



## New phylogenetic lineage of *Calonectria* causing leaf blight in *Eucalyptus urophylla* in Mato Grosso, Brazil

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**ABSTRACT:** *Calonectria*-leaf-blight (CLB) caused by *Calonectria* spp. is one of the main foliar disease of eucalypt plantation in Brazil. Recent studies have shown the potential impact of this disease and the hyper-diversity in the pathogen population. However, there are no studies addressing the occurrence and identification of *Calonectria* species in Mato Grosso state, Brazil. The aims of this study were to conduct surveys of soils and trees in eucalypt plantation in southeast of Mato Grosso and identify which species are dominant by DNA sequence analyses. The phylogenetic analyses were performed by using partial sequences of translation elongation factor 1- $\alpha$  (*tef1a*),  $\beta$ -tubulin (*tub2*), calmodulin (*cal*) and histone H3 (*his3*) gene regions. A great variability in *Calonectria* was observed in the Mato Grosso state, since different phylogenetic groups were found in a survey from a single collection place. Based on multigene phylogenetic analyses, a new phylogenetic lineage is proposed (*Calonectria* sp. new.), *Calonectria pteridis* is reported in *Eucalyptus urophylla* in Mato Grosso, and the occurrence of *Calonectria pseudometrosideri* causing CLB on *Eucalyptus* spp. is reported for the first time in the world.

**Keywords:** forest pathology, *Cylindrocladium*, eucalyptus farming, planted forest.

## Nova linhagem filogenética de *Calonectria* causando mancha foliar em *Eucalyptus urophylla* em Mato Grosso

**RESUMO:** A mancha-de-calonectria causada por *Calonectria* spp. é uma das principais doenças foliares da cultura do eucalipto no Brasil. Estudos recentes têm mostrado o potencial impacto dessa doença e a enorme variabilidade existente na população do patógeno. No entanto não há estudos sobre ocorrência e a identificação de espécies de *Calonectria* no estado de Mato Grosso. Assim, os objetivos desse trabalho foram fazer um levantamento das espécies de *Calonectria* que ocorrem no sudeste de Mato Grosso e identificar por meio de análises de sequência de DNA, quais espécies são predominantes. As análises filogenéticas foram realizadas com sequências parciais dos genes fator de alongação (*tef1a*),  $\beta$ -tubulina (*tub2*), calmodulina (*cal*) e histona H3 (*his3*). Foi observada uma grande variabilidade de *Calonectria* no estado de Mato Grosso, uma vez que foram encontrados diferentes grupos filogenéticos em isolados obtidos a partir de um único local de coleta. Com base na análise filogenética multi-gênica, uma nova linhagem filogenética é proposta (*Calonectria* sp. new.), *Calonectria pteridis* é encontrada em *Eucalyptus urophylla* no Mato Grosso, e relata-se pela primeira vez no mundo a ocorrência de *Calonectria pseudometrosideri* causando a mancha-de-calonectria em *Eucalyptus* spp.

**Palavras-chave:** patologia florestal, *Cylindrocladium*, floresta plantada, mancha-foliar.

### 1. INTRODUCTION

The market of planted forests in Mato Grosso has grown exponentially in recent decades, mainly by planting species of the genus *Tectona* and *Eucalyptus*. This growth is largely due to the scarcity of wood from native species, the legal restriction for commercial exploitation and environmental awareness of society, where sustainability is no longer fashionable, but a business strategy (IBÁ, 2015). Such expansion of plantation using genotypes not adapted or selected to edaphic and climatic

conditions for the Mato Grosso state; the implementation of new management techniques and successive stand rotations in the same area have favored the incidence of several biotic and abiotic diseases.

Among these diseases, calonectria-leaf-blight (CLB) stand out, because the defoliation occurring in early stages of the plant development may reduce the volume increase due to interference in the internal physiological processes, especially photosynthesis, metabolism of nutrients, hormones, and water uptake (KOZŁOWSKI, 1969). As consequence, the defoliation

caused by CLB may reduce more than 45 % of the wood volume, especially in hot and humid regions which are highly favorable to the pathogen (GUIMARÃES et al., 2010).

