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Colletotrichum SPECIES ASSOCIATED WITH ANTHRACNOSE OF CASHEW TREE

LAVRAS – MG

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título de Mestre.

Orientador

Dr. Ludwig Heinrich Pfenning

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MATEUS J. COMÉ

Colletotrichum SPECIES ASSOCIATED WITH ANTHRACNOSE OF CASHEW TREE

(ESPÉCIES DE *Colletotrichum* ASSOCIADAS A ANTRACNOSE DO CAJUEIRO)

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título de Mestre.

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2014

Á *Hanleidy A. Comé*, a razão de tudo
o que tenho feito!
Dedico.

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ABSTRACT

Anthracnose caused by members of the *Colletotrichum gloeosporioides* species complex is an important disease in cashew nut producing countries worldwide. In this study, we investigated the phylogenetic definition of a set of 61 *Colletotrichum* isolates associated with the cashew tree, belonging to this species complex. Single spore isolates were obtained from symptomatic cashew leaves collected in Brazil and Mozambique. Phylogenetic analyses were performed through Bayesian Inference and Neighbor-Joining based on ApMAT dataset. BI was carried out using concatenated partial sequences of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin (ACT) and ITS-5.8S rDNA. The ApMat-based phylogeny provided the best resolution in the delimitation of six lineages, namely, *C. siamense sensu lato*, *C. asianum, C. tropicale, C. fructicola*, *C. theobromicola*, and a still undesignated *Colletotrichum* sp., potentially a novel species. Morphological characters of the taxa in the species complex could not clearly separate species within the complex. Selected isolates of all identified species were pathogenic, triggering typical symptoms of cashew anthracnose on leaves when tested for pathogenicity toward cashew seedlings. Yet, two taxa (*C. siamense sensu lato* and the still undesignated taxon) are considered to be dominant groups of species because were the majority in number and were represented by both isolates from Brazil and Mozambique. All known species found in this study are reported to have worldwide geographic distribution. These findings show that cashew anthracnose is caused by at least six different species, five of them known from other tropical trees and different regions of the world in association with plant diseases in various agriculturally important crops. *Colletotrichum gloeosporioides stricto sensu* was not found causing cashew anthracnose.

Keywords: *Anacardium occidentale*. *Colletotrichum gloeosporioides* species complex. Anthracnose. Pathogenicity test. Morphological markers. Multigene-based phylogenetic analyses.

RESUMO

A antracnose, causada por representantes do complexo *Colletotrichum gloeosporioides*, é uma doença importante em todos os países produtores da castanha do caju no mundo. Buscou-se, através do presente estudo, investigar a definição filogenética de uma amostra de 61 isolados de *Colletotrichum* associados ao cajueiro, pertencentes a este complexo de espécies. Foram obtidos isolados monospóricos de folhas sintomáticas de cajueiro colectadas no Brasil e em Moçambique. Análises filogenéticas foram efetuadas através da Inferência Bayesiana (IB) e análise Neighbor-Joining baseada em sequências da região ApMAT. A IB foi efetuada usando sequências concatenadas da região GAPDH, actina e da região ITS-5.8S do rDNA. A filogenia molecular baseada na região ApMAT forneceu melhor resolução na delimitação de seis linhagens, a saber, *C. siamense sensu lato*, *C. asianum, C. tropicale, C. fructicola*, *C. theobromicola,* e uma nova linhagem ainda sem designação. Os caracteres morfológicos de cada taxon não puderam separar claramente as espécies do complexo. Todos os isolados selecionados para testes de patogenicidade, representantes de todas as linhagens filogenéticas identificadas, foram capazes de induzir sintomas típicos de antracnose em folhas de mudas de cajueiro. Dois táxons (*C. siamense sensu lato* e o taxon ainda não designado) podem ser considerados grupos dominantes pois, foram a maioria em termos numéricos e foram representados por isolados obtidos no Brasil e em Moçambique. Todas as espécies conhecidas identificadas neste trabalho possuem distribuição mundial, o que sugere que elas podem estar associadas a doenças em várias culturas de importância econômica. Portanto, a antracnose do cajueiro é causada por pelo menos seis espécies diferentes, cinco das quais são conhecidas de outras fruteiras tropicais e diferentes regiões do mundo. *Colletotrichum gloeosporioides* não é o agente causal da antracnose do cajueiro.

