them a more or less horse-shoe appearance (Srivastava ans Morgan-Jones 1996). *Melanographium citri* (Gonz. Frag. & Cif.) M.B. Ellis is clearly distinct from the new species by having conidiophores becoming increasingly thick towards the apex, and conidia which are smooth to verruculose (Ellis 1963). *Melanographium cookei* M.B. Ellis has longer conidiophores (up to 1700.0 μm) and wider conidia (up to 18.0 μm wide) than those of *Melanographium* sp. which are only formed at the apex of the conidiophores (Ellis 1963). *Melanographium fasciculatum* S. Hughes can be distinguished from *Melanographium* sp. nov. as by having conidiophores becoming wider towards the apex, conidia that are oval or limoniform, smooth or verruculose (Ellis 1963). *Melanographium indicum* Saikia & A.K. Sarbhoy can be distinguished from *Melanographium* sp. nov. by having shorter conidiophores with up to 7 septa that are usually confined to the lower part of the conidiophores. Its conidia are formed on the tip of the conidiophores (Saikia and Sarbhoy 1981). *Melanographium selenioides* (Sacc. & Paol.) M.B. Ellis has longer conidiophores (800.0 − 2500.0 μm) than those of *Melanographium* sp. nov. and becomes increasingly wider towards the apex, splaying out in a branch-like manner along the sides and specially towards the apex of the synemata. Its conidia arise only from the apex of the conidiophores and are smooth to verruculose (Ellis 1963). The available description of *Melanographium trachycarpi*. is very poor and information available does not allow for a proper comparison with the fungus on *E. edulis* but it assumed here that, as this fungus was described on a different host genus and species (*Trachycarpus fortunei* (Hook.) H. Wendl.) and was collected in Japan (Katumoto 1962), that it is highly unlikely that it is conspecific with the fungus from Brazil. Additionally, *Melanographium* sp.nov. is the only species associated with Arecaceae that has denticulate conidiogenous loci. This is the first species in this fungal genus described in association with *Euterpe edulis*.

Passalora eitenii R.B. Medeiros & Dianese   (Figure 5)

Lesions at the margins or apex of leaves, 2.0 – 20.0 cm, necrotic, pale brown. *Internal mycelium* intercellular, 1.5 – 2.0 μm, branched, septate, pale brown. *External mycelium* absent. *Stroma* rudimentary, composed of few brown angular cells. *Conidiophore* amphigenous, fasciculate, cylindrical to subcylindrical, 27.0 – 80.0 × 3.5 – 6.0 μm, 1 – 4 septate, unbranched, pale brown to brown, smooth. *Conidiogenous cell* terminal, integrated, sympodial, cylindrical to subcylindrical, 13.0 – 40.0 × 3.5 – 5.0 μm, pale brown. *Conidiogenous loci* conspicuous, 1 -3 per cell, rounded, 2.0 – 2.5 μm, slightly thickened, black. *Conidia* dry, solitary, holoblastic, subcylindrical, 22.0 – 65.0 × 3.5 – 4.5 μm, apex round, base truncate, 2.0 – 2.5 μm, 1 – 4 septate, conidial scar slightly thickened, eguttulate, pale brown, smooth to finely verruculose.

Specimens examined. BRAZIL. Minas Gerais: Carangola. On living leaves of *Euterpe edulis*, Aug 2009, F.B. Rocha. Herbarium accession number: FBR 114.

Note: *Passalora eitenii* is the sole species among more than 600 species included in *Passalora* (Farr and Rossmann 2006) that has been reported on a member of the Arecaceae. The specimen found associated with *Euterpe edulis* fits well into the description provided by Medeiros and Dianese (1994), for *P. eitenii* which was originally found associated with *Syagrus comosa* (Mart.) Mart. in Brasilia, Brazil. This is the first report of *P. eitenii* on *E. edulis*.

4. Discussion

Four anamorphic fungi have been found and described associated in association with *Euterpe edulis*. The morphological characters described above indicate that two of the species which were found are known species: *Bypolaris cynodontis* is a common species associated with several members of the Poaceae (Farr et al. 2006) but this is the first time that this species is reported on a member of the Arecaceae. *Passalora eitenii* was originally described as a parasite on *Syagrus comosa*. It is possible that *P. eitenii* is a polyphagous species in the Arecaceae and hence isn't threatened of co-extinction but experimental inoculation studies should be performed in order to confirm this. The newly described species of *Melanographium* and *Distocercospora* are unknown on other hosts and may

represent endangered fungal species if proven host-specific and host-dependent. This hypothesis requires experimental verification. The elucidation of the taxonomic status of the fungi collected on *E. edulis* represents only the first step towards the listing of fungi on this host which may deserve being regarded as endangered of extinction.

5. Acknowledgements

6. Bibliography

Braun U, Freire FDO, 2004. Some cercosporoid hyphomycetes from Brazil – III. *Cryptogamie, Mycologie*. 25, 221-244.

Braun U, Hill F, Schubert K, 2006. New species and new records of biotrophic micromycetes from Australia, Fiji, New Zealand and Thailand. *Fungal Diversity* 22: 13-35.

Carmichael JW, Kendrick WB, Conners IL, 1980. Genera of Hyphomycetes. University of Alberta Press. 396p.

Craus PW, Braun U, 1994. *Cercospora* species and similar fungi occurring in South Africa. *Sydowia* 46, 204-224.

Dhingra OD, Sinclair JB, 1995. Basic plant pathology methods, 2nd ed. Lewis, Boca Raton

Dighton J, 2003. Fungi in ecosystem processes. Vol. 17. CRC Press, Mycology series. USA.

Dulymamode R, Cannon P, Peerally A, 2001. Fungi on endemic plants of Mauritius. *Mycological Research*. 105, 1472-1479.

Dunn RR, Harris NC, Colwell RK, Koh LP, Sodhi NS, 2009. The sixth mass coextinction: are most endangered species parasites and mutualists?. *Proceedings of the Royal Society B*. 276, 3037-3045.

Ellis MB, 1963. Dematiaceous Hyphomycetes. V. *Mycological Papers* 93, 1-33.

Ellis MB, 1971. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute Kew, Surrey, England. 608p.

Farr DF, Rossman AY, 2006. Fungal Databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. http://nt.ars-grin.gov/fungaldatabases. Accessed February 03, 2011.

Katumoto K, 1962. Notes on fungi from western Japan. 5. *Journal of Japanese Botany* 37, 295-299.

Kirk PM, Cannon PF, Minter DW, Stalpers JA, 2008. Dictionary of the Fungi. 10th edition. CABI Europe – UK. 771p.Lorenzi H, Noblick LR, Kahn F, Ferreira E, 2010. Flora brasileira Lorenzi: Arecaceae (palmeiras). Instituto Plantarum, Nova Odessa, SP. 284pp.

Medeiros RB, Dianese JC, 1994. *Passalora eitenii* sp. nov. on *Syagrus comosa* in Brazil and a key to *Passalora* species. *Mycotaxon*. 51, 509-513.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J, 2000. Biodiversity hotspots for conservation priorities. *Nature* 403, 853-858.

Ministério do Meio Ambiente – MMA, 2002. Avaliação de áreas e ações prioritárias para a conservação, utilização sustentável e repartição dos benefícios da biodiversidade nos biomas brasileiros. Brasília: MMA/SBF. 404p.

Moore D, Nauta MM, Evans SE, Rotheroe M, 2001. *Fungal Conservation: Issues and Solutions*, Cambridge University Press, Cambridge,MA, USA. 262 pp.

Pereira JM, Barreto EC, Maffia LA, 2003. *Corynespora cassiicola* f. sp. *lantanae*: a potential biocontrol agent for *Lantana camara* from Brazil. *Biological Control* 26, 21–31.

Pons N, Sutton BC, 1988. *Cercospora* and similar fungi on yams (*Dioscerea* species). *Mycological Papers* 160, 1-78.

Ribeiro MC, Metzger JP, Martensen AC, Ponzoni FJ, Hirota MM, 2009. The Brazilian Atlantic Forest: How much is left, and how is the remaining forest

distributed? Implications for conservation. *Biological Conservation* 142, 1141-1153.