Use of resistant genotypes is considered the best strategy to control this disease. However, the correct identification of the causal agent of the disease is the first step in a breeding program to select resistant genotypes (FONSECA et al., 2010).

Currently, it is known that among the species of *Calonectria* described in the world, 45 occur in Brazil, with emphasis on species of the *Ca. pteridis* complex and *Ca. candelabra* complex (ALFENAS et al., 2015). The species of the *Ca. candelabra* complex are commonly found in tree nurseries causing leaf blight, and *damping-off* (CROUS, 2002). As for the species of the *Ca. pteridis* complex, these are found causing leaf blight and intense defoliation on plants in field (ALFENAS et al., 2015).

Morphological characteristics of *Calonectria* are useful for the identification of species complexes. However, nowadays, it is necessary to adopt the polyphase taxonomy, especially for the description of cryptic species where only the DNA sequence may assist in the accurate identification of species (ALFENAS et al., 2015).

Although recent studies adopting polyphasic taxonomy for identification *Calonectria* (ALFENAS et al., 2013a, b, 2015) have been published in Brazil, studies focused on CLB in the Mato Grosso state are still preliminary, even with climate conditions highly favorable for the *Calonectria* spp.

Furthermore, the accurate identification mainly based on molecular analyses of *Calonectria* spp. occurring in Mato Grosso is fundamental to support disease control measures in field. Therefore, the aims of this study were to conduct surveys of soils and trees in eucalypt plantation in southeast of Mato Grosso and identify which species are dominant in Mato Grosso by DNA sequence analyses.

## 2. MATERIAL AND METHODS

### 2.1. Sample collection

Leaf samples with typical symptoms incited by *Calonectria* were collected in clonal plantations of *Eucalyptus urophylla* in Primavera do Leste, Mato Grosso, Brazil. In each collection site, soil samples were also collected (at 0-20 cm layer). The samples were analyzed in the laboratory of Microbiology/Pathology of the Federal University of Mato Grosso, *Campus Sinop*.

Isolation of single spore cultures was performed as described by CROUS et al. (2009). Subsequently, isolates were stored following Castellani's method and in mineral oil at room temperature.

### 2.2. DNA extraction

Genomic DNA was extracted from single spore cultures grown in MEA (Malt Extract Agar) at 5 days of age using the Wizard® Genomic DNA purification Kit (Promega Corporation,

WI, USA) following the manufacturer's recommendations. For PCR reaction, DreamTaq™ Master Mix (MBI Tools, Vilnius, Lithuania) was used. Fragments of the genetic regions Calmodulin (*cal*), translation elongation factor (*tefla*), Histone (*his3*) and  $\beta$ -tubulin (*tub2*) were amplified using the primers set described in Table 1. The PCR conditions were the same for all the primer as follow: 96 °C for 30 seconds for denaturation, followed by 50 °C for 30 seconds for annealing and 72 °C for 1.5 minutes for elongation, in 30 cycles, with extension in the final step of 72 °C for 4 minutes to complete the reaction. PCR products were confirmed in agarose gel 1.5% and purified using the GFX purification kit illustrates PCR DNA and Gel Band Purification Kit® (GE Healthcare Life Sciences).

### 2.3. Sequencing and phylogenetic analyses

Sequencing reactions were performed with the same pairs of primers used for amplification, following the protocol BigDye® (AppliedBioSystems, Foster City, CA, USA) on a ABI Prism sequencer.

After checking the quality of sequences using the Electropherogram quality analyses (<http://www.biomol.unb.br/phph>) (TOGAWA et al., 2013), contigs were assembled in Mega v.6 (TAMURA et al., 2013). Then, a Blastn ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) for each sequence was made in order to obtain similar sequences from GenBank in order to perform phylogenetic analyses. Alignment of sequences was performed in MAFFT v. 7.0 (KATO; STANDLEY, 2013), and the quality of the alignment was checked in the Mega v. 6 (TAMURA et al., 2013).