Palavras - chave: *Anacardium occidentale.* Complexo *Colletotrichum gloeosporioides.* Antracnose. Testes de patogenicidade. Marcadores morfológicos. Filogenia baseada em análise multigênica.

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1 INTRODUCTION

Cashew tree (*Anacardium occidentale* L.) is an important fruit tree in all producing countries worldwide (SANTOS et al., 2007). Originary of Northeastern Brazil, it is nowadays produced in tropical, sub-tropical and temperated regions, although it is commercially produced only in tropical areas, such as East Africa, Northeast Brazil, Southeast Asia, India and Australia (PAIVA; BARROS; CAVALCANTI, 2009). Brazil is one of the leading producing countries, and Mozambique a historically important cashew producing country (ZHENG; LUO, 2013), with cashew being an important component of its economy.

Benefits of cashew sector in the economy of all cashew nut producing countries may decrease because of many reasons, from which one of the most important is anthracnose, a disease caused by *Colletotrichum* species (FREIRE et al., 2002; MENEZES, 2005). Cashew anthracnose is characterized by formation of necrotic and irregular lesions on leaves and terminal branches, burning and falling flowers, deformation and atrophy of young fruits, necrotic lesions on mature fruits and postharvest fruit rot (MENEZES, 2005; MEDEIROS; BARRETO; FREIRE, 2011; UACIQUETE; KORSTEN; VAN DER WAALS, 2013a). Temperature around 25°C and humid over 80% are favourable conditions for development of these symptoms.

The genus *Colletotrichum* has been reported as one of the most important genera of phytopathogenic fungi (LIMA et al., 2013), and almost every cultivated plant is susceptible to one or more species of this genus (DEAN et al., 2012), the causal agents of anthracnose. This disease causes losses in important crops, especially in fruit plants, legumes, and ornamentals. Those damages and the ability of *Colletotrichum* species to act as postharvest plant pathogens are because of latent infections, which are initiated before harvest,

and do not become active until after fruits have been stored or appear on the market to be sold (DEAN et al., 2012; HYDE et al., 2009a; LIMA et al., 2013).

Because of the definition of *C. gloeosporioides* based on morphology, associations with anthracnose and the state of its taxonomy, Phoulivong et al. (2010), by analysing sequence data of five gene regions of *Colletotrichum* spp. from tropical fruits and by comparing them with the *C.gloeosporioides* epitype, they have outlined that *C. gloeosporioides* is not a common pathogen on tropical fruits. In fact, they have resolved *Colletotrichum* species in the "*gloeosporioides*" complex as distinct phylogenetic lineages with high statistical support. This finding was realized after phylogenetic studies based on DNA sequences of several gene markers, and has marked a new era on which multigene-based phylogeny analyses are required to delimitate species complexes, such as *Colletotrichum gloeosporioide*s species complex, recently highlighted in Weir, Johnston and Damm (2012).

One has to bear in mind that the first step for these studies was the epi-typification of *Colletotrichum gloeosporioides sensu stricto* (CANNON et al., 2008), the causal agent of common anthracnose of tropical fruits (PHOULIVONG et al., 2010). This was followed by the formal descriptions of dozens of new species of the *C. gloesporioides* species complex, based on multi-gene phylogenetic analyses. This state of affairs was summarized in a monograph of the complex in Weir, Johnston and Damm (2012) where 22 species plus one subspecies within the *C. gloeosporioides* complex were recognized.

Although considerable advances in research about species belonging to the *Colletotrichum gloeosporioides* species complex have been achieved (WEIR; JOHNSTON; DAMM, 2012), taxonomy of this group is still in the state of flux, because there are a lot of remaining uncertainties about their phylogenetic position (HYDE et al., 2009a; DEAN et al., 2012). Thus, because

of their importance, their unique intracelular hemibiotrophic lifestyle, and their ease with which they may be cultured and manipulated, beyond availability of new research technologies, species belonging to this complex have a long and distinguished history as model plant pathogens for fundamental future studies (DEAN et al., 2012; SHARMA et al., 2013).