Robert V, Stegehuis G, Stalpers J, 2005. The MycoBank engine and related databases. http://www.mycobank.org. Accessed February 03, 2011.

Rocha FB, Barreto RW, Bezerra JL, Meira Neto JAA, 2010. Foliage mycobiota of *Coussapoa floccosa*, a highly threatened tree of the Brazilian Atlantic Forest. *Mycologia*. 102, 1240-1252.

Saika UN, Sarghoy AK, 1981. Hyphomycetes of north-eastern India - IV. The genus Melanographium. *Indian Phytopathology* 34, 356-367.

Siboe GM, Kirk PM, David JC, Cannon PF, 2000. Necrotrophic fungi from Kenyan endemic and rare plants. *Sydowia*. 52, 286-304.

Sivanesan A, 1987. Graminicolous species of *Bipolaris*, *Curvularia*, *Drechslera*, *Exserohilum* and their teleomorphs. *Mycological Papers*. 159, 1-261.

Srivastava N, Morgan-Jones G, 1996. Notes on hyphomycetes LXX. A new species of *Melanographium* from india, with comments on the genus. *Mycotaxon* 57, 195-200.

7. Figures and Tables

Figure 1. *Euterpe edulis* individual (a); individual of *E. edulis* felled for extraction of heart of palm (b).
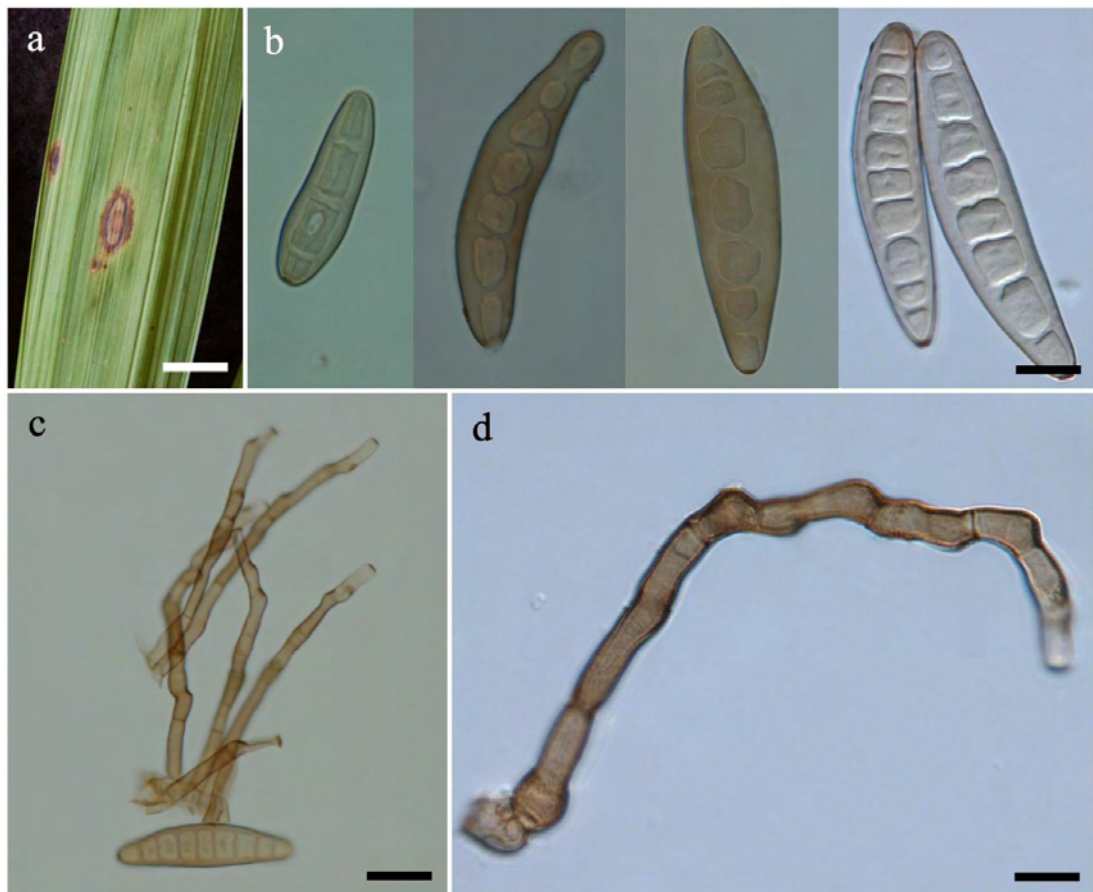