Phylogenetic analyses was performed by Bayesian Inference (BI) in Mr Bayes v. 3.2.5 (RONQUIST, HUELSENBECK, 2003) using the Markov Chain Monte Carlo (MCMC) algorithm. Models of substitution of nucleotides were previously selected in MrModeltest v. 2.3 according to Akaike Information Criterion (AIC) (NYLANDER, 2004). Then, the analyses was generated until the average standard deviation reached a value below 0.01, with trees being saved every 1000 generations. The phylogenetic tree was edited in the Figtree software v.1.4.2 (RAMBAUT, 2014).

## 3. RESULTS AND DISCUSSION

Among the 21 isolates obtained, two from soil and 19 from leaves with typical symptoms of CLB. However, only 16 samples had satisfactory quality in the DNA sequencing, allowing the phylogenetic analyses thereof.

Amplifications of approximately 450 base pairs were obtained for *his3* and 500 base pairs for *tub2*, *tefla* and *cal*.

Congruency tests with 162 taxa including the *outgroup* revealed no conflicts in tree topologies for the four gene regions used in each of the four separate datasets and were therefore combined, resulting in a data set with 1986 characters including gaps. Among these, 1261 were conserved and 612

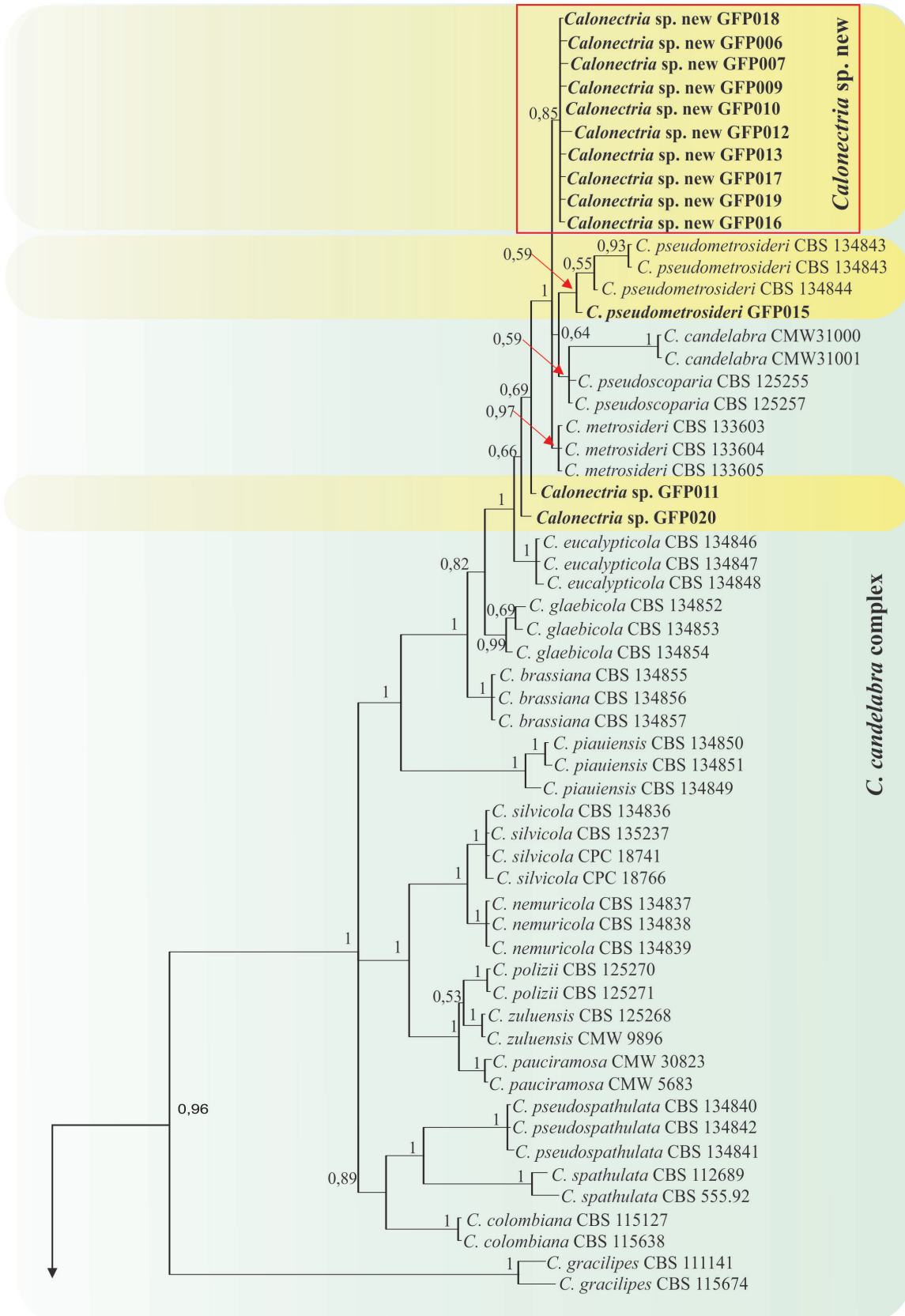
Table 1. Genetic regions and primers used for the sequencing reaction.

