GLÁUCIA MARA MOREIRA

DIVERSIDADE DE ESPÉCIES DE *Clonostachys* NO BRASIL

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para a obtenção do título de Doutora.

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Moreira, Gláucia Mara.
Diversidade de espécies de *Clonostachys* no Brasil / Gláucia Mara Moreira. – Lavras : UFLA, 2016.
83 p. : il.

Tese (doutorado) – Universidade Federal de Lavras, 2016.
Orientador(a): Ludwig Heinrich Pfenning.
Bibliografia.

1. *Bionectria*. 2. Bionectriaceae. 3. Concordância genealógica. 4. Filogenia molecular. 5. Hypocreales. I. Universidade Federal de Lavras. II. Título.

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APROVADA em 19 de fevereiro de 2016.

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*Aos meus pais, Sebastião Elco Moreira (*in memoriam*) e Maria Aparecida Moreira*

Às minhas irmãs, Renata e Vanessa

Ao meu sobrinho, Artur

Ao meu amor, Maurício

DEDICO

AGRADECIMENTOS

A Deus, por me guiar nas minhas escolhas e momentos de dúvidas e incertezas.

À Universidade Federal de Lavras, aos Departamentos de Biologia e Fitopatologia e ao programa de Pós-Graduação em Microbiologia Agrícola pela oportunidade. Aos professores pelos valiosos ensinamentos.

À CAPES, pela concessão da bolsa de estudos no Brasil e no exterior, concedida pelo programa de Doutorado Sanduíche.

Ao meu orientador, Prof. Ludwig H. Pfenning, pela orientação, confiança e ensinamentos.

Ao meu coorientador, Prof. Lucas Magalhães de Abreu, pelos ensinamentos, conversas, disponibilidade e ajuda em todas as etapas desse trabalho.

Ao meu supervisor no Agricultural Institute of Slovenia - Eslovênia, Dr. Hans-Josef Schroers, pela confiança e conhecimentos transmitidos.

Aos professores Dra. Patrícia Gomes Cardoso, Dr. Luís Roberto Batista e Dr. Flávio Henrique Vasconcelos de Medeiros, que se dispuseram a participar da banca examinadora e pela grande contribuição.

Aos amigos da UFLA, do Laboratório de Sistemática e Ecologia de Fungos e do Agricultural Institute of Slovenia pelo companheirismo e boa convivência. Agradecimento especial à estagiária Amanda Rodrigues.

À minha mãe, pelo incentivo e apoio incondicional. Às minhas irmãs e sobrinho queridos, pelo amor, companheirismo e alegria. Renata, sem seu apoio não estaria aqui hoje.

Ao Maurício, pelo amor, paciência, conselhos, apoio e ajuda em tudo que preciso, compartilhando comigo todos os momentos, de alegria, de dúvidas e de dificuldades.

RESUMO GERAL

O objetivo desse trabalho foi delimitar espécies filogenéticas de uma coleção de 106 isolados de *Clonostachys* oriundos de diferentes substratos e biomas do Brasil utilizando filogenia molecular multilocus, realizar a caracterização fenotípica dos isolados e descrever novas espécies. Os fungos foram cultivados em MEA e incubados a 10, 15, 20, 25, 30 e 35 ºC para avaliação do crescimento durante sete dias. Os isolados foram avaliados quanto à coloração da colônia após sete dias de crescimento em BDA e quanto às suas características micromorfológicas em OA e SNA contendo fragmentos de folha de cravo. Para todos os isolados foi sequenciado um fragmento de DNA do gene ATP citrato liase (*acl1*). Representantes de cada linhagem filogenética identificada foram selecionados para obtenção de sequências de fragmentos de DNA dos genes da maior subunidade da RNA polimerase II (*rpb1*), fator de elongação 1-α (*tef1*) e beta tubulina (*tub*). Foram obtidas árvores filogenéticas pelos métodos de inferência Bayesiana (BI) e máxima verossimilhança (ML) para cada região separadamente e para sequências combinadas. A filogenia utilizando sequências combinadas das quatro regiões gênicas para 54 isolados do subgênero *Bionectria* confirmaram a identificação de *C. chloroleuca* sp. nov. e das espécies conhecidas *C. byssicola*, *C. rosea*, *C. rhizophaga*, *C. pseudochroleuca* e *C. rogersoniana*, que formaram grupos monofiléticos com alto suporte estatístico. Os 13 isolados de *C. pseudochroleuca* se distribuíram em três subclados, sendo um dos clados representado por um morfotipo, que se diferencia dos demais. Na filogenia preliminar com sequências do gene *acl1* de 52 isolados, dois clados distintos se formaram dentro do subgênero *Bionectria* compostos por isolados com marcadores morfológicos semelhantes àqueles de *C. compactiuscula*, denominados *C.* cf. *compactiuscula*. Sequências de referência de *C. compactiuscula* foram incluídas nas análises filogenéticas do gene *tub.* As duas linhagens nomeadas *C.* cf. *compactiuscula* não se agruparam com sequências desta espécie, representando prováveis novas espécies. Foram observados também representantes dos subgêneros *Zebrinella* e *Epiphloea*. Árvores combinadas dos genes *acl1-tef1-tub* foram obtidas pelos métodos BI e ML, assim como árvores individuais para cada região gênica, confirmando a identificação da nova espécie *Clonostachys* sp. 2, inserida no subgênero *Epiphloea*, juntamente com a espécie já conhecida *C. candelabrum*. *Clonostachys* sp. 3 representa uma linhagem distinta no subgênero *Zebrinella*. Os resultados indicam que há grande diversidade de *Clonostachys* no Brasil, com alto potencial para a descoberta de novas espécies ou espécies ainda pouco estudadas.

Palavras-chave: *Bionectria*. Bionectriaceae. Concordância genealógica. Filogenia molecular. Hypocreales.

GENERAL ABSTRACT

The objective of this work was to delineate phylogenetic species from a collection of 106 strains of *Clonostachys* isolated from different substrates and biomes in Brazil based on multilocus DNA sequence analyses, to determine morphological markers, and describe new species. The fungi were cultivated on MEA and incubated at 10, 15, 20, 25, 30, and 35 ºC during seven days for evaluating growth rate. Pigmentation of the colony was evaluated after seven days on PDA and the micromorphological characters on OA and SNA with fragments of carnation leaves. A DNA fragment of ATP citrate lyase gene (*acl1*) was sequenced for all strains. Representatives of each identified phylogenetic lineage were selected to obtain DNA sequences of the genes largest subunit of RNA polymerase II (*rpb1*), translation elongation factor 1-α (*tef1*), and beta tubulin (*tub*). Phylogenetic trees were obtained by Bayesian inference (BI) and maximum likelihood (ML) for each gene region and also for concatenated sequences. The phylogeny of concatenated sequences for the four loci using 54 strains of subgenus *Bionectria* confirmed the identification of *C. chloroleuca* sp. nov. and the known species *C. byssicola*, *C. rosea*, *C. rhizophaga*, *C. pseudochroleuca*, and *C. rogersoniana*, which formed monophyletic groups with high statistical support. The 13 strains of *C. pseudochroleuca* formed three subclades, being one represented by a morphotype which differs from the other strains. In the preliminary phylogeny of the 52 strains based on *acl1* gene two distinct clades were observed within the subgenus *Bionectria*, with strains morphologically similar to *C. compactiuscula*, named as *C.* cf. *compactiuscula*. Reference sequences of *C. compactiuscula* were included in the phylogeny of *tub* gene. The two lineages denominated *C.* cf. *compactiuscula* did not group with sequences of this species, representing a putative new species. Representatives of subgenera *Zebrinella* and *Epiphloea* were observed. Combined trees of genes *acl1-tef1-tub* were obtained by BI and ML methods, as well as individual trees for each region gene, confirming the identification of new species *Clonostachys* sp. 2, inserted in subgenus *Epiphloea*, together with the known species *C. candelabrum*. *Clonostachys* sp. 3 represented a disctinct lineage in the subgenus *Zebrinella*. The results showed that there is large diversity of *Clonostachys* in Brazil, with high potential for the discovery of new species or species poorly studied.

Keywords: *Bionectria*. Bionectriaceae. Genealogical concordance. Molecular phylogeny. Hypocreales.

SUMÁRIO

PRIMEIRA PARTE

1 CARACTERIZAÇÃO DO PROBLEMA

O gênero *Clonostachys* Corda (Bionectriaceae, Hypocreales, Ascomycota) é composto por espécies comumente isoladas de amostras de solo e restos de plantas, na fase sexuada ou assexuada, em regiões de clima temperado e tropical (Domsch et al., 2007; Schroers, 2001; Schroers et al., 1999). Os trabalhos relacionados ao gênero normalmente são concentrados em *C. rosea*, espécie facilmente isolada e conhecida por sua capacidade de antagonizar e parasitar outros fungos, nematoides e insetos (Baloyi et al., 2012; Chatterton & Punja, 2009; Cota et al., 2008, 2009; Toledo et al., 2006). Entretanto, outras espécies possuem também esta capacidade (Moreira, 2012; Schroers, 2001).

A monografia de *Clonostachys* é atualmente o trabalho mais completo, no qual são discutidas e identificadas praticamente todas as espécies conhecidas até o momento, baseado na morfologia e filogenia das regiões beta tubulina (*tub*) e rDNA (ITS + 28S parcial), que se mostraram pouco informativas para algumas espécies. No entanto, o número de isolados sequenciados é pequeno, se comparado com os avaliados pela morfologia, sendo que para algumas espécies o isolado tipo não tem sequências disponíveis (Schroers, 2001). Além disso, a maioria das sequências disponíveis no banco de dados são advindas dessa monografia.

Resultados alcançados em trabalho anterior, utilizando análises filogenéticas de sequências de *tub* e ITS-LSU rDNA de isolados de *Clonostachys* obtidos de diferentes regiões do Brasil, evidenciaram a existência de dez linhagens filogenéticas em uma amostra de apenas 44 isolados. Dentre as linhagens, quatro revelaram-se prováveis novas espécies (*Clonostachys* sp. 1-4),

duas dessas representadas por um único isolado cada (*Clonostachys* sp. 3 e 4). Dentre as espécies identificadas, *C. byssicola* formou um grupo polifilético. Isolados de *C. pseudochroleuca* e *C. rogersoniana* formaram três e dois subclados, respectivamente, sendo que o clado de *C. pseudochroleuca* teve baixo suporte estatístico dependendo do método de análise filogenética utilizado (Moreira, 2012). Esses resultados foram comparados com perfis de proteínas obtidos por espectrometria de massas utilizando o método MALDI-TOF, que se mostrou eficiente na identificação de espécies de *Clonostachys*. No dendograma resultante de MALDI-TOF isolados de *C. byssicola* se agruparam em um único grupo distinto, enquanto que isolados de *C. rogersoniana* formaram dois grupos (Abreu et al., 2014).

Como resultado, foram delineadas as seguintes questões: a) a inclusão de outras regiões gênicas melhoraria a resolução das árvores filogenéticas? b) *C. byssicola*, *C. pseudochroleuca* e *C. rogersoniana* representam mais de uma espécie? c) isolados denominados *Clonostachys* sp. 1 e 2 por Moreira (2012) correspondem a novas espécies? e d) Existem mais espécies novas a serem descritas?

A partir dessas questões foram delineadas as seguintes hipóteses: a) análises de sequências de DNA multilocus melhoram a resolução das árvores filogenéticas e b) existem espécies a serem descritas.

A fim de confirmar as hipóteses e com base no conhecimento sobre a diversidade de espécies de *Clonostachys* ocorrendo naturalmente em diferentes biomas do Brasil, além do potencial de emprego como agentes de biocontrole de doenças, foi dada continuidade ao trabalho já iniciado. Coletas e isolamentos adicionais foram conduzidos a partir de amostras de solo e liteira aumentando a coleção de trabalho para 116 fungos. Também foram adquiridos 19 isolados de referência, representantes de 12 espécies de *Clonostachys*, utilizados por

Schroers (2001). Todos os isolados foram depositados na Coleção Micológica de Lavras (CML), Departamento de Fitopatologia, UFLA.

Os objetivos do presente trabalho foram delimitar as espécies de uma coleção de isolados de *Clonostachys* oriundos de diferentes substratos e regiões do Brasil através de filogenia molecular multilocus, realizar a caracterização fenotípica dos isolados e descrever novas espécies. Parte do trabalho foi executada durante o doutorado sanduíche sob a supervisão do Dr. Hans-Josef Schroers, especialista em taxonomia e sistemática filogenética de *Clonostachys* e autor da monografia do gênero.

Esta tese está dividida em duas partes. A primeira diz respeito ao estado da arte, no qual são descritas as características do gênero *Clonostachys* e os métodos para reconhecimento de espécies em fungos. A segunda parte está subdivida em dois artigos, que serão submetidos a revistas especializadas na área. O primeiro se refere ao estudo do subgênero *Bionectria* com a descrição de uma nova espécie e o segundo testa o gene ATP citrato liase (*acl1*) como *barcode* para *Clonostachys* com a descrição de outras novas espécies.

2 ESTADO DA ARTE

2.1 Características do gênero *Clonostachys*

O gênero *Clonostachys* é composto por espécies comumente encontradas no solo e em restos de plantas, tanto em regiões de clima temperado como tropical, incluindo endófitos, micoparasitas, parasitas de invertebrados, promotores de crescimento e, raramente, fitopatógenos (Baloyi et al., 2012; Abang et al., 2009; Zhang et al., 2008; Toledo et al., 2006; Domsch et al., 2007; Schroers, 2001; Schroers et al., 1999).

As espécies de *Clonostachys* na fase assexuada são caracterizadas pela formação de conídios em formato assimétrico, ligeiramente curvado, o que é determinado pela sua extrusão lateral a partir das fiálides. Podem apresentar massa de conídios de coloração branca, laranja-clara ou verde e, muitas vezes, formam conidióforos dimórficos, chamados de primários (verticilados) e secundários (penicilados), sendo que os últimos podem ser formados em esporodóquio e seus conídios são arranjados em cadeias imbricadas (Schroers, 2001; Schroers et al., 1999).

A fase sexuada do gênero *Clonostachys* (anteriormente denominada *Bionectria* Speg.) pode ser encontrada em cascas de árvores recentemente mortas, formando um estroma enrumpente sobre o qual há a formação de peritécios agrupados, que podem ser de coloração laranja, laranja-amarelado, laranja-amarronzado ou marrom. A parede dos peritécios pode consistir de três regiões distintas formadas por diferentes tipos celulares e sua superfície pode ser lisa, rugosa ou verrucosa. Os ascos são clavados e os ascósporos são elipsoidais, com um septo, podendo ser lisos, verrucosos ou estriados (Schroers, 2000, 2001).