Figure 2. *Bipolaris cynodontis*. *Euterpe edulis* leaf showing lesion caused by *B. cynodontis* (a); conidia (b); and geniculate conidiophores (c, d). Bars: a = 2.0 cm; b= 15.0 µm; c= 30.0 µm; d= 10.0 µm.
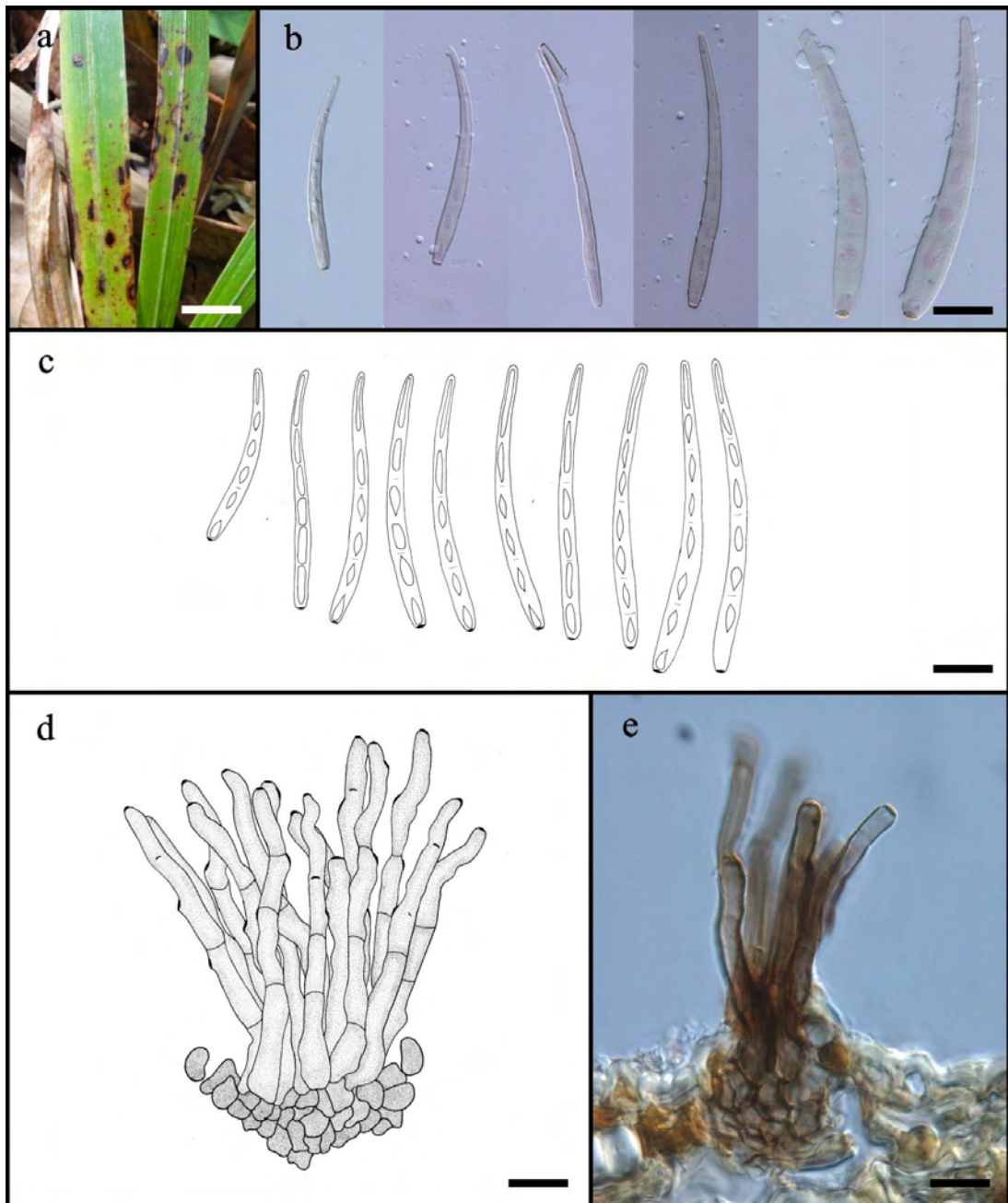
Figure 3. *Distocercospora* sp. *Euterpe edulis* leaf presenting lesions (a); conidia (b, c); and fasciculate conidiophore (d, e). Bars: a= 5.0 cm; b= 10.0 μm; c= 10 μm; d= 10.0 μm; e= 10.0 μm.
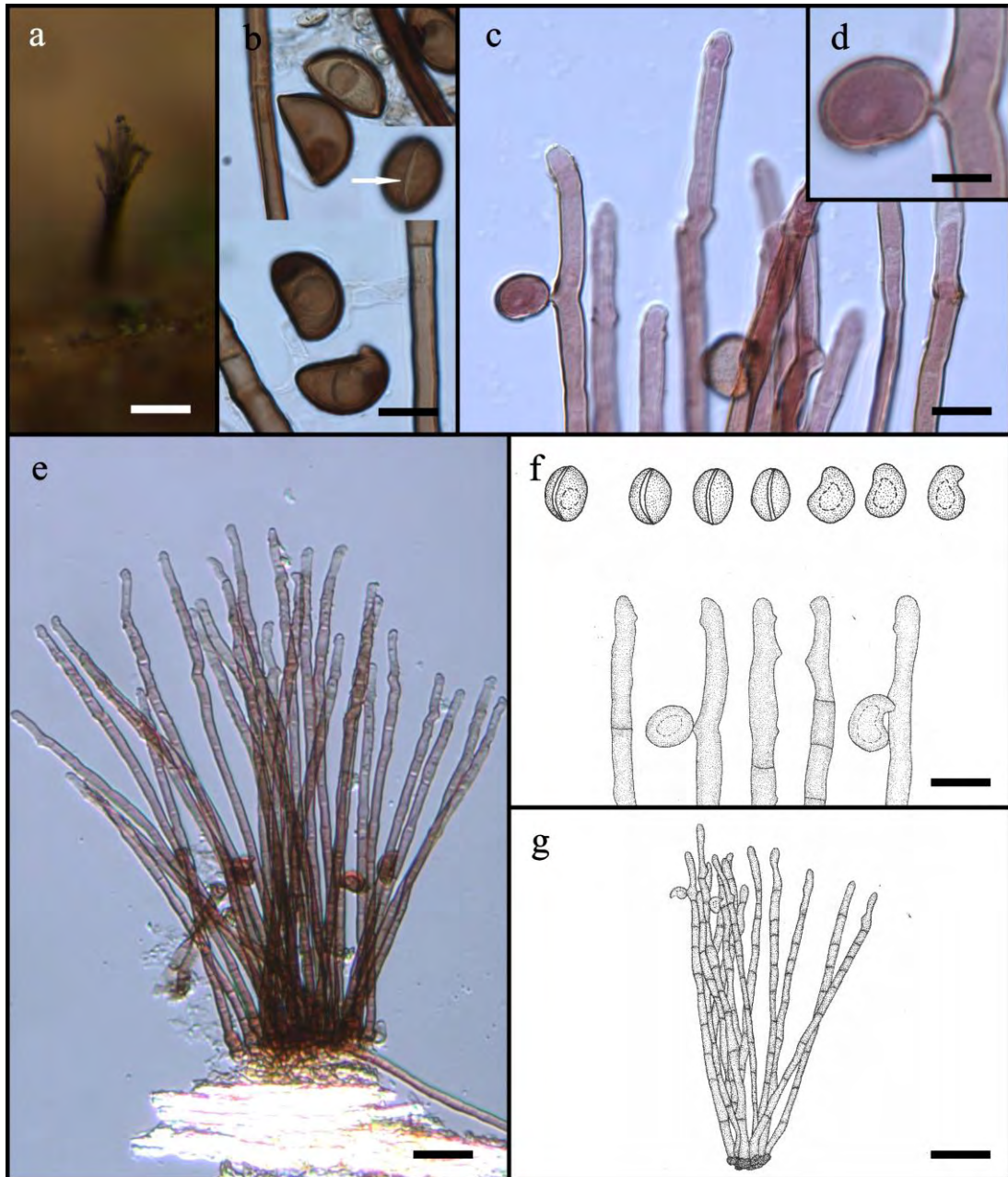
Figure 4. *Melanographium* sp. Synemium on leaf of *Euterpe edulis* (a); reniform conidia with germ slit (arrowed) (b); conidia attached to a denticle (c); fasciculate conidiophores (e); drawing of conidia and conidiophores (f, g).Bars: a= 100.0 µm; b= 10.0 µm; c= 10.0 µm; d= 5.0 µm; e= 35.0 µm; f= 10.0 µm; g= 50.0 µm.
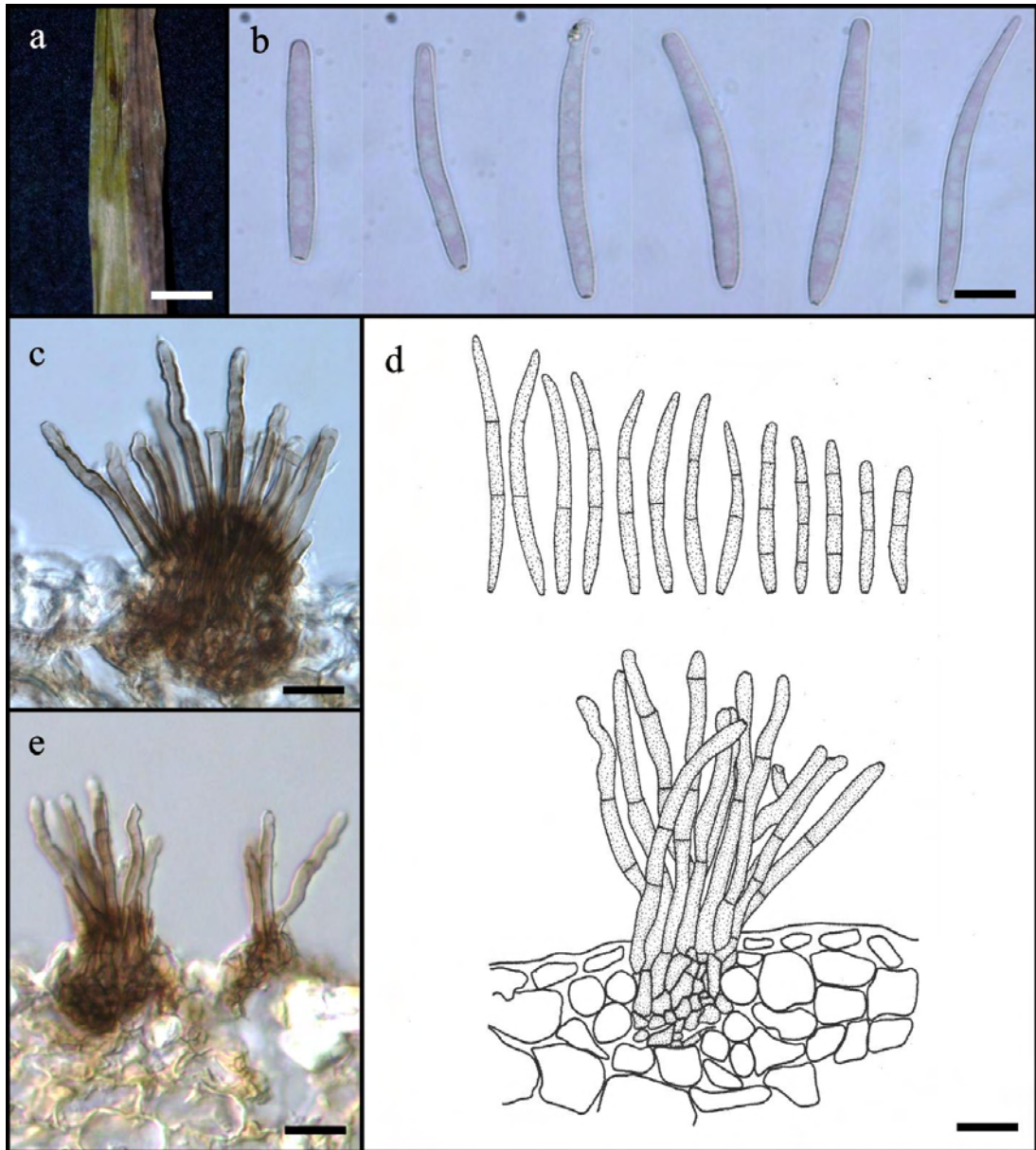
Figure 5. *Passalora eitenii*. Necrotic lesion to which *P. eitenii* was associated (a); conidia (b); conidiophores (c, e); and drawing of conidia and conidiophores (d). Bars: a= 2.5 cm; b= 10.0 μm; c= 15.0 μm; d= 10.0 μm; e= 25.0 μm.