Gene	Primer	Sequence	Direction	Reference
Calmodulin ( <i>cal</i> )	CAL-228F	5'-GAGTTCAAGGAGGCCTTCTCCC-3'	Foward	Carbone & Kohn (1999)
	CAL-737R	5'-GCATCATRAGYTRGACRAACTCG-3'	Reverse	Carbone & Kohn (1999)
Translation elongation factor ( <i>tefla</i> )	EF1-728F	5'-CATCGAGAAGTTCGAGAA-3'	Foward	Carbone & Kohn (1999)
	EF-2	5'-GGARGTACCAGTSATCATGTT-3'	Reverse	Carbone & Kohn (1999)
Histone 3 ( <i>his3</i> )	CYLH3F	5'-AGTCCACTGGTGGCAAAG-3'	Foward	Crous et al. (2004)
	CYLH3R	5'-AGCTGGATGTCCTTGGACTG-3'	Reverse	Crous et al. (2004)
$\beta$ - tubulin ( <i>tub2</i> )	T1	5'-AACATGCGTGAGATTGTAAGT-3'	Foward	O'Donnell & Cigelnik (1997)
	CYLTUB1R	5'-AGTTGTCCGGACGGAAAGAG-3'	Reverse	Crous et al. (2004)

were parsimony-informative. The best evolutionary model of nucleotide substitution HKY+G for *tub2*, GTR+G for *cal*, GTR+I+G for *his3* and *tefla* were selected and incorporated in the Bayesian inference.

Based on the multigene analyses, it was found that the isolates collected in the southeast of Mato Grosso belongs to the *Ca. candelabra* and *Ca. pteridis* complexes (Figure 1).

However, the isolates from Mato Grosso state belongs to the *Ca. candelabra* complex formed a well-supported separated clade from other species described in this complex (Figure 1). Thus, this indicates a new phylogenetic lineage within this complex. Therefore, pathogenicity trials are, however, needed to determine if this species present a potential risk to eucalypt plantations in Mato Grosso. As well as, more surveys are



Continued from Figure 1.