Com as mudanças relativas à nomenclatura dos fungos pleomórficos no Código Internacional de Nomenclatura de Algas, Fungos e Plantas (ICN) foi proposto o uso do nome *Clonostachys* para holomorfos. Anteriormente o nome *Bionectria* era utilizado para isolados na fase sexuada e *Clonostachys* para isolados na fase assexuada. A proposta foi feita porque *C. rosea* é um nome já estabelecido na literatura, tem um conceito de espécie bem definido e é comumente utilizado para diversos estudos de controle biológico, enquanto que o nome *Bionectria* é poucas vezes citado, o tipo, *B. tonduzi*, não é bem caracterizado e nunca foi cultivado (Rossman et al., 2013).

Por muito tempo *Clonostachys* foi considerado como sinônimo de *Gliocladium* e diversos trabalhos foram publicados com estudos utilizando *G. roseum* (*C. rosea*) como agente de controle biológico. Posteriormente, aumentaram as evidências de que se tratava de dois gêneros distintos, baseado em estudos sobre características morfológicas e moleculares (Schroers, 2001; Schroers et al., 1999; Rehner & Samuels, 1994).

Schroers (2001) revisou o gênero *Clonostachys*, na qual delimitou espécies utilizando isolados obtidos a partir de conídios e ascósporos e espécimes de herbário, correlacionando isolados da fase assexuada aos da fase sexuada, comparando-os com os espécimes tipo e dados de trabalhos anteriormente publicados. Realizou análises filogenéticas utilizando sequências do DNA ribossômico (rDNA) e gene β-tubulina (*tub*). Schroers (2001) dividiu o gênero *Clonostachys* em seis subgêneros: *Bionectria*, *Zebrinella*, *Astromata*, *Myronectria*, *Epiphloea* e *Uniparietina*, e classificou 44 espécies, sendo 9 táxons sem fase sexuada conhecida. Posteriormente, a espécie *C. pseudostriatopsis* foi descrita no Japão (Hirooka & Kobayashi, 2007) e a fase sexuada de *C. intermedia* foi descrita na China (Luo & Zhuang, 2009).

Diferentes espécies de *Clonostachys* podem ser parasitas de outros fungos. Algumas são encontradas na natureza infectando outras espécies,

incluindo insetos e nematoides (Moreira, 2012; Schroers, 2001). No entanto, a espécie mais estudada e conhecida como um micoparasita destrutivo é *C. rosea*, nas suas duas formas *C. rosea* f. *rosea* e *C. rosea* f. *catenulata*. Esta espécie também apresenta outros modos de ação que a torna um agente promissor para uso na agricultura, que inclui competição, produção de enzimas líticas, promoção de crescimento e indução de resistência (Gan et al., 2007; Sutton et al., 1997). Recentemente, foi sequenciado o genoma de um importante isolado de *C. rosea* (IK726) (Karlsson et al., 2015). Para outro isolado dessa espécie (67-1) foi anunciado o sequenciamento do genoma (Sun et al., 2015).

No mercado existem produtos de amplo espectro utilizados para o controle de fitopatógenos em diversas culturas, de plantas herbáceas até árvores. Os bioprodutos, formulados a partir *C. rosea*, são Prestop®, Clonosnat®, Clonotri®, EndoFine®, Kamoi (Bettiol et al., 2012; Chatterton et al., 2008; Kapongo et al., 2008; Fravel, 2005).

2.2 Reconhecimento de espécies em fungos

Três conceitos podem ser utilizados para a delimitação de espécies em fungos. O conceito de espécie morfológica define espécies como grupos que compartilham características morfológicas e fisiológicas consistentes, diferentes daquelas apresentadas por grupos correlatos. Já o conceito de espécie biológica define grupos de indivíduos de compatibilidade sexual que cruzam entre si e são reprodutivamente isolados de outros grupos. O conceito de espécie filogenética define espécie como o menor agrupamento de táxons que possuem um ancestral recente comum e que partilham de diferentes características (fenotípicas e genotípicas) derivadas de tal ancestral, formando grupos monofiléticos (Taylor et al., 2000).

Idealmente, uma espécie de fungo deveria se enquadrar nesses três conceitos de espécies. Entretanto, uma espécie morfológica pode compreender mais de uma espécie biológica e filogenética. O conceito de espécie biológica é restrito a alguns grupos, pois não pode ser aplicado para isolados homotálicos, aos fungos que somente cruzam na natureza e aqueles que não são cultiváveis (Taylor et al., 2000).

Dadas as limitações dos conceitos de espécies morfológica e biológica, o conceito de espécie filogenética se tornou o paradigma para o reconhecimento de espécies de fungos. No entanto, se determinado gene analisado contiver diferentes alelos entre indivíduos de uma espécie, na filogenia pode haver a separação desses indivíduos em diferentes ramos, porém, não indicando espécies distintas, mas apenas variações intraespecíficas. Para evitar a subjetividade da determinação do limite das espécies, devem-se utilizar diferentes *loci* polimórficos nas análises filogenéticas comparando as diferentes genealogias de genes. Quando há uma concordância entre as diferentes árvores geradas a partir de diferentes genes, elas apresentam a mesma topologia (Rosenberg & Nordborg, 2002; Taylor et al., 2000). Esse método para reconhecimento de espécies é denominado concordância genealógica, que utiliza do conceito em inglês *Genealogical Concordance Phylogenetic Species Recognition* (GCPSR), introduzido por Taylor et al. (2000). Tal estratégia tem sido utilizada com sucesso para a definição de espécies da ordem Hypocreales (Salgado-Salazar et al., 2013; Hirooka et al., 2012; Sung et al., 2007; O'Donnell et al., 2004).

A concordância genealógica permite a identificação de diversas espécies distintas que compreendem uma única espécie morfológica (Salgado-Salazar et al., 2015). Esses são os chamados complexos de espécies e existem vários exemplos para o gênero *Fusarium* (COSTA et al., 2016; Herron et al., 2015; Laurence et al., 2014; O'Donnell et al., 2009). Análises baseadas em GCPSR para *Fusarium* nos últimos anos resultaram na identificação de 300 espécies

filogenéticas distintas, 20 complexos de espécies e 9 linhagens monotípicas (O'Donnell et al., 2015). Os genes mais informativos para diagnóstico e inferência filogenética são fator de elongação 1-α (*tef1*), maior e segunda maior subunidade da RNA polimerase II (*rpb1* e *rpb2*) (O'Donnell et al., 2015; Geiser et al., 2004).

A filogenia multilocus tem sido também empregada para a delimitação de espécies em *Trichoderma*, que frequentemente tem acrescentadas novas espécies e outras mais sendo recombinadas. Os genes *tef1*, *rpb2*, *cal1* (calmodulina), *acl1* (ATP citrato liase), *chi18-5* (endoquitinase) são utilizados para a delimitação das espécies (Chaverri et al., 2015; Jaklitsch & Voglmayr, 2015; Jaklitsch et al., 2013; Druzhinina et al., 2012).

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 Multilocus phylogeny of *Clonostachys* subgenus *Bionectria* from Brazil and description of *Clonostachys chloroleuca* sp. nov.

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Manuscrito preparado para submissão à revista Mycologia (JCR 2014/2015: 2,471)

ABSTRACT

The multilocus phylogeny of *Clonostachys* subgenus *Bionectria* occurring in Brazil was investigated using a collection of 65 strains. Phylogenetic analyses were based on fragments of ATP citrate lyase (*acl1*), beta tubulin (*tub*), the largest subunit of RNA polymerase II (*rpb1*), translation elongation factor 1-α (*tef1*), and combined *acl1-tef1-rpb1-tub* dataset. In addition, colony morphology and growth rates were assessed. The results allowed the recognition of a new species, *Clonostachys chloroleuca*. The multigene phylogeny supported the monophyly and recognition of the species *C. byssicola*, *C. rosea*, *C. rhizophaga*, *C. pseudochroleuca*, and *C. rogersoniana*. Strains of *C. pseudochroleuca* clustered in three subclades, of which one exhibiting distinct morphology.

Keywords: Bionectriaceae, GCPSR, neotropical fungi, phylogeny, taxonomy

1 INTRODUCTION

Clonostachys (Bionectriaceae, Hypocreales) species are common soil fungi, also isolated as endophytes and epiphytes. The teleomorphic phase of these taxa is often observed on dead plants (Domsch et al., 2007; Schroers, 2001). Generally regarded as saprothrophs, several members of *Clonostachys* are known mycoparasites (Schroers, 2001).

The ubiquitous *Clonostachys rosea* is a destructive parasite of other fungi, including plant pathogens, that has been extensively evaluated for biological control agent of plant diseases (Krauss et al., 2013; Cota et al., 2009; Chatterton et al., 2008). There are also reports of *C. rosea* parasitizing chitin-containing invertebrates, including some nematodes and insects (Zhang et al., 2008; Toledo et al., 2006). Biofungicides based on strains of *C. rosea* are marketed for the biocontrol of plant pathogens in different parts of the world (Kapongo et al., 2008; McQuilken et al., 2001). Recently, the genome sequence of the important strain IK726 of *C. rosea* was published (Karlsson et al., 2015), and the genome sequencing of another strain, 67-1, was announced (Sun et al., 2015).

C. rosea is classified in the subgenus *Bionectria*, which is composed by 22 species. These are characterized by dimorphic conidiophores, intercalary phialides rarely produced on secondary conidiophores, conidia with a laterally displaced hilum and conidial masses that can be pigmented either greenish, white or pale yellow-salmon. The species are found on bark and recently dead plant tissues, as endophytes, and as soil fungi. The other subgenera are *Zebrinella*, *Epiphloea*, *Astromata*, *Uniparietina*, and *Myronectria*, distinguished from each other on the bases of sexual and assexual characters (Schroers, 2001).

Species within the subgenus *Bionectria* formed a monophyletic group in phylogenetic trees constructed with DNA sequences of partial beta tubulin (*tub*)

and ITS-28S rDNA markers. However, not all recognized species inside this group formed well supported clades in these two-gene phylogenies (Schroers, 2001; Abreu et al., 2014).

We have employed MALDI-TOF mass spectrometric fingerprinting of biomarkers and molecular phylogenetics to assess the diversity of *Clonostachys* species isolated from different substrates and locations in Brazil. This polyphasic approach allowed the identification of five species inside subgenus *Bionectria*, including an undescribed species morphologically similar to *C. rosea* f. *catenulata* (Abreu et al., 2014).

Here we present an expanded analysis of *Clonostachys* subgenus *Bionectria* occurring in Brazil using multilocus phylogenetic analyses of four protein-coding gene sequences (*acl1*, *tef1*, *rpb1*, *tub*), and the description of *Clonostachys chloroleuca* sp. nov.

2 MATERIALS AND METHODS

2.1 Fungal strains

The *Clonostachys* strains used in this study were obtained from different substrates or were isolated as parasites overgrowing colonies of other fungi collected in different regions of Brazil (Table 1). The strains are deposited in the Coleção Micológica de Lavras (CML), Departamento de Fitopatologia, Universidade Federal de Lavras, Minas Gerais, Brazil (http://www.dfp.ufla.br/cml/). Reference and type strains included in this research were purchased from the Centraalbureau voor Schimmelcultures Fungal Biodiversity Center, Utrecht, The Netherlands (CBS).

2.2 Morphological characterization

The colony pigmentation was assessed according to Kornerup and Wanscher (1978). A disk of mycelium of 5 mm of diameter for each strain was inoculated on the center of vented plastic Petri dishes of 90 mm containing potato dextrose agar (PDA, Difco, USA) and the plates were incubated during 14 d at 25 ºC in darkness and under near UV light (315-400 nm). The evaluation of growth rate was made with the same procedures, but the strains were inoculated on malt extract agar (MEA, Oxoid, Basingstoke, UK) and incubated at 10, 15, 20, 25, 30, and 35 ºC during seven days.

Table 1 Strains of *Clonostachys* subgenus *Bionectria* used in this study.

Taxon name	**Code CML**[a]	**CBS code**[b]	**Substrate**	**Locality**	**GenBank Accession No.**			
					acl1	***tub***	***rpb1***	***tef1***
C. chloroleuca	1213		Soil, native Cerrado	Montividiu, GO[c]	x	KF871173	x	x
	1912		Soil, native Cerrado	Montividiu, GO	x	KF871168	x	x
	1916		Soil, cotton field	Montividiu, GO	x	KF871174	x	x
	1917		Soil, native Cerrado	Montividiu, GO	x	KF871169	x	x
	1918		Soil, native Cerrado	Montividiu, GO	x	x	x	x
	1919		Soil, native Cerrado	Montividiu, GO	x	KF871167	x	x
	1920		Soil, native Cerrado	Montividiu, GO	x	x	x	x
	1921		Soil, native Cerrado	Montividiu, GO	x	KF871166	x	x
	1922		Soil, native Cerrado	Montividiu, GO	x	KF871170	x	x
	1927		Soil, soybean field	Montividiu, GO	x	KF871171	x	x
	1941		Soil, native Cerrado	Montividiu, GO	x	KF871172	x	x
	2537		Bryophyte	Itumirim, MG	x	x	x	x
C. byssicola	0422		Soil, secondary forest	Benjamin Constant, AM	x	KF871150	x	x
	1943		Soil, Amazon Forest	Benjamin Constant, AM	x	KF871151	x	x
	2309		*Fragaria ananassa*	Bento Gonçalves, RS	x	KF871149	x	x
	2510	364.78 T[d]	Bark	Venezuela	x	AF358153	x	x
	1942		Soil, Amazon Forest	Benjamin Constant, AM	x	KF871148	x	x
	2311		Mycoparasite	Lavras, MG	x	KF871152	x	x
	2402		Fruit, *Annona squamosa*	Januária, MG	x	x	x	x
	2404		Fruit, *Annona x atemoya*	Jaíba, MG	x	KF871153	x	x