Table 1. Main morphological characters of *Distocercospora*

Characters		*Distocercospora* on *Euterpe edulis*	*D. africana*	*D. livistonae*	*D. pachyderma*
Stromata		- or small	+	+	- or small
Conidioph.	Length	52.5 - 130.0	15.0 - 80.0	40.0 - 280.0	16.0 - 400.0
	Width	3.75 - 5.0	3.0 - 10.0	3.0 - 6.0	4.0 - 6.0
	Branches	unbranched	branched	unbranched	branched
	Surface	smooth	not inf.	not inf.	Smooth to verrucose
Conid. cell	Length	12.5 - 47.5	not inf	10.0 - 30.0	18.0 - 55.0
	Width	3.5 - 5.0	not inf.	not inf.	3.0 - 5.0
Conidia	Length	26.0 - 79.0	30.0 - 110	20.0 - 85.0	18.0 - 82.0
	Width	2.5 - 5.0	3.0 - 5.0	4.0 - 7.0	1.5 - 5.0
	Septae	3.0 - 7.0	1.0 - 6.0	2.0 - 5.0	1.0 - 4.0
	Base	2.5 - 5.1	not inf.	2.0 - 3.0	1.5 - 5.0
	Shape	slightly curved	obclavate	obclavate	very curved sometimes
	Surface	smooth	not inf.	not inf.	finely verrucose
	Conid. loci	2.5 - 5.0	not inf.	2.0 - 2.5	1.0 - 2.0

Table 2. Main morphological characters of *Melanographium* associated Arecaceae

		Melanog. on *E. edulis*	*M. anceps*	M. *calami*	*M. citri*	*M. cookei*	*M. fasciculatum*	M. indicum	M. selenioides
Conidio phores	Length	227.5 - 407.5	600 - 1000	100 - 300	150 - 1200	160 - 1700	150 - 600	120 - 210	800 - 2500
	Width	3.75 - 6.25	4.0 - 5.0	5.0 - 7.0	3.0 - 5.0	4.0 - 6.0	4.0 - 6.0	4.5 - 6.0	4.0 - 7.0
	Septae	7.0 - 14.0	not inf.	up to 6	not inf.	not inf.	not inf.	2.0 - 7.0	not inf.
	Branc hes	unbranched	not inf.	unbranch ed	unbranch ed	unbranc hed	not inf.	unbranc hed	branched-like strutures
	Surfac e	smooth	not inf.	smooth	smooth	smooth	not inf.	smooth	smooth
Conidia	Length	11.0 - 21.0	not inf.	16.0 - 25.0	14.0 - 19.0	16.0 - 23.0	13.0 - 20.0	10.5 - 15.0	15.0 - 23.0
	Width	9.0 - 13.0	3.0 - 4.0	10.0 - 13.0	8.0 - 13.0	12.0 - 18.0	8.0 - 11.0	7.5 - 9.5	8.0 - 14.0
	Shape	Reniform	not inf.	Horse-shoe shape	Renifor m	Renifor m to pyriform	not inf.	not inf.	not inf.
	Surfac e	smooth	not inf.	smooth	smooth to verrucul ose	not inf.	smooth to verruculose	not inf.	verruculos e

3. CHAPTER THREE

Endangered parasites: gathering evidence of threat of coextinction of plant pathogenic fungi on two rare plant species from the Atlantic forest in Brazil

Fabiano B. Rocha, Robert W. Barreto, Olinto Liparini Pereira, Eliane Mayumi Inokuti and João A. A. Meira Neto

Abstract

Plant pathogenic fungi are normally labeled as noxious organisms, mainly because of the relative minority that are agricultural pests. In fact, most cause no direct harm to human interests and provide valuable ecosystem services as bioregulators. They also represent a significant part of the world's biodiversity. As such, it is clear that, in the ongoing process of mega-extinction resulting from human activities, fungi in general, and plant pathogenic fungi, in particular, are not being spared. Nevertheless they have been completely neglected in conservation initiatives. Those many plant pathogenic fungi which are strictly specialized to endangered host plant species, provide a clear example of fungal species that are candidates to becoming coextinct. Unlike fungi, many plant species are listed in official red lists. Two examples are *Coussapoa floccosa* and *Euterpe edulis,* which are native from the shrinking ecosystem known as the Brazilian Atlantic Forest. These plants support a diverse mycobiota which is only being described now. The objective of the present work was to assess the host range of selected fungi associated with those two plant species aiming at determining the degree of specialization of each fungus in order to clarify the level of dependency of each fungal species on their threatened hosts for survival..

1. Introduction

Plant pathogenic fungi are normally labeled as noxious organisms, mainly because of the relative minority that are agricultural pests. In fact, most cause no direct harm to human interests and provide valuable ecosystem services as bioregulators. They also represent a significant part of the world's biodiversity. As such, it is clear that, in the ongoing process of mega-extinction resulting from human activities, fungi in general, and plant pathogenic fungi, in particular, are not being

spared. Such organisms can become victims of coextinction events that occurs when an organism depending upon an endangered organism to survive becomes extinct because of the disappearance of this key species (Dunn et al. 2009). This implies that some organisms that maintain a strict relationship, such as mutualism, parasitism or other, can become extinct if its unique substrate become extinct for some reason. Koh et al. (2004b) have reported events where the loss of a host species led associated species to extinction. Among the organisms that could become coextinct are highly host-specific plant parasitic fungi. At present there is no formal recognition of any of these organisms as endangered and, as a consequence no conservation measures are being taken to preserve those unique species *in situ* or *ex situ.* Generating convincing scientific evidence of this condition for plant pathogenic fungi is a necessity in order to build a case in favor of including such organisms in biodiversity conservation policies. Organisms such as pollinators, parasites and mutualistic symbionts fit well into the group of organisms that can become extinct as a consequence to the poss of their hosts. Published evidences of that are available for some cases, such as pollinating *Ficus* wasps and species of *Ficus*, butterflies and their laval host plants, and parasites and their hosts (Koh et al. 2004b). There are also examples where coextinction occurred locally, like butterflies and its hosts plants in Singapore (Koh et al. 2004a). Although reports of coextinction are still scarce, it is possible predict the importance of coextinction by estimating the loss of affiliates according to the general specific parasite average per host and them estimating how many host species are endangered (Dunn 2005, Koh et al. 2004b). Besides coextinction event reports, some evidences indicate that there is a decrease of parasites diversity as a host-species slides towards extinction (Altizer et al. 2007, Gibson et al. 2010). This indicate that even before a host plant become extinct their parasites species richness declines.