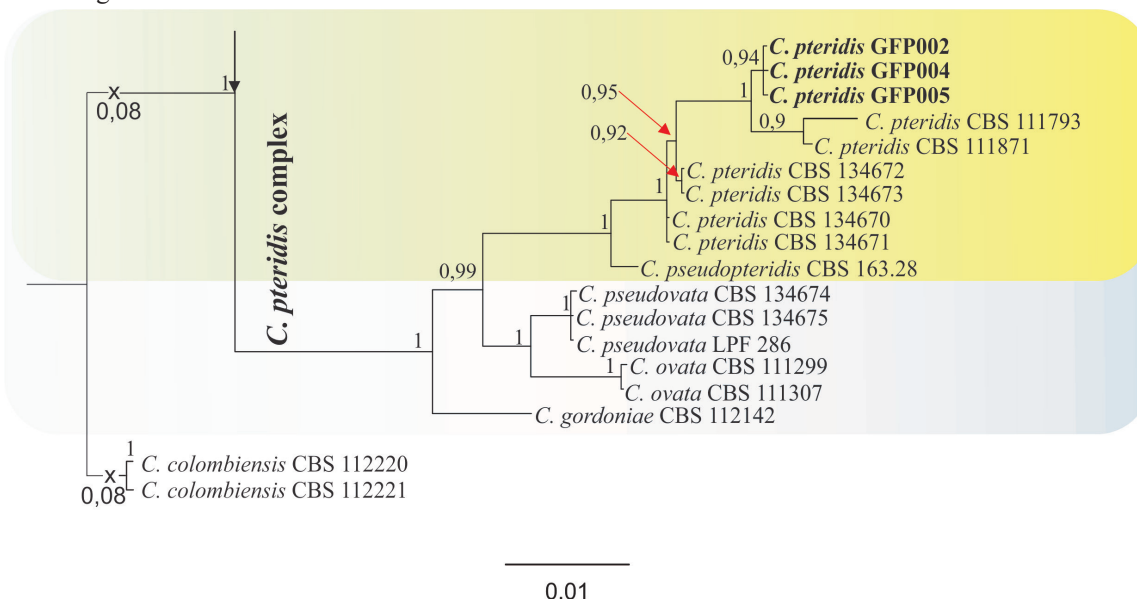


Figure 1. Phylogenetic tree resulting from the Bayesian analysis of combined sequences of the four genes *tub*, *his3*, *tefla* and *cal* to the complex *Calonectria candelabra* and *Ca. pteridis*. *Calonectria colombiensis* was used as *outgroup*.

required to obtain a clearer view on the species diversity of *Calonectria*. Two isolates, GFP011 and GFP020, also belonging to the *Ca. candelabra* complex did not fit into any sub-clade of the complex. However, it is necessary to collect more isolates in order to confirm the new phylogenetic lineage of *Calonectria* occurring in Mato Grosso. The isolate GFP015 clustered with the species *Ca. pseudometrosideri*, being the first occurrence of this species in eucalypt plantation in the world. Until now *Ca. pseudometrosideri* was associated with leaf spots and root rot in seedlings of *Metrosideros* spp. nursery (ALFENAS et al., 2015).

Among the collected isolates, three (GFP02, GFP04 and GFP05) belong to the species *Ca. pteridis* (Figure 1). In a recent study, Alfenas et al., (2015) analyzed 1067 isolates collected in different regions of Brazil and observed that *Ca. pteridis* is the most commonly found species in eucalypt plantation, featuring one of the most important fungal diseases of eucalypts. However, there was no one isolate collected in the Mato Grosso state, and this is, therefore, the first report of this species in *Eucalyptus urophylla* in Mato Grosso.

Although morphological characteristics have a great importance for the identification of species of *Calonectria*, the polyphasic identification has solved many questions in species complexes. The use of molecular techniques has revolutionized and clarified many enigmas in fungal taxonomy, especially when working with the anamorph and the teleomorph, which exhibit morphological differences, but share the same DNA (WINGFIELD et al., 2012). Therefore, the teleomorph of *Cylindrocladium*, in the taxonomy of *Calonectria*, at present, only the name *Calonectria* is adopted, even when the teleomorph is not observed, since phylogenetic studies have shown that all species of *Cylindrocladium* have connection with *Calonectria* (LOMBARD et al., 2010a).

In recent years, adopting the species concept proposed by LOMBARD et al. (2010a), more than 40 new species of *Calonectria* have been described, and the majority can only be identified based on phylogenetic inference (LOMBARD et al., 2010b, c; CHEN et al., 2011; XU et al., 2012; ALFENAS et al., 2013a, b; ALFENAS et al., 2015; LOMBARD et al., 2015).