Table 1, continued

Taxon name	Code CML	CBS code	Substrate	Locality	GenBank Accession No.			
					acl1	*tub*	*rpb1*	*tef1*
	2511	365.78	Wood	Venezuela	x	AF358154	x	x
	2533		Bryophyte	Itumirim, MG	x	x	x	x
	2541		Litter	Itumirim, MG	x	x	x	x
	2552		*Piper nigrum*	Montes Claros, MG	x	x	x	x
	2654		Litter	Barroso, MG	x	x	x	x
	2665		Litter	Lavras, MG	x	x	x	x
C. rosea f. *catenulata*	2516	154.27 T	Soil	United States	x	AF358160	x	x
	2517	443.65	Soil	United States	x	AF358166	x	x
C. rosea f. *rosea*	0817		*Lychnophora pinaster*	Ingaí, MG	x	KF871147	x	x
	2310		*Fragaria ananassa*	Caxias do Sul, RS	x	KF871146	x	x
	2518	710.86 T	Soil	Netherlands	x	AF358161	x	x
	2519	194.57	Decaying bulb of *Lilium auratum*	United States	x	AF358165		x
	2549		Litter	Itumirim, MG	x	x	x	x
C. rhizophaga	1210		Soil, soybean plantation	Montividiu, GO	x	KF871156	x	x
	1984		Soil, native Cerrado	Montividiu, GO	x	KF871155	x	x
	2312		Mycoparasite	Lavras, MG	x	KF871157	x	x
	2514	361.77	Mycoparasite	Switzerland	x	AF358158	x	x
	2522		Soil	Lavras, MG	x	x	x	x
C. pseudochroleuca	018		*Solanum tuberosum*	S. Rita de Caldas, MG	x	KF871159	x	x
	0520		*Phoradendron perrottettii*	Lavras, MG	x	KF871160	x	x

Table 1, continued

Taxon name	Code CML	CBS code	Substrate	Locality	GenBank Accession No.			
					acl1	*tub*	*rpb1*	*tef1*
	0824		*Lychnophora pinaster*	Ingaí, MG	x	KF871162	x	x
	1940		*Phoradendron perrottettii*	Lavras, MG	x	KF871161	x	x
	1982		Soil, Amazon Forest	Benjamin Constant, AM	x	KF871165	x	x
	1983		Soil, Amazon Forest	Benjamin Constant, AM	x	KF871163	x	x
	2406		*Saccharum officinarum*	Piracicaba, SP	x	KF871158	x	x
	2435		Mycoparasite	Lavras, MG	x	x	x	x
	2440		Mycoparasite	Lavras, MG	x	x	x	x
	2513	187.94 T	Base of decaying palm frond	French Guiana	x	KF871188	x	x
	2523		Soil	Lavras, MG	x	x	x	x
	2524		Unidentified	MG	x	x	x	x
	2526		*Magifera indica*	Janaúba, MG	x	x	x	x
	2562	192.94	Bark	French Guiana	x	AF358171	x	x
	2670		Unidentified	Lavras, MG	x	x	x	x
C. rogersoniana	1216		Soil, native Cerrado	Montividiu, GO	x	KF871178	x	x
	1914		Soil, Amazon Forest	Benjamin Constant, AM	x	KF871180	x	x
	1923		Soil, native Cerrado	Montividiu, GO	x	KF871175	x	x
	1926		Soil, native Cerrado	Montividiu, GO	x	KF871176	x	x
	1944		Soil, secondary forest	Benjamin Constant, AM	x	KF871181	x	x
	2536		Bryophyte	Itumirim, MG	x	x	x	x
	2547		Litter	Itumirim, MG	x	x	x	x

Table 1, continued

Taxon name	Code CML	CBS code	Substrate	Locality	GenBank Accession No.			
					acl1	*tub*	*rpb1*	*tef1*
	2557	920.97 T	Soil under *Araucaria* sp.	SP	x	x	x	x
	2558	582.89	Soil, Amazon Forest	Capitão Poço, PA	x	AF358189	x	x
	2646		Litter	Barroso MG	x	x	x	x
	2656		Litter	Barroso MG	x	x	x	x
	2666		Litter	Lavras, MG	x	x	x	x

[a]CML = Coleção Micológica de Lavras, Universidade Federal de Lavras, Lavras, MG, Brazil; [b]CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; [c]Brazilian state = AM: Amazonas, GO: Goiás, MG: Minas Gerais, PA: Pará, RS: Rio Grande do Sul, SP: São Paulo. [d]T = ex-type strain.

The micromorphological characterization was made with seven to 14 d old colonies on 90 mm vented plastic Petri dishes containing oatmeal agar (OA, Difco, USA) and synthetic nutrient-poor agar with pieces of carnation leaves (abbreviated combination SNA/CL) incubated at 24 ºC. Slides were prepared with colony fragments and lactic acid 85 - 90 %. The size and shape of conidia, metulae and phialides, the presence, size and pattern of ramification of primary and/or secondary conidiophores, and the presence of intercalary phialides were observed. Images were recorded with Zeiss AxioCam MRc5 digital camera. Measurements were made with Axio Vision SE64 Rel. 4.9.1 software based on at least 20 independent measurements for each structure.

2.3 DNA extraction, PCR, and sequencing

Strains were grown for 7 d on OA at 25 ºC in darkness. The biomass was harvested in 2 mL tubes and subjected to DNA extraction using the NucleoSpin® Plant II kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Fragments of ATP citrate lyase (*acl1*), beta tubulin (*tub*), the largest subunit of RNA polymerase II (*rpb1*), and translation elongation factor 1-α (*tef1*) genes were amplified using the following primer pairs, respectively: acl1-230up and acl1-1220low (Gräfenhan et al., 2011), T1 and T2 (O'Donnell & Cigelnik, 1997), Fa and R8 (O'Donnell et al., 2010), EF1-728F and EF2 (Carbone & Kohn, 1999; O'Donnell et al., 1998). PCRs were performed with 50-100 ng of DNA in 50 mL reaction volumes containing 1x of PCR buffer, 2 mM of $MgCl_2$, 0.02 U/µL of Taq polymerase (*Taq* DNA Polymerase [native], Thermo Scientific, Massachusetts, USA), 0.8 mM of dNTP (Promega, Wisconsin, USA), and 1 µM of each primer in a Sure Cycler 8800 Thermal Cycler (Agilent Technologies, Santa Clara, California, USA). Cycling conditions for *acl1* were according to Gräfenhan et al. (2011). The amplification

of *tub* and *tef1* followed the conditions: 95 ºC for 3 min, 5 cycles of 95 ºC for 45 s, 56 ºC of 45 s, 72 ºC of 2 min, 35 cycles with annealing temperature of 52 ºC, and a final extension at 72 ºC for 10 min. The cycling conditions for *rpb1* were: 95 ºC for 3 min, 5 cycles of 95 ºC for 45 s, 60 ºC of 45 s, 72 ºC of 2 min, 5 cycles with annealing temperature of 58 ºC, 30 cycles with annealing temperature of 54 ºC, and a final extension at 72 ºC for 10 min. The fragments of DNA amplified were sent for sequencing by a commercial service.

2.4 Sequence alignment and phylogenetic analyses

Consensus sequences of *acl1*, *tub*, *rpb1*, and *tef1* were assembled and edited from forward and reverse trace files using SeqAssem (Hepperle, 2004). The poorly aligned positions of the introns in *acl1*, *tef* and *tub* gene fragments were removed before analyses using Gblocks program (Castresana, 2000). Sequences were aligned using ClustalW, as implemented in the software MEGA 6 (Tamura et al., 2013). Sequences from fragments of the four loci used for *Clonostachys* resulted in 2,579 bp in concatenated alignment: 709 bp for *acl1*, 341 bp for *tef1*, 1,024 bp for *rpb1*, and 505 bp for *tub*. *Clonostachys candelabrum* (CBS 504.67) was used as outgroup. Phylogenetic analyses were done using maximum likelihood (ML) and Bayesian inference (BI) methods conducted for each gene partition and for the concatenated *acl1-tub-rpb1-tef1* dataset. Models of sequence evolution were estimated using jModelTest (Darriba et al., 2012). The following models were implemented in the Bayesian phylogenetic analyses: SYM+G for *acl1* and *tef1*, SYM+I+G for *rpb1*, K80+G for *tub*. Bayesian analyses were performed with MrBayes 3.2 (Ronquist et al., 2012). For each dataset, two independent analyses were run for $2x10^6$ generations and sampled every 500 generations. Fifty-percent majority-rule consensus trees were constructed after discarding 25 % of the initial trees.

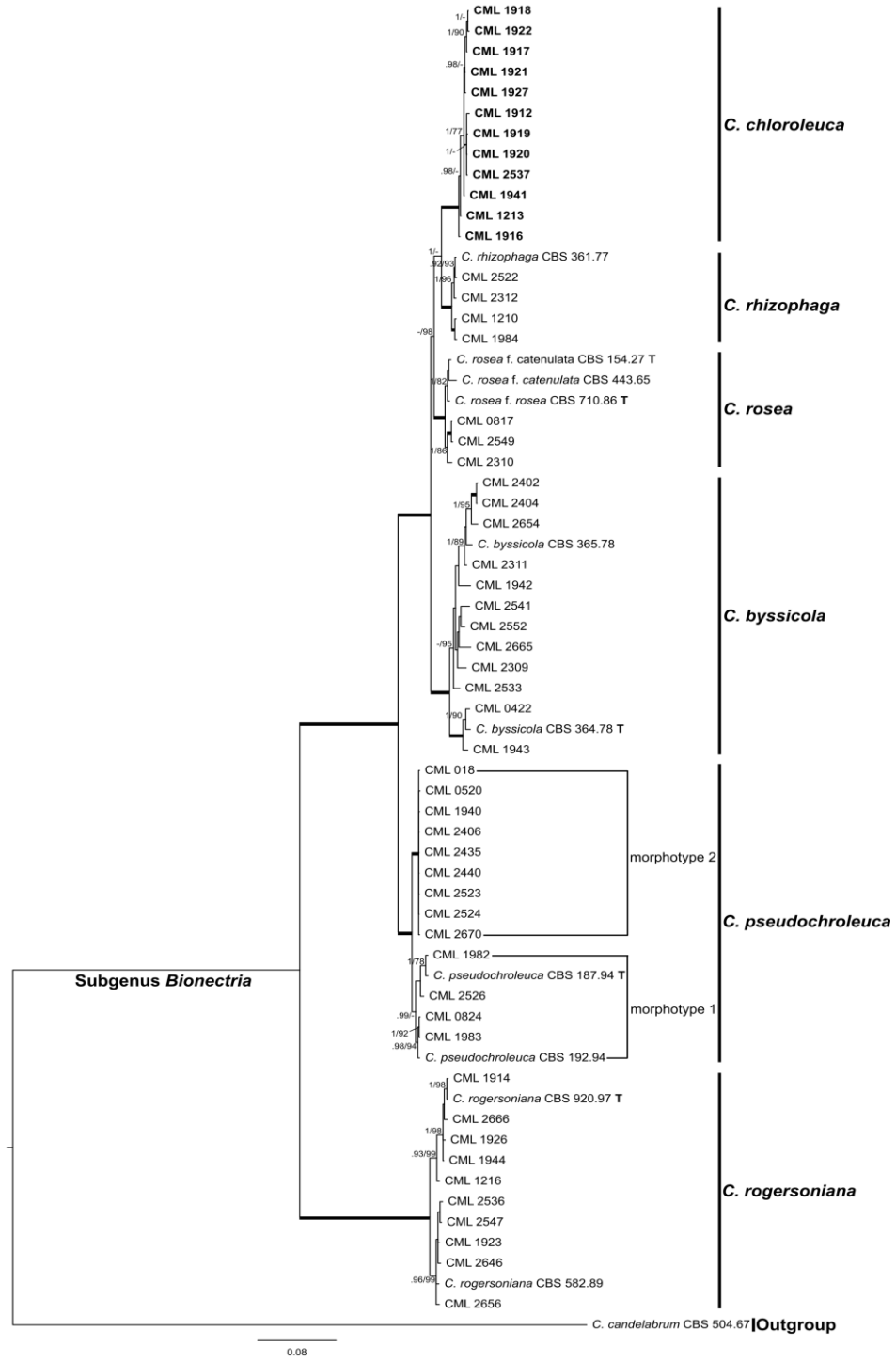
Phylogenetic trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and edited using InkScape 0.91 (www.inkscape.org). ML-based analyses were inferred using MEGA 6 with GTR+G for *acl1* and *tef1*, GTR+I+G for *rpb1*, K80+G for *tub* and 1,000 bootstrap pseudoreplicates.

3 RESULTS

The topology of combined trees obtained using ML and BI methods were congruent and BI tree is showed in Figure 1. Five clades corresponded to the known species: *C. byssicola*, *C. rhizophaga*, *C. rosea*, *C. pseudochroleuca*, *C. rogersoniana*. One additional well supported clade was composed by strains identified as belonging to a new species, described in this paper as *Clonostachys chloroleuca* (Fig. 1).

The *C. pseudochroleuca* clade was composed by three subclades. The one containing most of strains corresponded to a distinct morphotype (morphotype 2), differing from the original species description (Schroers, 2001). These strains formed bright yellow colonies on all media, with yellow reverse on OA and sulphur to greenish yellow (1A5; 1A8) on PDA, where they secret a diffusible yellow pigment. Primary and secondary conidiophores produced by these strains have adpressed branches and phialides (Fig. 2, p. 43).

Figure 1 Bayesian phylogenetic consensus tree of species of *Clonostachys* subgenus *Bionectria* based on concatenated DNA sequences of *acl1*, *tub*, *rpb1*, and *tef1* genes. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥70 % are in the nodes. Branches with ≥ 0.99 PP and 100 % bootstrap values are thickened. This tree is rooted with sequences of *C. candelabrum* (CBS 504.67). Codes in bold represent the newly described *C. chloroleuca*. Letter (T) following strain numbers indicates type strain.