The host-specificity interaction has a complex genetic basis that together with environment outlines the coevolution among species over time (Wolinska and King 2009). Host-specific interaction between plant parasitic fungi and their hosts can be rather sophisticate (Ferreira et al. 2006; Mangan et al, 2010) and construct a permanent link of dependency of the parasitic fungus to its host-species (Agrios 2004). It is important to highlight that it is not sufficient for a plant parasitic organism to simply have a known association with an endangered host to allow for

the conclusion that it is also consequently also endangered of becoming co-extinct. The key question in coextinction is the level of specificity of the affiliate species. Will it be able to survive and maintain a viable population on a different host or substrate? Two interesting examples illustrate the importance of knowing the identity and the host range of such organisms. A case in point is that of *Campanulotes defectus* Teneiro and *Columbicola extinctus* Malcomson. These parasitic lice were originally described as two distinct species. The former associated with commom pigeon and the latter in association with an already extinct migratory pigeon, *Ectopistes migratorius* (L.). Later it was recognized that *C. extinctus* was a synonym to the common (not threatened) species *C. defectus* (Clayton and Price 1999, Price et al. 2000).

The efforts towards recognition of fungal species in danger of extinction are mostly restricted to researchers involved in the study of macrofungi and lichens (Balmford et al. 2000, Dahlberg 2010). Few publications address to conservation issues involving microfungi (Dulymamode et al. 2001, Rocha et al. 2010, Siboe et al. 2000). Once the identity of a parasitic fungus associated with an endangered plant species has been resolved, the next issue to be investigated in order to clarify its conservation status is its level of host-dependency (host-specificity, host–range, saprophytic survival…). This will then allow for a more precise understanding of the conservation status of each plant parasitic fungal species.

The present publication aims at pioneering yielding evidence along those lines for fungi associated with two endangered tree species of the Brazilian Atlantic raiforest, itself an important biodiversity hot-spot (Myers et al. 2000, Ribeiro et al. 2009), namely: *Coussapoa. floccosa* Akkermans & C.C Berg (Cecropiaceae) - recognized as vulnerable in "The IUCN Red List of Threatened Species" (IUCN 2010) – and threatened because of habitat destrucion and . *Euterpe edulis* Mart. (Arecaceae) - officially listed as endangered in Brazil (Ministério do Meio Ambiente 2008) - originally (contrarily to *C. floccosa*) common throughout the Brazilian Atlantic forest but overexploited as a source of heart of palm. Herbarium database searched (splink.cria.org.br) combined with intensive field searches resulted in only twelve *C. floccosa* individuals occurring in nature (with no sign of recruitment plants at any site) confirming its rarity. None of the individuals that were located occurred in protected areas and no living specimen is known under cultivation in botanic

gardens. This scenario indicates that the present status in IUCN is wrong and the situation for this particular species is far worse and it is not an exaggeration to consider this on the brink of extinction. The objective of the present work was that of clarifying the host range of microfungi previously found associated with *C. flocossa* (Rocha et al. 2010) and *E. edulis* (unpublished data, chapter 2 of this manuscript) through host-specificity testing and searches for occurrences of each fungal species on other plants in the vicinities of plants bearing colonies of those fungi in the field.

2. Material and methods

2.1. Fungi associated with Coussapoa floccosa and Euterpe edulis

The fungi previously collected on *C. floccosa* and described in Rocha et al (2010) were: *Pseudocercospora atrofuliginosa* F.B. Rocha, U. Braun & R.W. Barreto, *Pseudocercospora coussapoae* F.B. Rocha & R.W. Barreto, *Tripospermum acrobaticum* F.B. Rocha & R.W. *Mycosphaerella coussapoae* F.B. Rocha & R.W. Barreto, *Dennisiella coussapoae* F.B. Rocha, J.L. Bezerra & R.W. Barreto and *Pseudoallosoma nervisequens* F.B. Rocha, J.L. Bezerra & R.W. Barreto. The following fungal species were found in association with *E. edulis*: *Bipolaris cynodontis* (Marignoni) Shoemaker, *Passalora eitenii* R.B. Medeiros & Dianese, *Distocercospora* sp. nov. and *Melanographium* sp. nov...

2.2. Field occurrences and distribution of the fungi

Surveys were concentrated on a forest fragment which was central in our earlier studies (Rocha *et al.* 2010). The area is a small forest fragment of well preserved Atlantic Forest (Seasonal Semideciduous Montane Forest) situated in a private farm known as "Fazenda Bom Sucesso" (Figure 1a). In this small stretch of forest few large individuals of *C. floccosa* remain (Figure 1b and 1c) and also a group of *Euterpe edulis* individuals (Figure 1d and 1e). Additionally other areas in the states of Espirito Santo (ES), Minas Gerais (MG) and Rio de Janeiro (RJ) were also visited.

Coussapoa floccosa and *Euterpe edulis* individuals were located and carefully examined. Only twelve *C. floccosa* trees were located. All sites for which there were herbarium records of occurrence of *C. floccosa* were visited (splink.cria.org.br). Seven individuals were found in municipality of Viçosa (MG),

four in municipality of Carangola (MG) and one in municipality of Santa Maria do Jetibá (ES). Samples of *E. edulis* were obtained from selected areas of the three states (ES, MG and RJ) São Paulo. Samples of fresh leaves seemingly infected with fungi were collected using single rope technique to climb the trees (ter Steege and Cornelissen 1988) or with a long-poled prunner. Hand sections of fungal structures or fungal material scraped from leaves with a scalpel were mounted in lactophenol (20 g phenol, 20 ml lactic acid, 40 ml glycerin and 40 ml water). Observations were made with an Olympus BX 51 light microscope for observation of morphology and identification.

2.3. Host-specificity evaluations

Investigations on host-specificity followed three complementary approaches for fungi occurring on *C. floccosa*: 1. Collection and observation of selected plants (Table 1) occurring in the vicinities of *C. floccosa* individuals bearing fungal colonies in the field; 2. Inoculation of non-biotrophic fungi on test plants (Tabs 2 and 3) under controlled conditions; 3. Exposure of selected plants to natural inoculum (as sentinels or trap-garden). In the case of *E. edulis* only the second method was utilized due to practical limitations.

2.3.1. Gathering field evidence of host-specificity

Occurrence of fungi was searched for on plants with varying degrees of evolutionarily relatedness to *C. floccosa* but concentrated on members of the Rosales. A previous floristic work of the area of Fazenda Bom Sucesso where most of the individuals of *Coussapoa floccosa* was available and served as the basis for the previous selection of plants species belonging to Rosales that occur in the area and might be located and screened in search for the fungi occurring on *C. floccosa* (Tab. 1). Surveys on these plants were performed four times in a year, at different seasons (September/2008, November/2008, April/2009, August/2009). For the fungi associated with *Euterpe edulis*, the survey area at Fazenda Bom Sucesso harbored only few individuals of species in the order Arecales, namely *Astrocaryum aculeatissimum* (Schott) Burret. At least thirty from each treefresh leaves were collected, with special attention to those seemingly carrying fungal colonies. The leaves were examined under a stereomicroscope and a microscope as described above.

2.3.2. Inoculation tests under controlled conditions

The inoculum was obtained from growing cultures on PDA, incubated at 25 °C under a 12h photoperiod. When not sporulating an attempt to stimulate sporulation was made by flooding the plates with fully grown sterile cultures with sterile water and scraping their surface with a rubber spatula and keping them partially open in an incubator. Once sporulation was obtained the spores were collected by adding a solution of Tween 20 ^{TM} (0.5%) in sterile water to the plates, scraping the surface of cultures with a rubber spatula and filtering the resulting suspension through two layers of cheese cloth. Inoculum concentrations were measured adjusted to 10^6 spores/ml with the help of a Neubauer chamber. Inoculations were performed (depending on the amount of volume available) by spraying healthy test plants until runoff or by spreading the suspension on both sides of leaves with a clean and sterile painting brush. For fungi that did not sporulate in culture mycelial plugs were taken from the margin of actively growing cultures were used as inoculum. Three plugs were deposited on the upside leaves as an inoculum. None of the fungi grown in culture among those isolated from *C. floccosa* did sporulate and two (*Dennisiella coussapoae* and *Tripospermum acrobaticum*) were not included in the work as they failed to grow in culture even after repetitive attempts.