This is the first phylogenetic study with species of *Calonectria* carried out in Mato Grosso in which a new phylogenetic species is proposed. Furthermore, based on phylogenetic analyses we observed a large variability in the isolates from the Mato Grosso state, since different phylogenetic groups were found in isolates from a single site collection. This variability in the pathogen population is a major challenge for disease control in field.

#### 4. CONCLUSIONS

A new *Calonectria* phylogenetic lineage is proposed based on multigene phylogenetic inference. However, the pathogenicity trials are needed to determine if this species present a potential risk to eucalypt plantation in Mato Grosso.

The morphological features should be studied to rise this new phylogenetic lineage to a species level

For the first time in the world, *Calonectria pseudometrosideri* is found causing leaf spot in clonal planting of *E. urophylla* in Mato Grosso.

The occurrence of *Calonectria pteridis* causing CLB in *Eucalyptus urophylla* is reported in Mato Grosso state.

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#### 6. REFERENCES

- ALFENAS, R. F.; PEREIRA, O.L.; FERREIRA, M. A.; JORGE, V. L.; CROWS, P. W.; ALFENAS, A. C. *Calonectria metrosideri*, a highly aggressive pathogen causing leaf blight, root rot, and wilt of *Metrosideros* spp. in Brazil. **Forest Pathology**, v. 43, n. 4, p. 257-265. 2013a. <http://dx.doi.org/10.1111/efp.12035>