CML 1918
CML 1922
CML 1917
CML 1921
CML 1927
CML 1912
CML 1919
CML 1920
CML 2537
CML 1941
CML 1213
CML 1916
C. chloroleuca
C. rhizophaga CBS 361.77
CML 2522
CML 2312
CML 1210
CML 1984
C. rhizophaga
C. rosea f. catenulata CBS 154.27 T
C. rosea f. catenulata CBS 443.65
C. rosea f. rosea CBS 710.86 T
CML 0817
CML 2549
CML 2310
C. rosea
CML 2402
CML 2404
CML 2654
C. byssicola CBS 365.78
CML 2311
CML 1942
CML 2541
CML 2552
CML 2665
CML 2309
CML 2533
CML 0422
C. byssicola CBS 364.78 T
CML 1943
C. byssicola
CML 018
CML 0520
CML 1940
CML 2406
CML 2435
CML 2440
CML 2523
CML 2524
CML 2670
morphotype 2
CML 1982
C. pseudochroleuca CBS 187.94 T
CML 2526
CML 0824
CML 1983
C. pseudochroleuca CBS 192.94
morphotype 1
C. pseudochroleuca
Subgenus Bionectria
CML 1914
C. rogersoniana CBS 920.97 T
CML 2666
CML 1926
CML 1944
CML 1216
CML 2536
CML 2547
CML 1923
CML 2646
C. rogersoniana CBS 582.89
CML 2656
C. rogersoniana
C. candelabrum CBS 504.67
Outgroup
0.08

The topologies of individual gene trees supported most of the clades recovered using the combined dataset (Supplementary Figs. 1 - 4, p. 53-56). *C. byssicola* strains formed a monophyletic group in all gene trees, except in the *tub* tree, and *C. rhizophaga* was split in into two groups in the *tef1* tree. Strains of *C. rogersoniana* were classified into two subclades in all analyses. The remaining taxa of the trees were similar to those described by Schroers (2001) and Abreu et al. (2014).

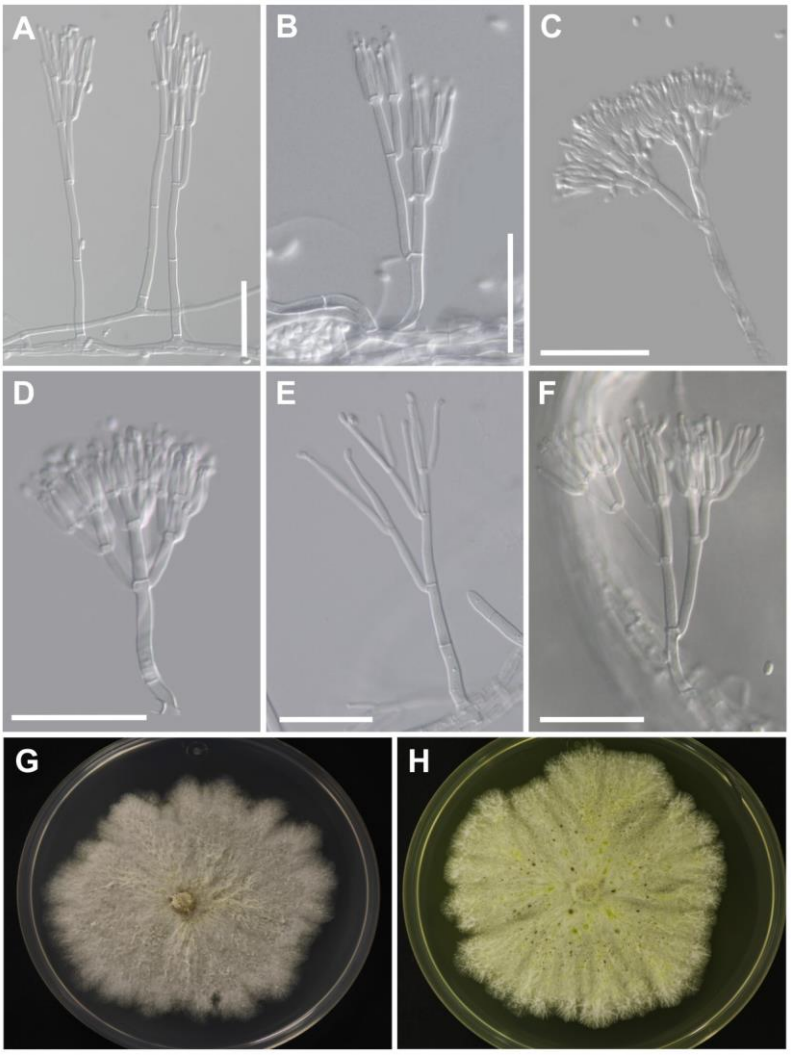


Figure 2 Morphology of *Clonostachys pseudochroleuca*. A-D. Morphotype 2. A-B. Primary conidiophores with adpressed branches and phialides; C-D. Secondary conidiophores with adpressed branches and phialides. E-F. Morphotype 1. E. Primary conidiophore with phialides and branches slightly divergent (CML 824). F. Secondary conidiophores with phialides slightly divergent (CML 824). G-H. Colonies 14 days old incubated at 24 ºC in the dark. Morphotype 1 (G) with pale yellow colony (CBS 187.94) and morphotype 2 (H) with yellow colony. Scale bars: 20 µm.

3.1 Taxonomy

Molecular phylogeny and morphological characters supported recognition of *Clonostachys chloroleuca* as distinct species within the *Bionectria* subgenus, which is formally described below.

Clonostachys chloroleuca Moreira, Abreu, Pfenning & Schroers, sp. nov.

(Fig. 3, p. 46)

Typification: BRAZIL. GOIÁS: Montividiu. Native soil of Cerrado; 2007; Vívian Carvalho; CML 1941.

Etymology: Greek chlorós = green + leukos = white, pale. Pale green coloration of the colony.

Colonies reaching 54 mm of diameter in 7 d at 25 ºC on MEA, optimum for growth 25 - 30 ºC (46 mm diam). On OA colony reverse light yellow or green, colony surface cottony because of strands of the aerial mycelium or granular; strong production of aerial mycelium. Surface unpigmented in the beginning becoming greenish grey to olivaceous because of conidial masses. On PDA incubated in darkness, colony reverse yellowish white or pale yellow (1A2-3; 2A2) and white surface with green points because of conidial masses on PDA, cottony colony. On PDA after incubation under UV, reverse greenish grey or orange white or orange grey (1B2; 5A2; 5B2), surface orange with green hues or pale green, velvety colony. Conidiophores dimorphic. Primary conidiophores verticillium-like, formed throughout the colony, stipes (43.8-)59-154.8(-226.6) µm long, (2.8-)3.5-4.7(-5.5) µm wide at base, (93.3-)125.6-246(-358.5) µm high, sometimes with short side branches arising from the upper part. Phialides

divergent, in whorls of 2 - 6 or singly from lower levels, (14.7-)18-36.4(-53) x (2-)2.4-3.5(-4.1) µm, straight, cylindrical, slightly tapering towards the tip. Secondary conidiophores solitary or aggregated, arising from strands of aerial mycelium or directly from the media, mainly on SNA/CL, bi- to quaterverticillate, (16.4-)29-87(-145) µm long, (3.3-)3.6-5.1(-5.6) µm wide at base, penicillus (83.2-)110-155(-210) µm high, usually with a side branch arising from the upper or middle part of stipe, divergent, terminating in less divergent metulae and adpressed phialides. Phialides (7.4-)8.2-15.32(-17.8) x (2-)2.3-3.6(-4.07) µm, in whorls of 2-6, almost cylindrical tapering in the upper part, straight to slightly curved; metulae (7.6-)9.4-17.6(-20.2) x (2.4-)2.6-3.7(-4.3) µm. Intercalary phialides rarely observed. Intermediate conidiophore with verticillium-like morphology but smaller phialides typical of secondary conidiophores were observed. Conidial masses transparent to greenish in watery heads in primary conidiophores and green abundantly formed and held in imbricate columns of aggregate secondary conidiophores. Conidia hyaline with laterally displaced or central hilum, one flattened side, sometimes ellipsoidal; those from secondary conidiophores (4.1-)5.6-7.4(-9.9) x (2.5-)3.5-4.4(-5.8) µm, from primary conidiophores (4.1-)4.4-5.8(-6.96) x (2.9-)3.2-4.1(-4.54) µm.

Habitat: soil, mosses.

Additional specimens examined: BRAZIL. GOIÁS: Montividiu. Native soil of Cerrado; 2007 (CML 1912, CML 1917, CML 1918, CML 1919, CML 1920, CML 1921, CML 1922) and July 2008 (CML 1213); soil cultivated with *Gossypium* sp. (CML 1916); soil cultivated with *Glycine max* (CML 1927); Vívian Carvalho. MINAS GERAIS: Itumirim. Bryophyte; 3 May 2013; Gláucia Mara Moreira (CML 2537).

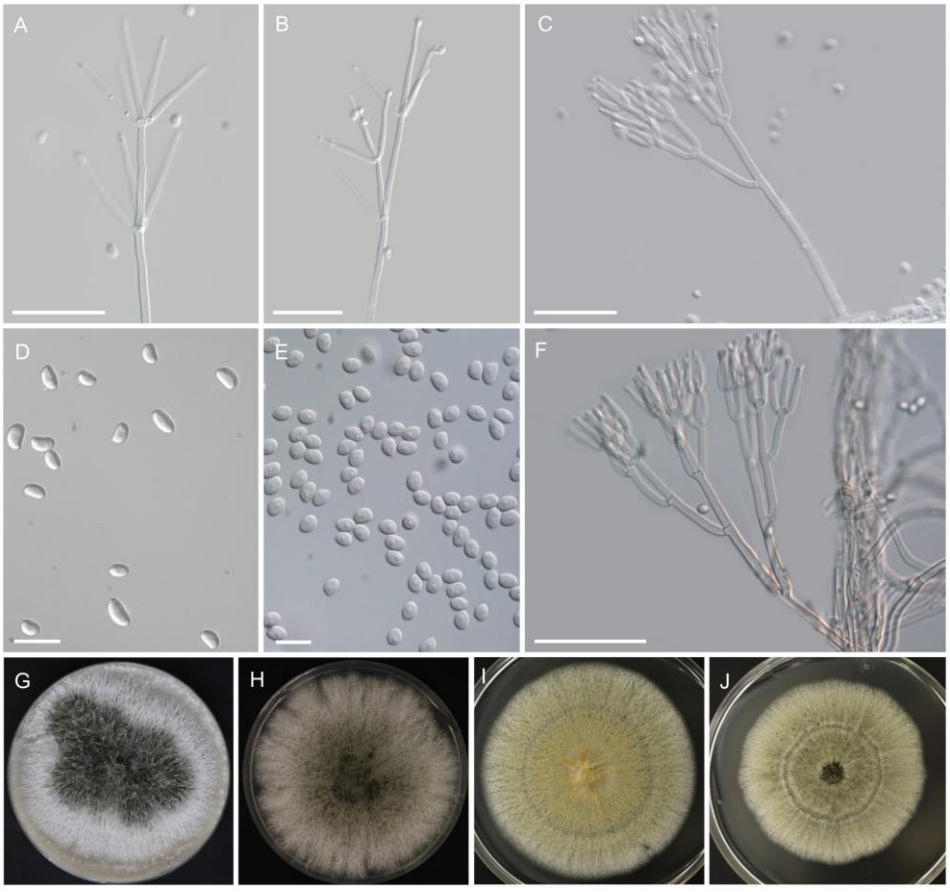


Figure 3 Morphology of *Clonostachys chloroleuca*. A-B. Primary conidiophores with divergent branches and phialides. C, F. Secondary conidiophores with divergent branches, terminating in less divergent metulae and adpressed phialides. D. Conidia from primary conidiophores. E. Conidia from secondary conidiophores. G-J. Colonies 14 days old at 24 ºC. G. OA; H. PDA incubated in the dark; I-J. PDA incubated under near UV light. Scale bars: A-C, F- 20 µm; D-E- 10 µm.

4 DISCUSSION

Clonostachys chloroleuca is formally described herein as new species based on phylogenetic analyses of four loci and morphological characters. The species formed monophyletic group in all phylogenetic analyses (Fig. 1, p. 41; Supplementary Figs. 1 - 4, p. 53-56) and has a distinct morphology when compared with known species (Fig. 3, p. 46). Results of mass profile analyses by MALDI-TOF MS also distinguished *C. chloroleuca* from the other closely related species (Abreu et al., 2014).

Among the species that form green conidia in *Clonostachys*, *C. chloroleuca* can be confounded with *C. rhizophaga* and *C. rosea* f. *catenulata*. *C. chloroleuca* is distinguished of *C. rhizophaga* because it has branches less divergent in the secondary conidiophores, the primary conidiophores are less branched, and form green conidia always. In contrast, in *C. rhizophaga* green conidia are not formed in all strains, but is inherent characteristic of *C. chloroleuca*. *C. rosea* f. *catenulata* produces longer conidia than *C. chloroleuca* and the branches of secondary conidiophores are adpressed, while in *C. chloroleuca* are divergent (Fig. 3, p. 46).

Most species in *Clonostachys* have been recognized based on the holomorphs, including morphological characteristics of teleomorphs commonly found on recently dead woody hosts (Schroers, 2001). No sexual state was observed in *C. chloroleuca*, since all strains included in this work came from plate isolations, where only the anamorphs are commonly produced (Domsch et al., 2007; Schroers, 2001). Tests of homothallism were conducted with monosporic cultures of *C. chloroleuca* using protocols adapted for *Fusarium* (Leslie & Summerell, 2006) and revealed no signs of perithecia or proto-perithecia (data not shown), suggesting that this species may be heterothallic.

The monophyly of *C. byssicola* and *C. pseudochroleuca* was not fully supported in previous works where phylogenetic analyses were based on *tub* and ITS-LSU sequences, and these species were also distinguished by morphology of holomorphs and mass fingerprints obtained by MALDI-TOF MS (Abreu et al., 2014; Schroers, 2001). The additional gene markers accessed in the present work (*acl1*, *rpb1*, and *tef1*) strongly supported both species as monophyletic. Nine out of the 13 strains identified as *C. pseudochroleuca* morphotype 2 grouped in distinct subclade in the combined tree, as well as in *acl1*, *rpb1*, and *tub* trees. These strains exhibit a slightly distinct morphology, characterized by bright yellow colonies and adpressed phialides in primary conidiophores, resembling the morphological description of *Clonostachys solani* (Schroers, 2001). The remaining six strains of *C. pseudochroleuca* (morphotype 1) produce paler colonies without yellow diffuse pigment and less adpressed conidiophores and phialides (Fig. 2, p. 43).

The clade of *C. rogersoniana* had high statistical support in all phylogenetic analyses and is split into two subclades (Fig. 1, p. 41; Supplementary Figs. 1 - 4, p. 53-56), that are well supported in the combined tree. However, some strains can change positions according to the gene used. The strain CML 1216 is grouped in the subclade together with the type strain in the *tub*, *rpb1*, and combined trees, while CML 1926 was not grouped with type strain only in the *tub* tree.