The list of plants chosen for inclusion in the tests was selected on basis of a centrifugal-phylogenetic scheme (Wapshere 1974), based on known evolutionary relationship of test plants with *C. floccosa* and *E. edulis* separately. Availability of plants for inclusion in the host-specificity tests also had to be taken into consideration. A list of plant species belonging to Arecaceae (Table 2) (Figure 2a) was prepared for testing the fungi found associated with *Euterpe edulis* and a list of plant species belonging to the order Rosales (Table 3) (Figure 2c) was selected to performing the tests involving the fungi on *Coussapoa floccosa*. Most of the plantlts were obtained from local nurseries in municipality of Viçosa (MG).

After inoculation the plantlets were maintained in a dew chamber exposed to indirect sunlight for 48 h and then left on a greenhouse bench at *ca.* 27 °C and automatic sprinkler irrigation. The plantlets were evaluated every week until the onset of the symptoms and appearance of typical fungal structures. At the end of the experiment slides were mounted with the fungal structures that appeared on the

lesions and identity of the fungus was checked by mounting slides containing the structures and examination of the morphology under a microscope (Olympus BX 51).

2.3.3. Sentinel plants

Three young and healthy potted individuals of each species in the same list of plants (Table 3) used in inoculation test of the fungi associated with *C floccosa* were maintained under one selected individual of *C. floccosa* individual known to supports the whole range of fungal species collected and described on this host. These plants were left under this *C. floccosa* for three months with an apparatus for drip irrigation (Fig 2. The purpose of the experiment was to allow a possible natural infection to take place through direct exposing the test-plants to the "rain of inoculum" through a range of environmental conditions that might be conductive to infection occurrence. The plantlets were taken to the field in December/2009, when the rain season is in at its climax in Viçosa, and were removed from the field on March/2010. Plants were carefully examined monthly for observation of any fungal colonies that might have emerged and any potential evidence of infection was carefully evaluated by removal of abnormal leaves for examination in the lab (as described above).

3. Results

3.1. Field distribution of fungi

Among the fungi associated with *C. floccosa*, only *Mycosphaerella coussapoae* was observed in association with all twelve *C. floccosa* individuals in the field. All other fungi were only found on plants occurring at Fazenda Bonsucesso in Viçosa. *Pseudocercospora atrofuliginosa*, *Pseudocercospora coussapoae* and *Pseudoallosoma nervisequens* were found associated with five individuals. *Pseudocercospora coussapoae* was also found associated with the isolate individual found at Santana do Jetibá (ES). *Tripospermum acrobaticum*, which may form mixed colonies with *Dennisiella coussapoae*, was found associated with four individuals, but only one of those individuals was supporting *D. coussapoae*. None of these fungal species was found on any of the other plant species in the field during the investigation at "Fazenda Bom Sucesso".

The fungi associated with *Euterpe edulis* were found each at a different location. *Bipolaris cynodontis* was found in a commercial nursery (Viveiro Antuérpia – Viçosa, MG) attacking young plants that appeared to be stressed because of direct exposition to sunlight. *Passalora eitenii* was found at Carangola - MG, and Nova Friburgo - RJ. Among the two newly described species *Distocercospora* sp. nov. was found at Nova Friburgo – RJ and Viçosa –MG, and *Melanographium* sp. nov. was collected at Carangola – MG and Nova Friburgo – RJ. It is, nevertheless, acknowledged that the survey on *E. edulis* was too preliminary to allow for a reasonable idea of the distribution of each of the fungi.

3.2. Host specificity evaluation

None of the fungi found associated with *Coussapoa floccosa* was found associated with other plants species belonging to Rosales (Table 1) in the forest fragment where those fungi were previously found. The host-specificity evaluation that was performed under controlled conditions for the fungi obtained from *C. floccosa* that were included in the work did not result in any confirmation of pathogenicity and is regarded here as of little value as not even the original host (*C. floccosa*) became infected with any of the fungi. This may be owing to the limitations of using only sterile colonies as inoculum. It is known that for many pathogenic fungi sterile mycelium is not infective. As for the study involving the sentinel plants, none of the plants became colonized by any of the fungi occurring on *C. floccosa* . Only insect feeding and damage of other kinds appeared on some of the plants. Unfortunately, it seems that this method was not adequate for host-specificity evaluation as no fungal colonies were formed on young *C. floccosa* left for an even longer period of time under the same plant in the field. Perhaps fungal dispersion in these taxa is restricted to the canopy with little inoculum reaching the forest floor.

Among the fungi isolated from *E. edulis*, *Bipolaris cynodontis*, *Distocercospora* sp. and *Melanographium* sp. were to be pathogenic to their original host. Additionally the host-range test allowed for the recognition that the isolate of *B. cynodontis* is polyphagous in the Arecaceae, On the other hand, *Distocercospora* sp., *Melanographium* sp. were shown to have a narrower host-range, *Distocercospora.* sp. only infected the original host *E. edulis* and *Melanographium* sp. was only capable of infecting the two test-plant species belonging to the genus *Euterpe* that

were included in this study (*E. edulis* and *Euterpe oleracea* Mart.). No infection was obtained in inoculations involving *Passalora eitenii* even on its original host *E. edulis*. It is possible that some methodological problem or loss of virulence in culture occurred in that case.

4. Discussion

Demonstrating a narrow host range of the parasitic fungi found associated with the endangered plant species, *Coussapoa floccosa* (Rocha et al. 2010) and *Euterpe edulis* would be an important step towards gathering acceptable scientific evidence of their status of endangered species by coextincion (Dunn et al. 2009). Although our results indicate that the distribution of the fungi associated with *C. floccosa*, with the exception of *Mycosphaerella coussapoae* and *Pseudocercospora coussapoae*, are restricted to a small forest fragment, it is possible that plant and its fungal associates occur in other forest areas that were not covered by surveys made by botanists. Nevertheless, it appears that both the host plant and the fungal species it supports (not to mention other organisms which may be associated witht this plant species) are in critical danger of extinction. Our surveys for fungi associated with *E. edulis* were far less comprehensive than that performed for *C. floccosa* and it is premature to draw any conclusion about the distribution range for the fungi found on this host. Evidence obtained from the observation of possible hosts for fungi found in *C. floccosa* in the centrifugal-phylogenetic scheme in the field yielded indications that all fungi on that host are rather host-specific. Inoculum produced on *C. floccosa* along the years would most likely have repeatedly met the other potential hosts and if compatible, such associations would have progressed towards infection and the formation of colonies. Their absence is a convincing evidence of host-specificity, which in this case translates into dependence on *C. floccosa* and danger of co-extinction. Of particular significance is the occurrence of the congeneric *C. microcarpa* individuals in close proximity with *C. floccosa* in the forest fragment at Fazenda Bonsucesso consistently bearing no colonies of the fungi found *C. floccosa.*

Additional attempts of evaluating the host range of the fungi associated with *Coussapoa floccosa* by inoculations under controlled conditions should be performed if sporulation is obtained under conditions other than those used until now. It is likely that the general failure in the evaluations that were performe are owing to lack

of infectivity of sterile mycelium colonies. As for fungi found in association with *E. edulis* it is clear that *Bipolaris cynodontis* is of no relevance for conservation as it is a broad spectrum pathogen of monocots. It is, nevertheless worthy of notice that this fungal species was not previously known to be pathogenic to members of the Arecaceae but rather as a parasite of many members of the Poaceae (Farr et al 2006). It nevertheless proved capable of infecting species of Arecaceae included in this work. Results obtained for *Passalora eitenii* were inconclusive. It was expected that this fungus would have a broad host-range within the Arecaceae as it was originally described from a different host genus (*Syagrus*). Nevertheless our isolate did not even infect its original host *E. edulis* and it is possible that it lost infectivity or environmental conditions during the experiment were not ameanable to infection. *Melanographium* sp. was proven capable to establish on both *E. edulis* and *Euterpe oleraceae* and it may less threatened in case it survives in nature on other species belonging to the genus even if the remaining populations of *E. edulis* are pushed towards extinction. The situation appears different for *Distocercospora* sp. as this was only capable of infecting *Euterpe edulis* in our experiment. *Distocercospora* sp. deserves hence special attention as a potentially threatened fungal species.