- ALFENAS, R. F.; PEREIRA, O. L.; JORGE, V. L.; CROUS, P. W.; ALFENAS, A. C. A new species of *Calonectria* causing leaf blight and cutting rot of three forest tree species in Brazil. **Tropical Plant Pathology**, Brasília, v. 38, n. 6, p. 513-521. 2013b. <http://dx.doi.org/10.1590/S1982-56762013000600007>
- ALFENAS, R. F.; LOMBARD, L.; PEREIRA, O. L.; ALFENAS, A. C.; CROUS, P. W. Diversity and potential impact of *Calonectria* species in *Eucalyptus* plantations in Brazil. **Studies in Mycology**, v. 80, p. 89-130. 2015. <http://dx.doi.org/10.1016/j.simyco.2014.11.002>
- CHEN S.; LOMBARD, L.; ROUX, J.; WINGFIELD, M. J.; ZHOU, X. D. Novel species of *Calonectria* associated with *Eucalyptus* leaf blight in Southeast China. **Personia**, Laiden, v. 26, p. 1-12. <http://dx.doi.org/10.3767/003158511X555236>
- CROUS, P. W. **Taxonomy and pathology of *Cylindrocladium* (*Calonectria*) and allied genera**. APS Press, St. Paul, Minnesota, U.S.A. 2002. 278p.
- CROUS, P. W.; VERKLEY, G. J. M.; GROENEWALD, E.; SAMSON, R. A. **CBS Laboratory Manual Series 1: Fungal Biodiversity**. Central Bureau voor Schimmelcultures, Utrecht, The Netherlands, 2009. 269p.
- FONSECA, S. M.; RESENDE, M. D. V.; ALFENAS, A. C.; GUIMARÃES, L. M. S.; ASSIS, T. F.; GRATTAPAGLIA, D. **Manual Prático de Melhoramento Genético do Eucalipto**. Universidade Federal de Viçosa, Viçosa. 2010. 220p.
- GUIMARÃES, L. M. S.; TITON, M.; LAU, D.; ROSSE, L.; OLIVEIRA, L. S. S.; ROSADO, C. C. G.; CHRISTO, G. G. O.; ALFENAS, A.C. *Eucalyptus pellita* as a source of resistance to rust, ceratocystis wilt and leaf blight. **Crop Breeding and Applied Biotechnology**, Viçosa, v. 10, n. 2, p. 124-131. 2010. <http://dx.doi.org/10.12702/1984-7033.v10n02a04>
- INDÚSTRIA BRASILEIRA DE ÁRVORES – IBÁ 2015. Disponível em: <[http://www.iba.org/images/shared/iba\\_2015.pdf](http://www.iba.org/images/shared/iba_2015.pdf)>. Acesso em: 01 out 2015
- KATOH, K.; STANDLEY, D. M. MAFFT - Multiple sequence alignment software version 7: improvements in performance and usability. **Molecular Biology and Evolution**, Chicago, v. 30, n. 4, p. 772-780. 2013. <http://dx.doi.org/10.1093/molbev/mst010>
- KOZLOWSKI, T. T. Tree physiology and forest pests. **Journal of Forestry**, Washington, v. 67, p. 118-123, 1969.
- LOMBARD, L.; CHEN, S. F.; MOU, X.; CROWS, P. W.; WINGFIELD, M. J. New species, hyper-diversity and potential importance of *Calonectria* spp. from *Eucalyptus* in South China. **Studies in Mycology**, v. 80, p. 151-188. 2015. <http://dx.doi.org/10.1016/j.simyco.2014.11.003>
- LOMBARD, L.; CROUS, P. W.; WINGFIELD, B. D.; WINGFIELD, M. J. Phylogeny and systematic of the genus *Calonectria*. **Studies in Mycology**, v. 66, p. 31-69. 2010c. <http://dx.doi.org/10.3114/sim.2010.66.03>
- LOMBARD, L.; CROUS, P. W.; WINGFIELD, B. D.; WINGFIELD, M. J. Multigene phylogeny and mating tests reveal three cryptic species related to *Calonectria pauciramosa*. **Studies in Mycology**, v. 66, p. 1-14. 2010b. <http://dx.doi.org/10.3114/sim.2010.66.01>
- LOMBARD, L.; CROUS, P. W.; WINGFIELD, B. D.; WINGFIELD, M. J. Species concepts in *Calonectria* (*Cylindrocladium*). **Studies in Mycology**, v. 66, p. 15-30. 2010. <http://dx.doi.org/10.3114/sim.2010.66.02>
- NYLANDER, J. A. A. Mrmodeltest v.2. Programme distributed by the author. **Evolutionary Biology Center**, Uppsala University. 2004.
- RAMBAUT, A. **FigTree: Tree Figure Drawing Tool, V. 1.4.2**. Institute of Evolutionary Biology, University of Edinburgh. 2014
- RONQUIST, F.; HUELSENBECK, J. P. MrBayes 3.2.5: Bayesian phylogenetic inference under mixed models. **Bioinformatics**, v. 19, n. 12, p. 1572-1574. 2014. <http://dx.doi.org/10.1093/bioinformatics/btg180>
- TAMURA, K.; PETERSON, D.; PETERSON, N. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, end maximum parsimony methods. **Molecular Biology and Evolution**, Chicago, v. 28, n. 10, p. 2731-2739. 2013. <http://dx.doi.org/10.1093/molbev/msr121>
- TOGAWA, R.C. E.; BRIGIDO, M. M. PHPH we based toll for simple electropherogram quality analysis. **1st International Conference on Bioinformatics and Computational Biology- IcoBiCoBi** 14th to 16th May 2003, Ribeirão Preto, SP, Brasil. 2013.
- WINGFIELD, M. J.; DE BEER, Z. W.; SLIPPERS, B.; WINGFIELD, B. D.; GROENEWALD, J. Z.; LOMBARD, L.; CROUS, P. W. One fungus, one name promotes progressive plant pathology. **Molecular Plant Pathology**, v. 13, n. 6, p. 604-613. 2012. <http://dx.doi.org/10.1111/j.1364-3703.2011.00768.x>
- XU, J. J.; QIN, S. Y.; HAO, Y. Y.; REN, J.; TAN, P.; BAHKALI, A.; HYDE, K. D.; WANG, Y. A new species of *Calonectria* causing leaf disease of water lily in China. **Mycotaxon**, v. 122, p. 177-185. 2012. <http://dx.doi.org/10.5248/122.177>