In the combined phylogeny, *C. rosea* formed a well supported clade split into two subclades statistically supported (Fig. 1, p. 41). Schroers (2001) reported that *C. rosea* strains from conidia or ascospore origin formed two subclades in phylogenetic analyses. This finding was supported only in the *tub* phylogeny where conidial strains grouped with ascospore derived (CBS 194.57). Although this strain is not present in the *rpb1* and combined trees, the subclades are different represented depending on the analyzed gene (Fig. 1, p. 41;

Supplementary Figs. 1 - 4, p. 53-56). *C. rhizophaga* was found polyphyletic in the *tef1* tree (Supplementary Fig. 4, p. 56), but formed well supported clade in the remaining analyses, what supports the monophyly of the group.

According to the genealogical concordance concept (GCPSR) (Taylor et al., 2000), the multilocus phylogenetic analyses of four protein-coding gene sequences allowed the recognition of five known species plus *C. chloroleuca* sp. nov. Phylogeny of *Clonostachy*s subgenus *Bionectria* showed the genetic variation within some species, what suggests heterotallic mating system, since sexual crosses result in recombination. The gene regions here included to delimit the *Clonostachys* species, *acl1* phylogeny showed the most genetic variation among species when compared with the phylogeny using combined dataset (Fig. 1, p. 41; Supplementary Fig. 1, p. 53). Our study presents an expanded phylogenetic framework for delimitation and recognition of *Clonostachys* species inside the important subgenus *Bionectria*, and presumably it will be useful for authentication of strains, in special those used for biocontrol purposes.

ACKNOWLEDGEMENTS

Part of this work was conducted during a stay at Agricultural Institute of Slovenia, Ljubljana, supported by a scholarship granted to the first author by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (Proc. 011622/2013-00). We thank Edson Rodrigues-Filho from UFSCar for purchasing reference strains from CBS, with support of SISBIOTA-Brasil (Proc. 563063/2010-6).

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SUPPLEMENTARY MATERIAL

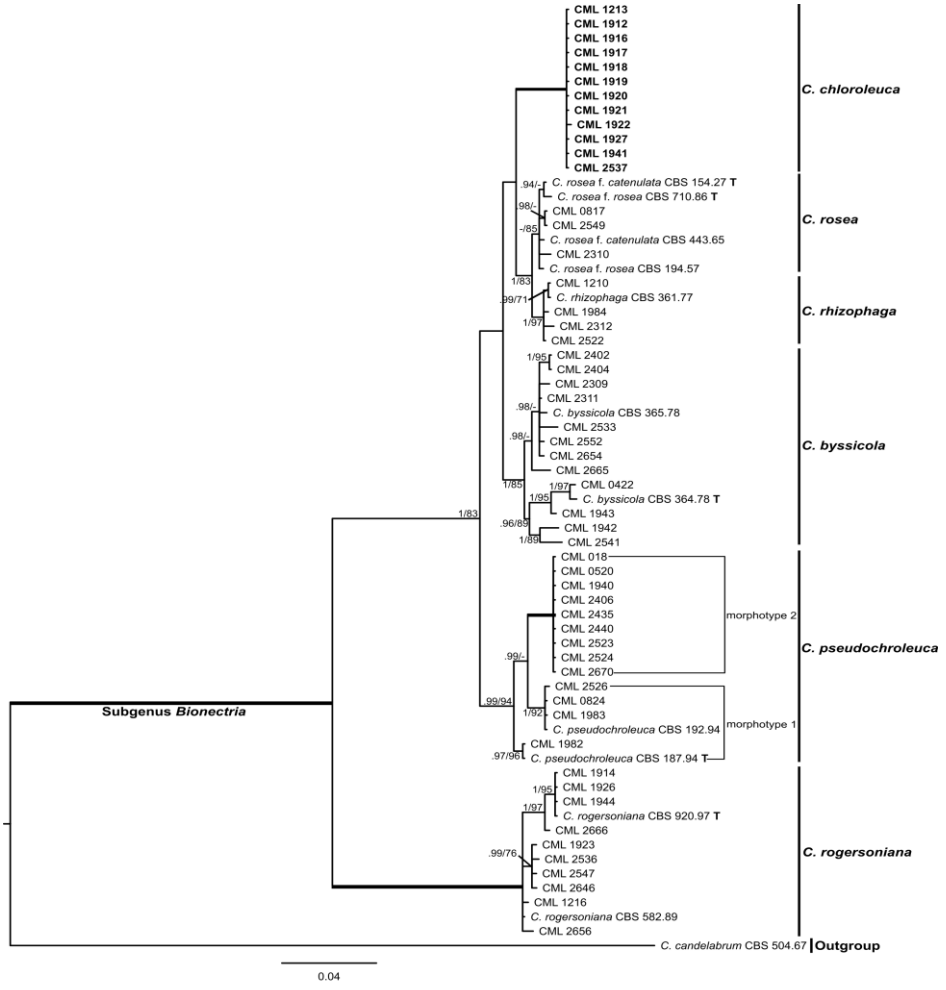

Figure 1 Bayesian phylogenetic consensus tree of species of *Clonostachys* subgenus *Bionectria* based on DNA sequences of *acl1* gene. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥ 70 % are in the nodes. Branches with ≥ 0.99 PP and 100 % bootstrap values are thickened. This tree is rooted with sequence of *C. candelabrum* (CBS 504.67). Codes in bold represent the newly described *C. chloroleuca*. Letter (T) following strain numbers indicates type strains.

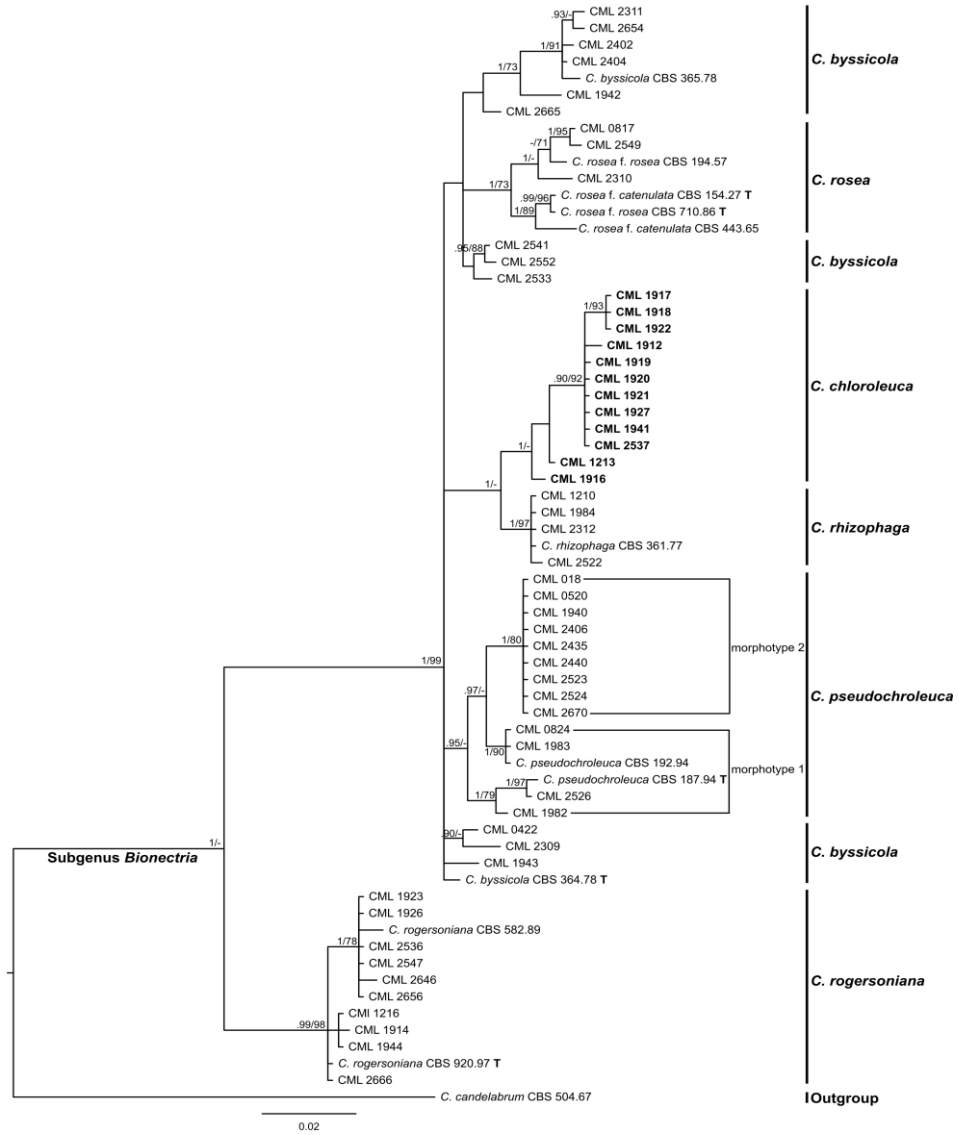

Figure 2 Bayesian phylogenetic consensus tree of species of *Clonostachys* subgenus *Bionectria* based on DNA sequences of *tub* gene. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥ 70 % are in the nodes. This tree is rooted with sequence of *C. candelabrum* (CBS 504.67). Codes in bold represent the newly described *C. chloroleuca*. Letter (T) following strain numbers indicates type strains.

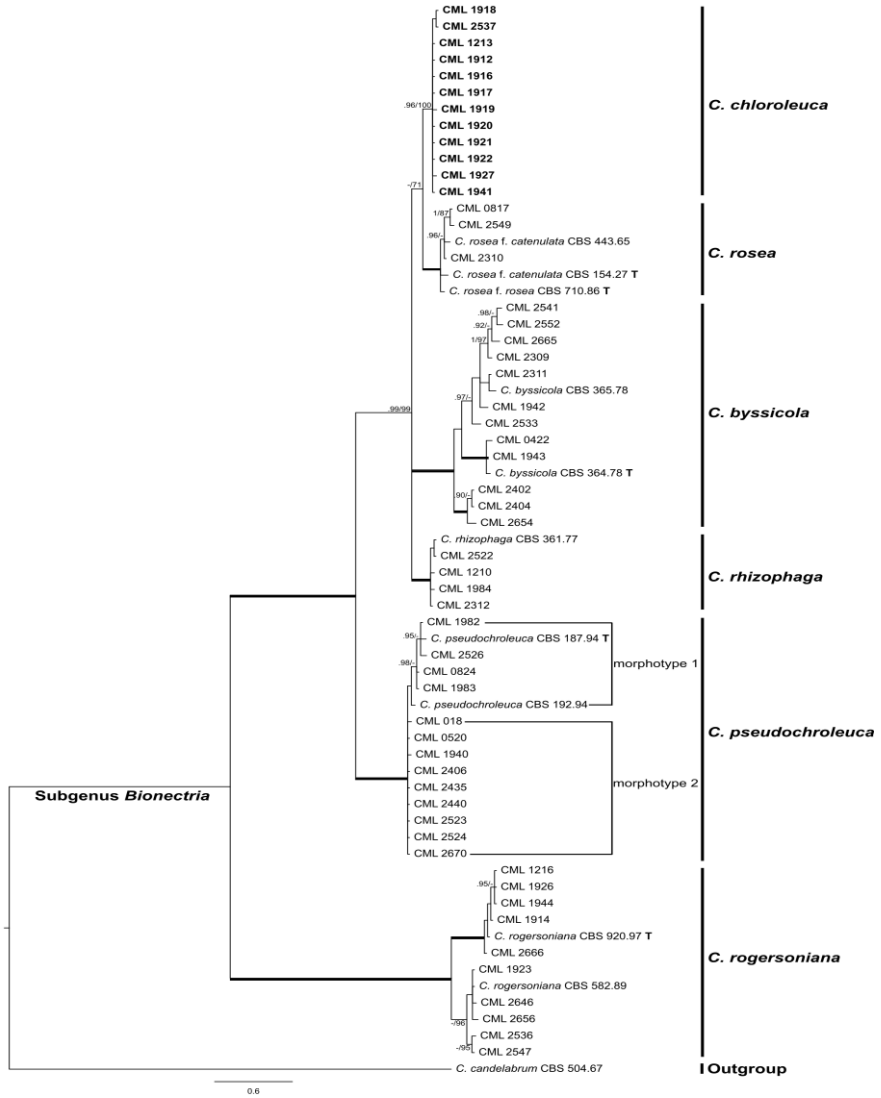

Figure 3 Bayesian phylogenetic consensus tree of species of *Clonostachys* subgenus *Bionectria* based on DNA sequences of *rpb1* gene. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥ 70 % are in the nodes. Branches with ≥ 0.99 PP and 100 % bootstrap values are thickened. This tree is rooted with sequence of *C. candelabrum* (CBS 504.67). Codes in bold represent the newly described *C. chloroleuca*. Letter (T) following strain numbers indicates type strains.

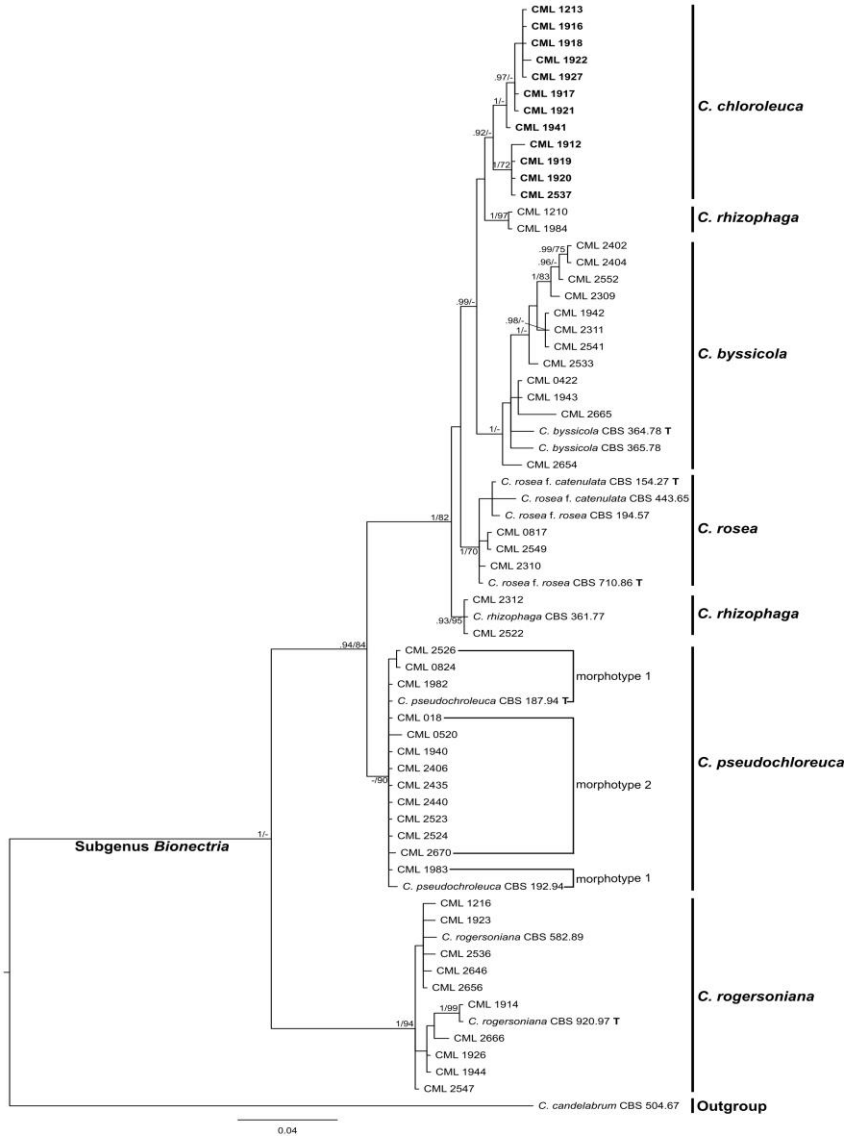

Figure 4 Bayesian phylogenetic consensus tree of species of *Clonostachys* subgenus *Bionectria* based on DNA sequences of *tef1* gene. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥ 70 % are in the nodes. This tree is rooted with sequence of *C. candelabrum* (CBS 504.67). Codes in bold represent the newly described *C. chloroleuca*. Letter (T) following strain numbers indicates type strains.