It is acknowledged here that this work does not exhaust the theme nor provide complete evidence of a threatened status for any of the fungal species included in this study. Perhaps such complete evidence will never be generated at a "beyond all doubt" level. Nevertheless, the mere recognition of the existance of specialized pathogenic microfungi in association with endangered plant species is in itself a good evidence of threat by coextinction to these fungal species. Additional work should further cover the possibility of each fungal species surving , in association with other plants as weak or opportunistic pathogens or as endophytes and additionally as saprophytes in the litter or in the forest soil. There are highly sensitive molecular tools available which could be used in such studies.

The study of endangered plant parasitic fungi is at its infancy and it is fully justifiable both in applied and academic arenas. Aditionally its exploitation has the potential to attract more general attention to the field of mycology and to lead to a more pro-active involvement of mycologists and plant pathologists in the neglected field of fungal conservation.

6. Bibliography

Agrios GN, 2004. Plant Pathology 5th ed. Elsevier, Oxford, UK. 922pp.

Altizer S, Nunn CL, Lindenfors P, 2007. Do threatened hosts have fewer parasites? A comparative study in primates. *Journal of Animal Ecology*. 76, 304-314.

Balmford A, Lyon AJE, Lang RM, 2000. Testing the higher-taxon approach to conservation planning in a megadiverse group: the macrofungi. *Biological Conservation*. 93, 209-217.

Clayton DH, Price RD, 1999. Taxonomy of New World Columbicola (Phthiraptera: Philopteridae) from the Columbiformes (Aves), with descriptions of five new species. *Annals of the Entomological Society of America* 92, 675-685.

Dahlberg A, Genney DR, Heilmann-Clausen J, 2010. Developing a comprehensive strategy for fungal conservation in Europe: current status and future needs. *Fungal Ecology*. 3, 50-64.

Dulymamode R, Cannon P, Peerally A, 2001. Fungi on endemic plants of Mauritius. *Mycological Research*. 105, 1472-1479.

Dunn RR, 2005. Modern Insect Extinctions, the Neglected Majority. *Conservation Biology* 19, 1030-1036.

Dunn RR, Harris NC, Colwell RK, Koh LP, Sodhi NS, 2009. The sixth mass coextinction: are most endangered species parasites and mutualists? *Proceedings of the Royal Society B*. 276, 3037-3045.

Farr DF, Rossman AY, 2006. Fungal Databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. http://nt.ars-grin.gov/fungaldatabases. Accessed February 03, 2011.

Ferreira RB, Monteiro S, Freitas R, Santos CN, Chen Z, Batista LM, Duarte J, Borges A, Teixeira AR, 2006. Fungal pathogens: the battle for plant infection. *Critical Reviews in Plant Sciences*. 25, 505-524.

Gibson AK, Mena-Ali JI, Hood ME, 2010. Loss of pathogens in threatened plant species. *Oikos*. 119, 1919-1928.

IUCN. 2010. IUCN Red List of Threatened Species. Available from http://www.iucnredlist.org/apps/redlist/details/34356/0 (accessed October 2010).

Koh L P, Sodhi NS, Brook BW, 2004*a*. Co-extinctions of tropical butterflies and their hostplants. *Biotropica* 36, 272–276.

Koh LP, Dunn RR, Sodhi NS, Colwell RK, Proctor HC, Smith VS, 2004b. Species coextinctions and the biodiversity crisis. *Science*. 305, 1632-1634.

Mangan AS, Herre EA, Bever JD, 2010. Specificity between Neotropical tree seedlings and their fungal mutualists leads to plant-soil feedback. *Ecology*. 91, 2594-2603.

Ministério do Meio Ambiente, 2008. Lista oficial das species da flora brasileira ameaçadas de extinção. www.mma.gov.br. (accessed October 2010).

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J, 2000. Biodiversity hotspots for conservation priorities. *Nature* 403, 853-858.

Price RD, Clayton DH, Adams RJ, 2000. Pigeon lice down under: taxonomy of Australian *Campanulotes* (Phthiraptera: Philopteridae), with a description of *C. durdeni* n.sp. *Journal of Parasitology* 86, 948-950.

Rocha FB, Barreto RW, Bezerra JL, Meira Neto JAA, 2010. Foliage mycobiota of *Coussapoa floccosa*, a highly threatened tree of the Brazilian Atlantic Forest. *Mycologia*. 102, 1240-1252.

Ribeiro MC, Metzger JP, Martensen AC, Ponzoni FJ, Hirota MM, 2009. The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. *Biological Conservation* 142, 1141-1153.

Siboe GM, Kirk PM, David JC, Cannon PF, 2000. Necrotophic fungi from Kenyan endemic and rare plants. *Sydowia*. 52, 286-304.

ter Steege H, Cornelissen JHC, 1988. Colleting and studying bryophytes in the canopy of standing rain forest trees. *Methods in bryology Proceeding of Bryology Methods Workshop*, 285-290.

Wapshere AJ, 1974. Strategy for evaluating safety of organisms for biological weed-control. *Annals of Applied Bioloby*. 77, 201-211.

Wolinska J, King KC, 2009. Enviroment can alter selection in host-parasite interactions. *Trends in Parasitology* 25, 236-244.
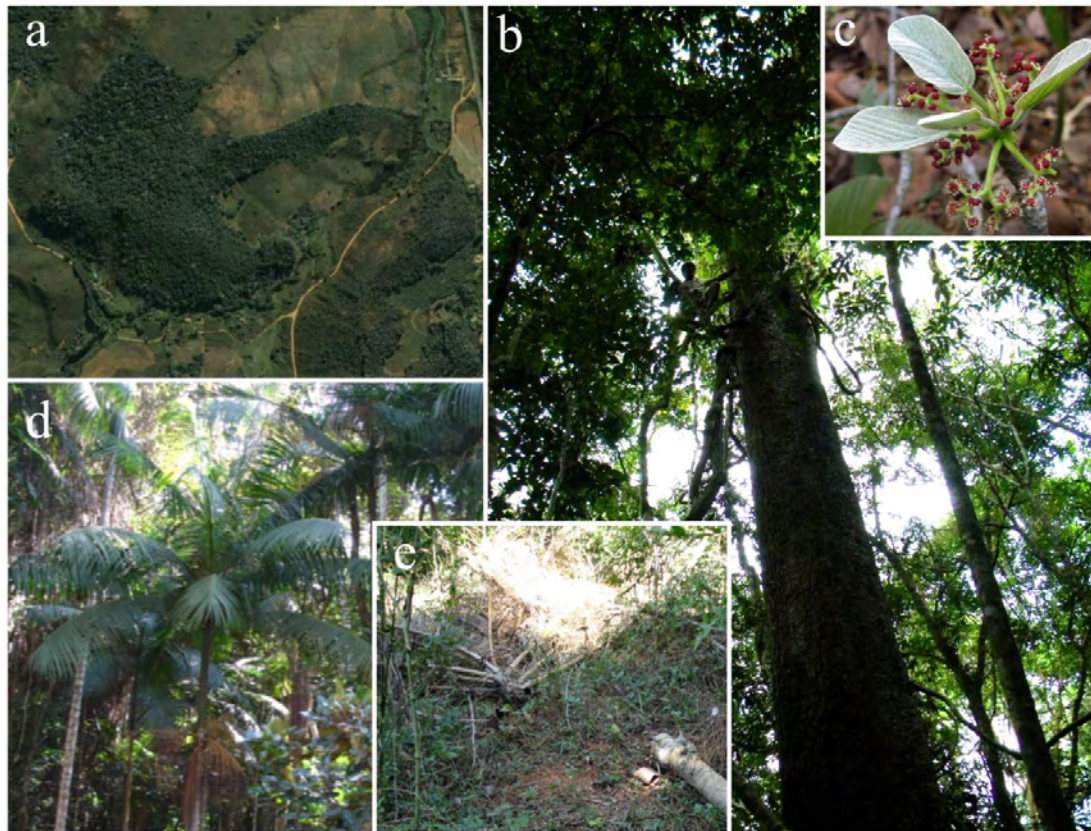
7. Figures and Tables