ARTIGO 2 Phylogeny of the genus *Clonostachys* from Brazil and description of new species

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Manuscrito preparado para submissão à revista Mycologia (JCR 2014/2015: 2,471)

ABSTRACT

We show an expanded phylogenetic analysis using ATP citrate lyase (*acl1*) gene for a large number of *Clonostachys* strains from Brazil. Representatives of the subgenus *Bionectria*, *Epiphloea* and *Zebrinella* are included. Six species from subgenus *Bionectria*, including two clades formed by strains morphologically similar to *C. compactiuscula*, were identified. In the subgenus *Epiphloea* it was identified *C. candelabrum* and a new species, *Clonostachys* sp. 2. The subgenus *Zebrinella* was represented by a single strain from an undescribed species, identified as *Clonostachys* sp. 3. The genealogical concordance using the genes *acl1*, beta tubulin (*tub*), and translation elongation factor 1-α (*tef1*) for selected strains, and morphological examinations, confirmed *Clonostachys* sp. 2 as a new species. Strains morphologically similar to *C. compactiuscula* did not group together with monophyletic group representing this species in the *tub* tree. These strains represent probable new species.

Keywords: Bionectriaceae, neotropical fungi, phylogeny, taxonomy

1 INTRODUCTION

The genus *Clonostachys* (Bionectriaceae, Hypocreales) comprises endophytes, mycoparasites, parasites of chitin-containing invertebrates, soil saprotrophs and, rarely, plant pathogens (Schroers, 2001; Domsch et al., 2007; Abang et al., 2009). The species are characterized by typical asymmetric conidia, determined by lateral extrusion from phialides, and the conidia are arranged in imbricate chains in the penicillus. All species form penicillate conidiophores and many of them also form verticillium-like conidiophores (Schroers, 2001). The well-known species *Clonostachys rosea* is a destructive mycoparasite used in biological control of plant pathogens and tested against nematodes and insects (Toledo et al., 2006; Chatterton et al., 2008; Kapongo et al., 2008; Zhang et al., 2008).

Species recognition in *Clonostachys* was made by Schroers (2001) connecting sexual-asexual morphs, based on morphological characters and molecular phylogeny of partial sequences of beta tubulin (*tub*) gene and ITS rDNA region. The author used few isolates in the phylogenetic analyses, and not all type strains were sequenced. Furthermore, the two-gene phylogenies showed inconsist for some groups. Abreu et al. (2014), studying different species of *Clonostachys*, observed the same result.

We investigated the species of subgenus *Bionectria* occurring in Brazil using multilocus phylogenetic analyses of four protein-coding gene sequences: ATP citrate lyase (*acl1*), largest subunit of RNA polymerase II (*rpb1*), elongation factor 1-α (*tef1*), and *tub* (Moreira et al. 2016 unpublished). The *acl1* and *rpb1* genes did not show any divergence between topologies and the concatenated tree. The groups formed in the trees were the same as those observed in the dendrogram of MALDI-TOF (Abreu et al., 2014) and corresponded to morphological character of their respective species. However,

acl1 showed more polymorphism, it was capable of generating a phylogeny of the genus comparable with the tree of concatenated sequences, and is easier to amplify, maybe it can be used as barcode.

We tested the ability of *acl1* as a phylogenetic marker and as a barcode for the genus *Clonostachys* examining a collection of 52 strains obtained along of the years from various substrates and locations in Brazil. We also did a detailed phylogenetic study using *acl1*, *tub* and *tef1* with isolates that had not grouped with known species, revealing three distinct species, described in this article.

2 MATERIALS AND METHODS

2.1 Fungal strains

The *Clonostachys* strains used in this study were recovered from the Coleção Micológica de Lavras (CML), Departamento de Fitopatologia, Universidade Federal de Lavras, Brazil (http://www.dfp.ufla.br/cml/). The isolates were obtained from different regions of Brazil corresponding to Amazon, Atlantic Rainforest and Cerrado biomes (Table 1). Reference and type strains included in this study were purchased from Centraalbureau voor Schimmelcultures Fungal Biodiversity Center, Utrecht, The Netherlands (CBS).

2.2 Morphological evaluations

Cultures were grown in 9 cm diam vented plastic Petri dishes on potato dextrose agar (PDA, Difco, USA) in darkness and under near UV (315-400 nm) light during 14 days at 25 ºC to evaluate the colony pigmentation, assessed according to Kornerup and Wanscher (1978). The growth rate was evaluated on malt extract agar (MEA, Oxoid, Basingstoke, UK) incubated at 10, 15, 20, 25, 30, and 35 ºC in darkness during seven days.

Micromorphological structures were examined with seven to 14 d old colonies in 90 mm vented plastic Petri dishes containing oatmeal agar (OA, Difco, USA) and synthetic nutrient-poor agar with pieces of carnation leaves (abbreviated combination SNA/CL) incubated at 24 ºC. Images were recorded with Zeiss AxioCam MRc5 digital camera. Measurements were made with Axio Vision SE64 Rel. 4.9.1 software. Nomarski differential interference contrast (DIC) was used for observations and measurements.

Table 1 *Clonostachys* strains used in this study.

Taxon name	Code CML[a]	Code CBS[b]	Substrate	Locality[c]	GenBank Accession No.		
					acl1	*tub*	*tef1*
Clonostachys sp. 2	1913		Soil, Amazon Forest	Benjamin Constant, AM	x	KF871183	x
	2315		Soil, Atlantic Rainforest	São Miguel Arcanjo, SP	x	KF871182	x
	2316		Soil, Atlantic Rainforest	São Miguel Arcanjo, SP	x	KF871184	x
C. candelabrum	2313		Soil, Atlantic Rainforest	Assis, SP	x	KF871186	x
	2512	504.67	Soil in wheat field	Netherlands	x	KF871189	x
	2546		Litter	Itumirim, MG	x	x	x
	2551		Litter	Itumirim, MG	x		
C. rossmaniae	2520	211.93 T[d]	Twig of recently dead tree	French Guiana	x	KF871190	x
C. subquaternata	2561	100003 T	Bark, recently died	Puerto Rico	x	x	x
Clonostachys sp. 3	1910		Soil, Amazon Forest	Benjamin Constant, AM	x	KF871185	x
C. cf. *compactiuscula* 1	2538		Bryophyte	Itumirim, MG	x		
	2545		Litter	Itumirim, MG	x		
	2548		Litter	Itumirim, MG	x	x	x
	2550		Litter	Itumirim, MG	x	x	x
C. cf. *compactiuscula* 2	2540		Litter	Itumirim, MG	x	x	x
	2542		Litter	Itumirim, MG	x		
	2544		Litter	Itumirim, MG	x	x	x
C. rogersoniana	1911		Soil, Amazon Forest	Benjamin Constant, AM	x		
	1925		Soil, native Cerrado	Montividiu, GO	x		
	2647		Litter	Barroso, MG	x		

Table 1, continued

Taxon name	Code CML	Code CBS	Substrate	Locality	GenBank Accession No.		
					acl1	*tub*	*tef1*
	2649		Litter	Barroso, MG	x		
	2650		Litter	Barroso, MG	x		
	2651		Litter	Barroso, MG	x		
	2652		Litter	Barroso, MG	x		
	2653		Litter	Barroso, MG	x		
	2655		Litter	Barroso, MG	x		
	2658		Litter	Barroso, MG	x		
	2660		Litter	Barroso, MG	x		
	2661		Litter	Lavras, MG	x		
	2662		Litter	Lavras, MG	x		
	2663		Litter	Lavras, MG	x		
	2664		Litter	Lavras, MG	x		
	2668		Litter	Lavras, MG	x		
	2669		Litter	Lavras, MG	x		
C. byssicola	2401		Fruit, *Annona squamosa*	Januária, MG	x		
	2403		Leaf, *Annona* x *atemoya*	Jaíba, MG	x		
	2405		Fruit, *Annona* x *atemoya*	Jaíba, MG	x		
	2407		Flower, *Annona squamosa*	Januária, MG	x		
	2525		*Piper nigrum*	Jaguaré, ES	x		
	2532		Bryophyte	Itumirim, MG	x		

Table 1, continued

Taxon name	Code CML	Code CBS	Substrate	Locality	GenBank Accession No.		
					acl1	*tub*	*tef1*
	2534		Bryophyte	Itumirim, MG	x		
	2535		Bryophyte	Itumirim, MG	x		
	2539		Bryophyte	Itumirim, MG	x		
	2553		Mycoparasite	Lavras, MG	x		
	2648		Litter	Barroso, MG	x		
	2657		Litter	Barroso, MG	x		
	2667		Litter	Lavras, MG	x		
C. rosea f. *rosea*	1670		*Theobroma cacao*	Ilhéus, BA	x		
	1820		*Theobroma cacao*	Manaus, AM	x		
	2659		Litter	Barroso, MG	x		
C. rhizophaga	1924		Soil, native Cerrado	Montividiu, GO	x		
	2515	125416	Bamboo	Italy	x		
	2832		*Cicer arietinum*	Cristalina, GO	x		
C. pseudochroleuca	1915		Soil, Amazon Forest	Benjamin Constant, AM	x		
	2436		Mycoparasite	Lavras, MG	x		
	2444		Mycoparasite	Lavras, MG	x		

[a]CML = Coleção Micológica de Lavras, Universidade Federal de Lavras, Lavras, MG, Brazil; [b]CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; [c]Brazilian state = AM: Amazonas, BA: Bahia, ES: Espírito Santo, GO: Goiás, MG: Minas Gerais, PA: Pará, RS: Rio Grande do Sul, SP: São Paulo. [d]T= ex-type strain.

2.3 DNA extraction, PCR, and sequencing

Isolates were grown for 7 d on OA at 25 ºC in darkness. The biomass was harvested in 2 mL tubes and subjected to DNA extraction using the NucleoSpin® Plant II kit (MACHEREY-NAGEL GmbH& Co. KG, Düren, Germany). Fragments of *acl1* were amplified using the primers acl1-230up and acl1-1220low (Gräfenhan et al., 2011). The primers T1 and T2 (O'Donnell & Cigelnik, 1997) were used to amplify fragments of *tub* gene. The *tef1* gene was amplified using the primers EF1-728F and EF2 (Carbone & Kohn, 1999; O'Donnell et al., 1998). PCRs were performed with 50 -100 ng of DNA in 50 mL reaction volumes containing 1 x of PCR buffer, 2 mM of $MgCl_2$, 0,02 U/µL of Taq polymerase (*Taq* DNA Polymerase [native], Thermo Scientific, Massachussetts, USA), 0,8 mM of dNTP (Promega, Wisconsin, USA), and 1 µM of each primer in a Sure Cycler 8800 Thermal Cycler (Agilent Technologies, Santa Clara, California, USA). Cycling conditions for *acl1* were according to Gräfenhan et al. (2011). The amplification of *tub* and *tef1* followed the conditions: 95 ºC for 3 min, 5 cycles of 95 ºC for 45 s, 56 ºC of 45 s, 72 ºC of 2 min, 35 cycles with annealing temperature of 52 ºC and a final extension at 72 ºC for 10 min. The fragments of DNA amplified were sent for sequencing by a commercial service.

2.4 Phylogenetic analyses

Consensus sequences of *acl1*, *tef1*, and *tub* were assembled and edited from forward and reverse trace files using SeqAssem (Hepperle, 2004). Sequences were aligned using ClustalW, as implemented in the software MEGA 6 (Tamura et al., 2013). Gblocks program was used to remove poorly aligned

positions of the introns (Castresana, 2000). Models of sequence evolution were estimated using jModelTest (Darriba et al., 2012).

All strains were submitted to phylogenetic analyses of *acl1* gene. Representatives of subgenus *Zebrinella* and *Epiphloea*, together with strains of *C.* cf. *compactiuscula* 1 and 2 and *C. rogersoniana* were used in the concatenated dataset (*acl1-tef1-tub*) analyses, having *Fusarium circinatum* (CBS 405.97) as the outgroup (Lombard et al., 2015). Additional *tub* gene sequences of reference strains (Schroers, 2001) were included in the phylogenetic analysis of this region, resulting in an alignment with 398 bp (Supplementary Table 1, p. 80).

The aligned sequences of *acl1* gene for all strains consisted of 600 bp. The dataset gene boundaries of selected strains for combined tree were 1-612 bp for *acl1*, 613-912 bp for *tef1* (300 bp), and 913-1,315 bp for *tub* (403 bp). Phylogenetic analyses were done using the methods of maximum likelihood (ML) and Bayesian inference (BI), for each gene partition and for the concatenated dataset. The model SYM+I+G was used for *acl1* phylogeny for all strains in the BI method. The models chosen for selected strains phylogeny were: SYM+I for *acl1*, SYM+I+G for *tef1*, K80+G for *tub*. Bayasian analyses were performed with MrBayes 3.2 (Ronquist et al., 2012). Two independent analyses were run for $2x10^6$ generations and sampled every 500 generations. Fifty-percent majority-rule consensus trees were constructed after discarding 25 % of the initial trees. Phylogenetic trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and edited using InkScape 0.9.1 (www.inkscape.org). ML-based analyses were inferred using MEGA 6 and clades support was inferred from 1,000 bootstrap replications. The model used for *acl1* tree with all strains was GTR+I+G. The models chosen for selected strains phylogeny were: GTR+I for *acl1*, GTR+I+G for *tef1*, and HKY+G for *tub*.

3 RESULTS

The *acl1* tree delimited six known species of subgenus *Bionectria* (*C. chloroleuca*, *C. byssicola*, *C. rosea*, *C. rhizophaga*, *C. pseudochroleuca*, *C. rogersoniana*) and two clades named as *C.* cf. *compactiuscula* 1 and 2. The subgenus *Zebrinella* included one unidentified taxa showing phylogenetic affinities to *C. subquaternata* (*Clonostachys* sp. 3). The subgenus *Epiphloea* is represented by *C. candelabrum* and a clade with three strains corresponded to *Clonostachys* sp. 2 in Abreu et al. (2014; Fig. 1, p. 69-70).

The tree of the concatenated sequences of *acl1*, *tef1*, and *tub* included representatives of subgenus *Zebrinella* and *Epiphloea* plus *C.* cf. *compactiuscula* 1 and 2. The result tree showed *Clonostachys* sp. 2 in a clade supported by high values of posterior probability (PP) in BI and bootstrap (BS) in ML (Fig. 2, p. 71). *Clonostachys* sp. 2 also formed a well supported monophyletic group in the individual trees (Supplementary Figs. 1 - 3, p. 81-83).

The strains named as *C.* cf. *compactiuscula* have similar morphology to *C. compactiuscula* (Fig. 3, p. 72). In the *tub* tree were included reference sequences of *C. compactiuscula*, however, the strains of *C.* cf. *compactiuscula* did not grouped with the species and continued split into two clades (Supplementary Fig. 3, p. 83). Strains of both of clades have the same morphological characters. Colonies reaching 35 mm of diameter in 7 d at 25 ºC on MEA. On OA reverse brown with light yellow pigmentation in the center, white colony. On PDA, colony reverse light or pastel yellow (2A5; 1A4) in the dark and pale yellow (2A3) with brown spots under UV. Colony surface cottony. Dimorphic conidiophores. Primary conidiophores verticillium-like, stipe 124-357.5 x 4-5.7 µm, 83-440 µm high; phialides divergent, 10.4-42.5 x 2-3.9 µm, in whorls of 3-6 or singly from lower levels. Secondary conidiophores bi- to terverticillate, with divergent branches, stipe 50-134 x 4-7.5 µm, penicillus 100-

375 µm high; adpressed metulae, 7-15 x 2-4.5 µm; 1-5 adpressed phialides, 5-16 x 2-3.9 µm; intercalary phialides arising below the terminal phialides. Conidial masses white or pale yellow formed in imbricate columns. Ellipsoidal to cylindrical conidia, with laterally displaced hilum, 5-8.1 x 1.5-3.3 µm.

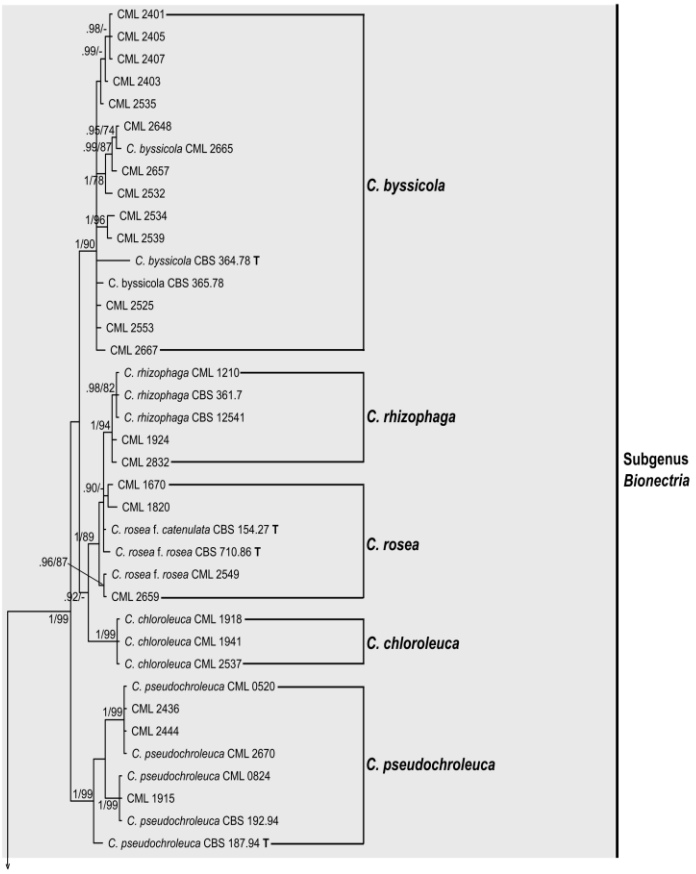
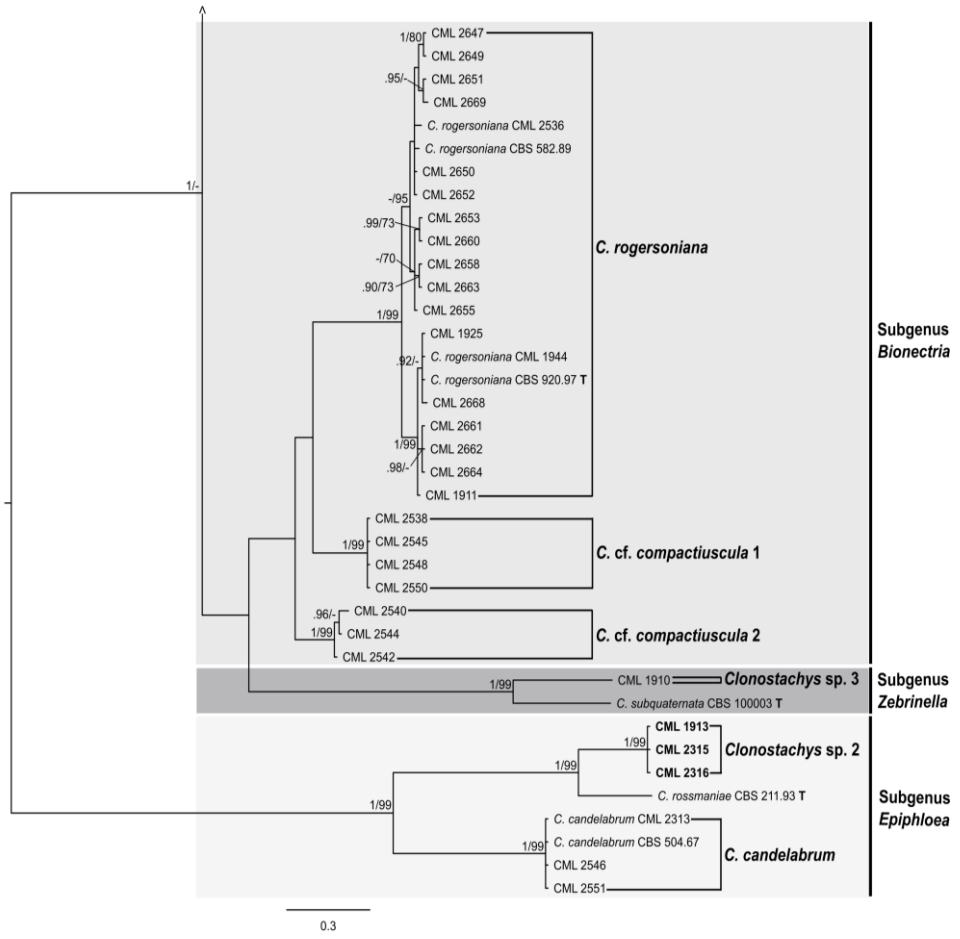


Figure 1 Bayesian phylogenetic consensus tree of species of *Clonostachys* based on DNA sequences of *acl1* gene. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥ 70 % are in the nodes. Branches with ≥ 0.99 PP and 100 % bootstrap values are thickened. Codes in bold represent the newly described *Clonostachys* sp. 2. Letter (T) following strain numbers indicates type strains.

(…continue…)


1/80
CML 2647
CML 2649
.95/-
CML 2651
CML 2669
C. rogersoniana CML 2536
C. rogersoniana CBS 582.89
CML 2650
1/-
-/95
CML 2652
.99/73
CML 2653
CML 2660
-/70
CML 2658
.90/73
CML 2663
CML 2655
1/99
C. rogersoniana
CML 1925
.92/-
C. rogersoniana CML 1944
C. rogersoniana CBS 920.97 T
CML 2668
CML 2661
1/99
CML 2662
.98/-
CML 2664
CML 1911
CML 2538
1/99
CML 2545
CML 2548
CML 2550
C. cf. compactiuscula 1
.96/-
CML 2540
1/99
CML 2544
CML 2542
C. cf. compactiuscula 2
Subgenus
Bionectria
1/99
CML 1910
Clonostachys sp. 3
C. subquaternata CBS 100003 T
Subgenus
Zebrinella
CML 1913
1/99
CML 2315
Clonostachys sp. 2
CML 2316
1/99
C. rossmaniae CBS 211.93 T
1/99
Subgenus
Epiphloea
C. candelabrum CML 2313
1/99
C. candelabrum CBS 504.67
C. candelabrum
CML 2546
CML 2551
0.3

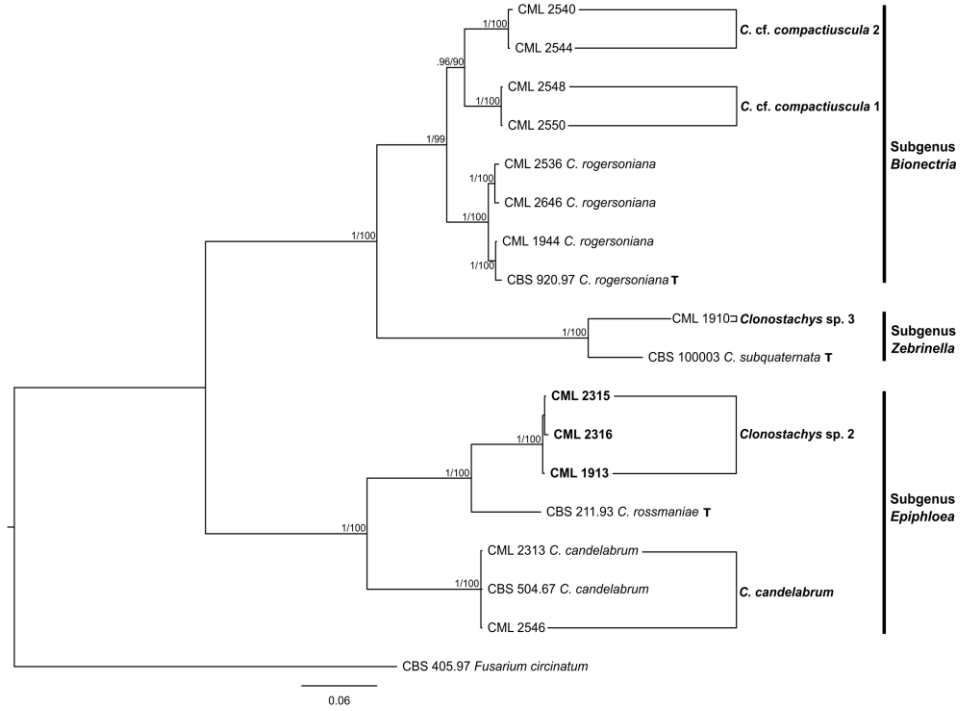


Figure 2 Bayesian phylogenetic consensus tree of species of *Clonostachys* based on concatenated DNA sequences of *acl1*, *tef1*, and *tub* genes. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥ 70 % are in the nodes. This tree is rooted with sequences of of *Fusarium circinatum* (CBS 405.97). Codes in bold represent the newly described *Clonostachys* sp. 2. Letter (T) following strain numbers indicates type strains.

Figure 3 Morphology of *Clonostachys* cf. *compactiuscula*. A. Primary conidiophore with divergent phialides (CML 2540). B-C. Secondary conidiophores with divergent branches and adpressed metulae and phialides (CML 2544). D. Ellipsoidal to cylindrical conidia (CML 2548). E. Colony on PDA 14 days old incubated at 24 ºC in the dark (CML 2544). Scale bars: A-D- 20 µm; E- 10 µm.

3.1 Taxonomy

Molecular phylogeny analyses and morphological characterization support the recognition of *Clonostachys* sp. 2 as a distinct species within *Clonostachys* subgenus *Epiphloea*, which is formally described below.

Clonostachys sp. 2 Moreira, Abreu, Pfenning & Schroers, sp. nov.

(Fig. 4, p. 74)

Typification: BRAZIL. SÃO PAULO: São Miguel Arcanjo, Parque Estadual Carlos Botelho. Native soil of Atlantic Rainforest; 16 Apr 2009; Cintya Souza; CML 2316.

Colonies reaching 33 mm of diameter in 7 d at 25 ºC in MEA, optimum temperature for growth. Minimum 15 ºC and maximum 30 ºC. Colony surface white and reverse yellowish white or greenish on OA. On PDA incubated in darkness the surface is white and the reverse is yellowish white (3A2), when incubated under UV the surface is pinkish white and the reverse is brownish to brown. Cottony or granular colonies, sometimes velvety on PDA, with conidiophores arising mainly from hyphae in the aerial mycelium. Monomorphic conidiophores, penicillate, bi- or terverticillate, stipe 27-128 µm long, 3.4-6.2 µm wide at base, penicillus 84-185.9 µm high, divergent or divergent at acute angles branches. Phialides cylindrical, but narrowing in the upper part, 5.5-12.3 x 2.4-4 µm long; metulae 4.6-11.6 x 2.6-4.1 µm long. Intercalary phialides below solitary terminal phialides, sometimes only intercalary phialides are remaining visible because of the collapse of the terminal phialides. Conidia ovoidal, with central hilum, 4.6-7.1 x 3-4.1 µm.

Habitat: soil.

Additional specimens examined: BRAZIL. AMAZONAS: Benjamim Constant. Native soil of Amazonia; May 2004; Lucas M. de Abreu; CML 1913. SÃO PAULO: São Miguel Arcanjo, Parque Estadual Carlos Botelho. Native soil of Atlantic Rainforest; 20 Jan 2010; Cintya Souza; CML 2315.