Figure 1. Location and hosts: 40 ha of Atlantic rain forest, the main study area where the most of *Coussapoa floccosa* and *Euterpe edulis* individuals are located (a), *Coussapoa floccosa* individual, detail to inflorescence (b, c),. *Euterpe edulis* individual (d);. a palm tree plant cut to extract the heart of palm (e).
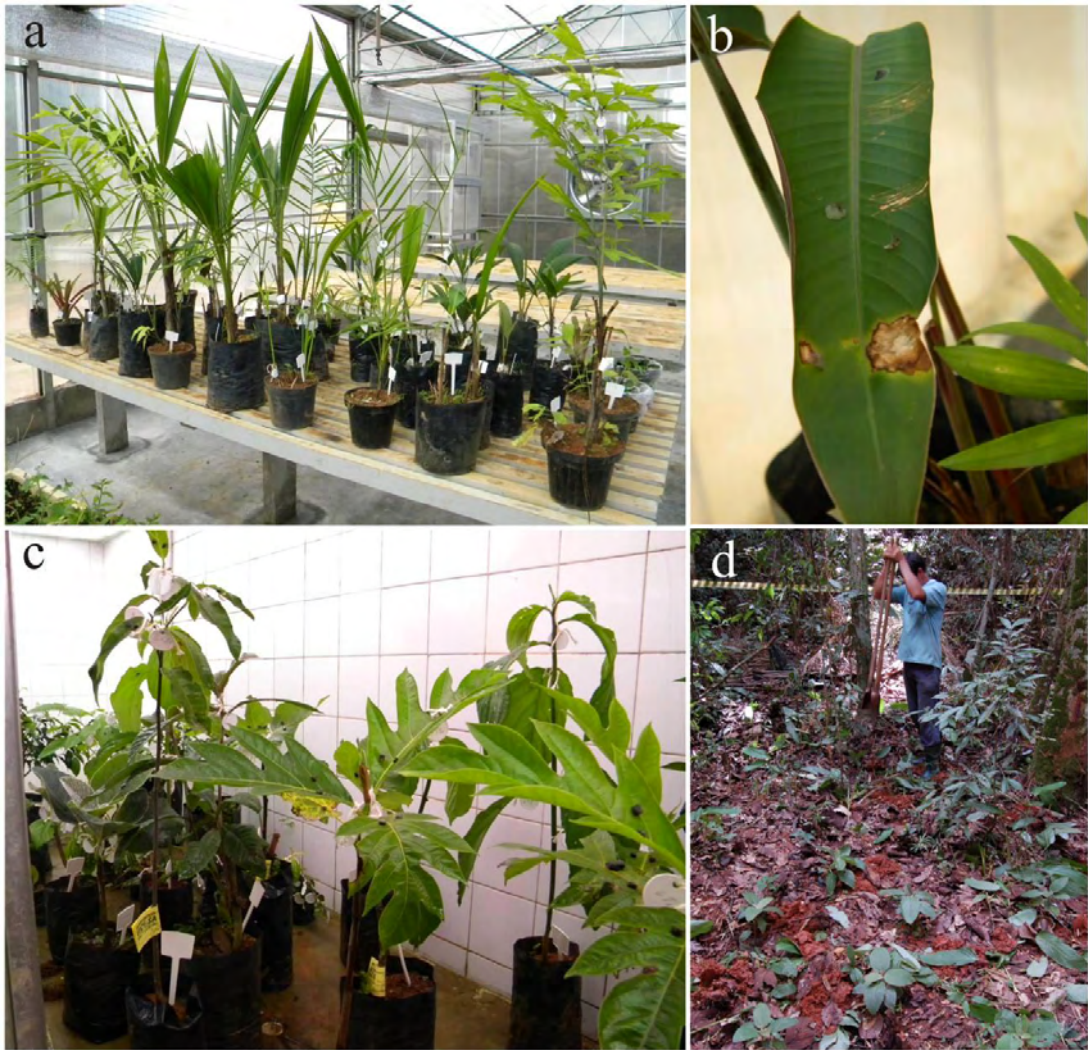
Figure 2. Specificity test: set of host close related to *Euterpe edulis* (a), *Bipolaris cynodontis* parasitizing *Musa ornata* (b), set of host close related to *Coussapoa floccosa* (c), sentinel plants being under *Coussapoa floccosa* individual (d).

Table 1. List of plant species belonging to order Rosales located in the same forest fragment where the fungi associated with *Coussapoa floccosa* were located.

Order	Family	Species
Rosales	Cecropiaceae	*Cecropia hololeuca*
		Coussapoa floccosa
		Coussapoa microcarpa
		Pourouma acutifolia
		Pourouma guianensis
	Moraceae	*Acantinophyllum ilicifolia*
		Brosimum glaziovii
		Brosimum guianense
		Ficus glabra
		Ficus gomelleira
		Ficus insipida
		Ficus luschnatiana
		Ficus mexia
		Helicostylis tomentos
		Maclura tinctoria
		Naucleopsis melobarretoe
		Sorocea bonplandii
		Sorocea hillari
	Rosaceae	*Prunus sellowii*
	Rhamnaceae	*Colubrina glandulosa*
	Ulmaceae	*Celtis iguinae*

Table 2. List of hosts belonging to superorder Lilianae used in the specificity test of the fungi associated with *Euterpe edulis*.

Order	Family	Species
Arecales	Arecaceae	*Euterpe edulis*
		Euterpe oleracea
		Chamaedorea elegans
		Phoenix canariensis
		Bactris acanthocarpa
		Cocos nucifera
		Caryota sp.
		Acrocomia aculeata
		Rhapis excelsa
		Syagrus coronata
		Roystonea oleracea
Poales	Cyperaceae	*Cyperus alternifolius*
	Bromeliaceae	Bromeliaceae
Commelinales	Pontederiaceae	*Eichhornia crassipes*
Zingiberales	Musaceae	*Musa ornata*

Table 3. List of hosts belonging to order Rosales used to specificity test of the fungi associated with *Coussapoa floccosa*.

Order	Family	Species
Rosales	Cecropiaceae	*Cecropia hololeuca*
		Cecropia glaziovi
		Pourouma cecropiifolia
	Moraceae	*Ficus benjamina*
		Morus alba
	Urticaceae	*Pilea microphylla*
		Boehmeria nivea
	Rosaceae	*Eriobotrya japonica*
	Rhamnaceae	*Colubrina sp.*
	Ulmaceae	*Trama micrantha*

CONCLUSÕES GERAIS

- Embora análises filogenéticas comprovem que a espécie *Trichoderma harzianum* é na verdade um complexo de espécies distintas e a diversidade morfológica, incluindo aspecto das colônias, ampla distribuição geográfica e diversidade de nichos corroborarem com essa hipótese, as análises morfológicas realizadas no presente trabalho não suportaram a distinção morfológica precisa entre os clados to complexo *Trichoderma harzianum* e sugerem que, para o momento o tratamento genérico deste táxon como *T. harzianum sensu lato* é o mais prudente até que delimitações mais consistentes sejam obtidas que permitam o reconhecimento taxonômico das formas agrupadas sob esta denomintação.

- Através da comparação morfológica entre as espécies de fungos encontradas associadas a *Euterpe edulis* e aquelas espécies descritas na literatura anteriormente, relatou-se pela primeira vez *Bipolaris cynodontis* associado a uma espécie de Arecaceae, e *Passalora eitenii* em *E. edulis*. Dois outros fungos encontrados, uma espécie de *Distocercospora* e outra de *Melanographium*, apresentaram diferenças morfológicas suficientes em relação às demais descritas para seus respectivos gêneros para serem reconhecidas como novas para a ciência.

- Embora seja uma conjectura lógica a de fungos fitopatogênicos parasitas altamente especializados e restritos a uma a uma única espécie vegetal, que por sua vez se encontra ameaçada de extinção estejam também ameaçados de extinção − em eventos de coextinção, as evidências obtidas para os fungos estudados no âmbito do presente trabalho ainda são preliminares. Há, no entanto, indícios de que os fungos associados a *Coussapoa floccosa* e dois dos fungos descritos associados a *Euterpe edulis* são específicos e poderão vir a ser reconhecidos como espécies ameaçadas. Estudos adicionais sobre a sobrevivência destes fungos como saprófitas no solo ou como endófitos em outros hospedeiros são ainda necessários para caracterizar plenamente esta condição.