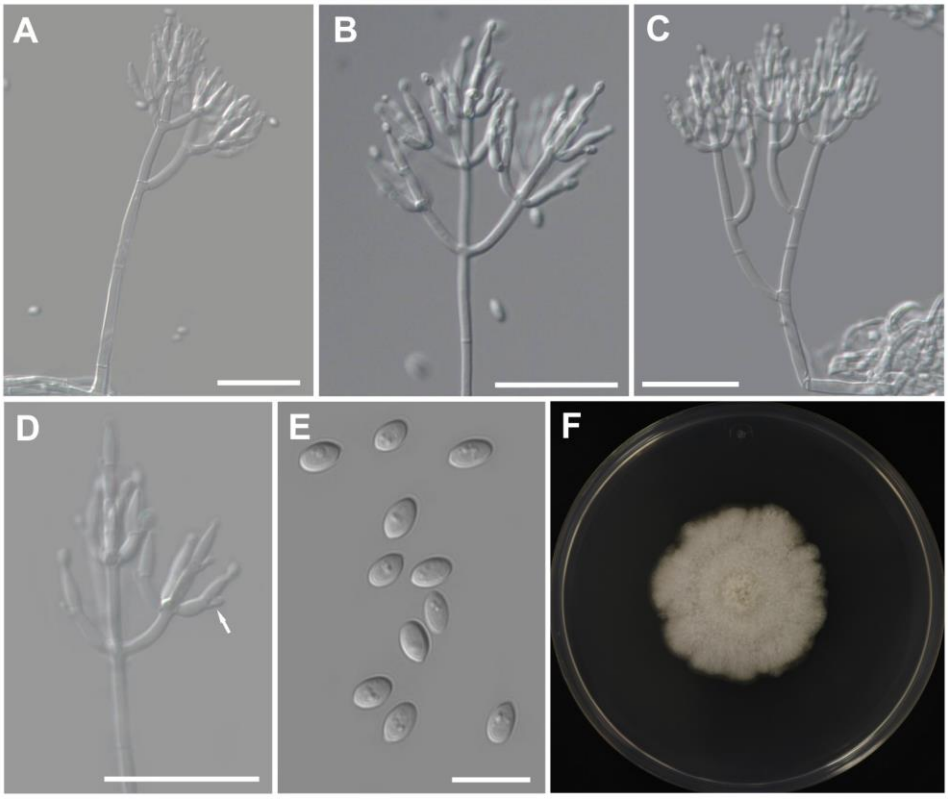


Figure 4 Morphology of *Clonostachys* sp. 2. A-D. Conidiophores with divergent branches. D. Arrow indicating solitary phialide and intercalar phialide that are released from each metulae. E. Ovoidal conidia. F. Colony on PDA 14 days old incubated at 24 ºC in the dark. Scale bars: A-D- 20 µm; E- 10 µm.

4 DISCUSSION

In this research we suggest *acl1* to define the *Clonostachys* species boundaries and describe *Clonostachys* sp. 2 based on phylogeny of three genes and morphological characterization. The *acl1* region was introduced as a phylogenetic marker by Gräfenhan et al. (2011) and have been used for *Fusarium* and nectriaceous fungi (Lombard et al., 2015, Laurence et al., 2014; Niessen et al., 2012; Schroers et al., 2011) and *Trichoderma* (Jaklitsch & Voglmayr, 2015; Jaklitsch et al., 2013) phylogenies.

Clonostachys sp. 2 formed a sister group of *C. rossmaniae* according to phylogenetic analyses (Figs. 1 - 2, p. 69-71; Supplementary Figs. 1 - 3, p. 81-83), but can be distinguished from that species because *Clonostachys* sp. 2 is characterized by solitary terminal phialides, rarely two are observed, and intercalary phialides present in each solitary matulae, ovoidal conidia with a L/W of 1.3-2(-3) (Fig. 4, p. 74). In contrast, *C. rossmaniae* does not possess intercalary phialides in all metulae, has ellipsoidal to ovoidal conidia, with higher L/W (2-2.3), and shorter stipes than *Clonostachys* sp. 2 (Schroers, 2001).

The name *C.* cf. *compactiuscula* was indicated because morphological characters (Fig. 3, p. 72), however the strains did not group with reference sequences included in the *tub* tree (Supplementary Fig. 3, p. 83) and formed two different clades in all other trees (Figs. 1 - 2, p. 69-71; Supplementary Figs. 1 - 2, p. 81-82). Although, there is similarity with conidia shape and colony pigmentation of *C. compactiuscula*, the secondary conidiophore of this species is more branched when compared with *C.* cf. *compactiuscula*, that have the penicillate conidiophores more similar to those from *C. rogersoniana*. Because there are no sequences available for *C. compactiuscula* in addition to *tub* gene and no reference strain was included in the morphological evaluations, we

cannot affirm that our strains belong to different species. Nevertheless, they probably represent two different species, since they form two distinct clades.

The strain identified as *Clonostachys* sp. 3 belongs to undescribed taxa according to phylogenetic analyses presented, supported by tub-ITS phylogeny and MALDI-TOF MS data (Abreu et al., 2014). The high diversity of *Clonostachys* species in a relatively small collection of isolates from Brazil, that represents a biodiversity-rich country, implies that more species can be discovered, especially in the subgenera with less number of species, like *Zebrinella* and *Epiphloea*.

ACKNOWLEDGEMENTS

Part of this work was conducted during a stay at Agricultural Institute of Slovenia, Ljubljana, supported by a scholarship granted to the first author by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (Proc. 011622/2013-00). We thank Edson Rodrigues-Filho from UFSCar for purchasing reference strains from CBS, with support of SISBIOTA-Brasil (Proc. 563063/2010-6).

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SUPPLEMENTARY MATERIAL

Supplementary Table 1 DNA sequences of beta tubulin gene of reference strains used in the phylogenetic analyses.

Taxon name	Strain	Genbank Accession No.	Notes
Clonostachys candelabrum	CBS 504.67[a] (=CML 2512[b])	KF871189	
C. compactiuscula	CBS 913.97	AF358194	ex-type
	CBS 592.93	AF358192	
	CBS 729.87	AF358193	
C. divergens	CBS 967.73b	AF358191	
C. grammicospora	CBS 209.93 (=CML 2556)	AF358206	ex-type
C. grammicosporopsis	CBS 115.87	AF358204	
C. levigata	CBS 948.97	AF358196	
C. rosea f. *rosea*	CBS 710.86 (=CML 2518)	AF358161	ex-neotype
C. samuelsii	CBS 699.97	AF358190	ex-type
C. sesquicillii	CBS 180.88	AF358214	ex-type
C. subquaternata	CBS 100003 (=CML 2561)	-	ex-isotype
C. rossmaniae	CBS 211.93 (=CML 2520)	KF871190	ex-type
	CBS 210.93	AF358213	

[a]CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; [b]CML = Coleção Micológica de Lavras, Universidade Federal de Lavras, Lavras, MG, Brazil.

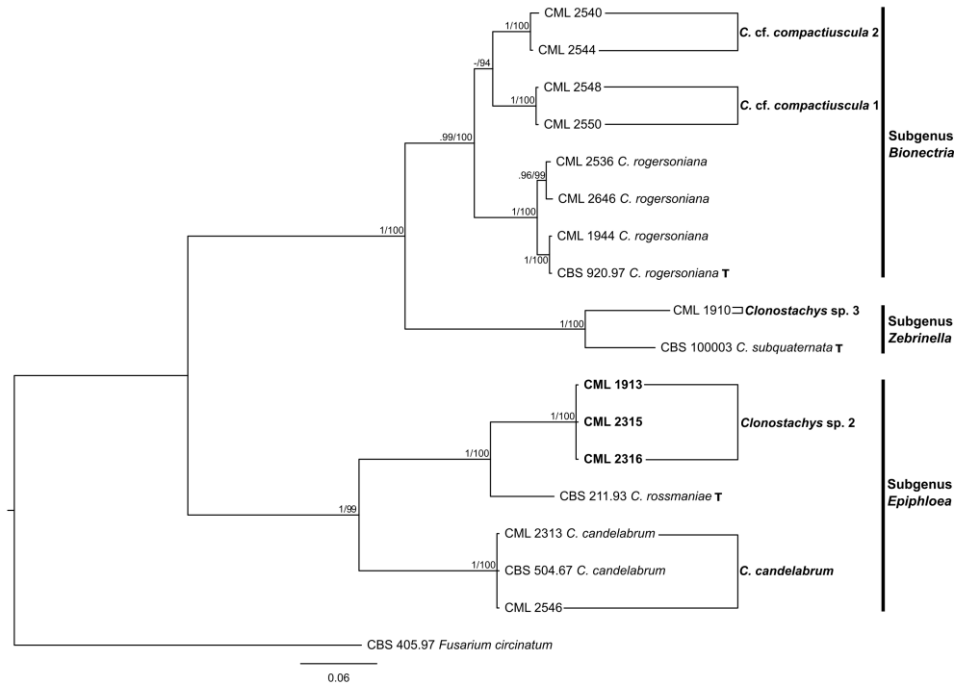

Figure 1 Bayesian phylogenetic consensus tree of species of *Clonostachys* based on DNA sequences of *acl1* gene. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥ 70 % are in the nodes. This tree is rooted with sequences of of *Fusarium circinatum* (CBS 405.97). Codes in bold represent the newly described *Clonostachys* sp. 2. Letter (T) following strain numbers indicates type strains.

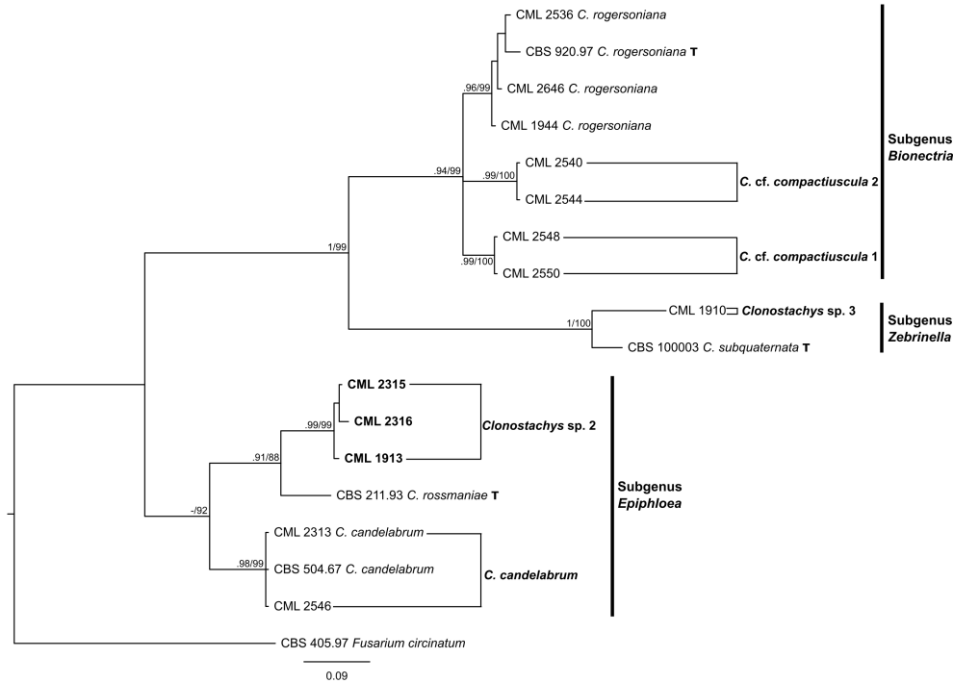

Figure 2 Bayesian phylogenetic consensus tree of species of *Clonostachys* based on DNA sequences of *tef1* gene. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥ 70 % are in the nodes. This tree is rooted with sequences of of *Fusarium circinatum* (CBS 405.97). Codes in bold represent the newly described *Clonostachys* sp. 2. Letter (T) following strain numbers indicates type strains.

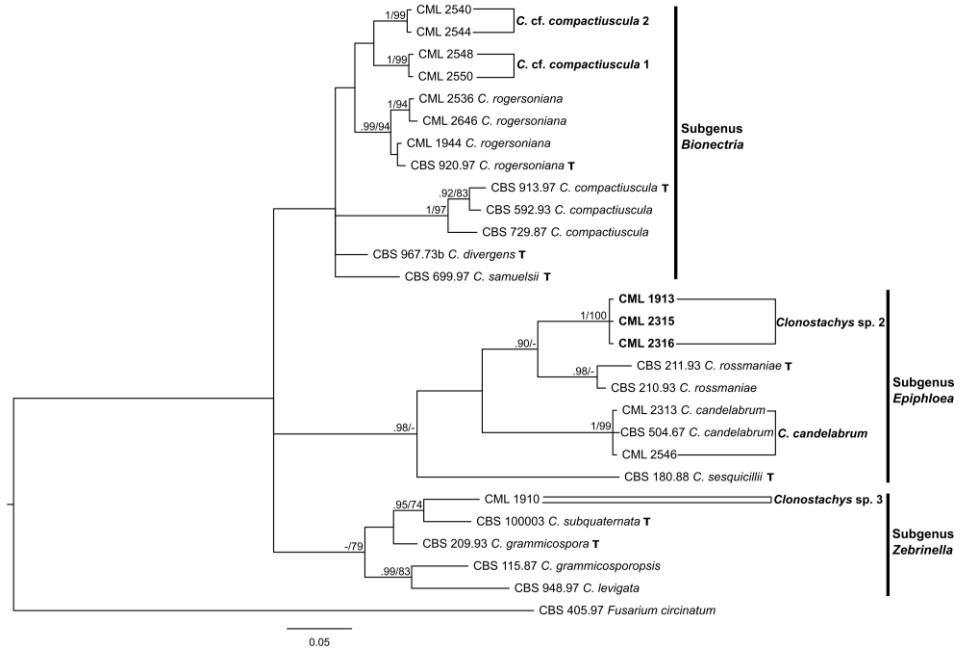

Figure 3 Bayesian phylogenetic consensus tree of species of *Clonostachys* based on DNA sequences of *tub* gene. Posterior probabilities ≥ 0.90 and Maximum Likelihood bootstrap values ≥ 70 % are in the nodes. This tree is rooted with sequences of *Fusarium circinatum* (CBS 405.97). Codes in bold represent the newly described *Clonostachys* sp. 2. Letter (T) following strain numbers indicates type strains.