Up to now, the etiological agents of cashew anthracnose have not been assessed using phylogenetic methods, despite of the importance of the disease in cashew nut producing countries in the world. Therefore, through this study we have sought for a response about phylogenetic definition of *Colletotrichum* isolates associated with the cashew tree, obtained from symptomatic leaves in producing regions of Brazil and Mozambique; aiming to perform their morphological, pathogenic and molecular characterization, through multi-gene phylogeny analyses involving glyceraldehyde-3-phosphate dehydrogenase (GAPDH), partial actin (ACT), intergenic region of APN2 and MAT1-2-1 genes (ApMAT) and internal transcribed spacer (ITS gene region), in order to define and delimit species within the species complex.

2 LITERATURE REVIEW

2.1 Major problems with the cashew cultivation

Cashew tree, *Anacardium occidentale*, is originary from Northeastern Brazil in the region between the Atlantic and Amazon rainforests (SANTOS et al., 2007). The cashew is now of pan-tropical distribution, and is grown commercially in many tropical areas of the world including East Africa, Southeast Asia, India and Australia, with Vietnam, India, Brazil, Nigeria and Tanzania currently the leading producing countries (PAIVA; BARROS; CAVALCANTI, 2009). Portuguese explorers first took cashew from Brazil to India (Goa) and then to Mozambique (Africa) in the 16th century. From these two areas, the cashew spread to other parts of East Africa and Angola, as well as throughout southeastern Asia and northern Australia (PAIVA; BARROS; CAVALCANTI, 2009; ZHENG; LUO, 2013).

Cashew is an important commercial crop in producing countries (ZHENG; LUO, 2013). Thus, cashew sector constitute an important component for the economy of those countries in the world (NAIR, 2010). However, its production can be limited because of many problems which may be, for instance: (i) low cashew production and productivity caused by frequency of cashew trees with low production or unproductive; (ii) desuniformity of plant sizes; (iii) unstability of production per plant; (iv) susceptibility to cashew anthracnose and other diseases; (v) unsuitability to different agro-climatic conditions; and (vi) restricted number of cashew genetic resources available in the germplasm collections, besides limited genetic basis of some accessions and low available variability for the important agronomic characters (PAIVA; BARROS; CAVALCANTI, 2009).

Furthermore, in many producing countries, cashew production is from non-uniform plantations, with low production, given the type of propagation used, the sexual way. Technological level that develops the culture is considered deficient, and socio-economic conditions of majority of producers involved in the cultivation of cashew do not permit them to irrigate and fertilize their orchards, besides non performing other management operations (ZHENG; LUO, 2013).

In addition, cashew trees are subject to pest and disease attack, which can severely reduce yields, mainly when plants are susceptible, and when they are under suitable conditions for incidence of these biotic causal agents (NAIR, 2010). The two major disease problems for cashew trees in all producing countries are powdery mildew and anthracnose (FREIRE et al., 2002). Both these fungal pathogens attack newly emerging panicles, flowers, young fruits, and leaves. Successful chemical control of diseases caused by fungi requires that all susceptible plant parts be thoroughly coated with the fungicide before infection occurs. Thus, the easiest method for avoiding disease problems is adopting a successful program of integrated management that comprise cultural cares and use of right amount of a recommended fungicide, timely applications before infection is most likely to occur (FREIRE et al., 2002; NAIR, 2010), and a comprehensive cashew breeding program (PAIVA; BARROS; CAVALCANTI, 2009).

2.2 Epidemiology of cashew anthracnose

Benefits that cashew sector provides to economy of cashew nut producing countries, such as Brazil and Mozambique, can be reduced with decrease of cashew nut production and quality (FREIRE et al., 2002; LOPEZ; LUCAS, 2010; UACIQUETE; KORSTEN; VAN DER WAALS, 2013a). When

it establishes a physical and/or physiological relation between the pathogen (*Colletotrichum* specie) and the host (cashew plant in case), it may occur anthracnose, if climatic conditions are favorable (CARDOSO; VIANA, 2011), resulting in a sequence of sucessive events that compose the cycle of disease.

According to Freire et al. (2002) and Uaciquete, Korsten, Van Der Waals (2013a), cashew anthracnose causes symptoms characterized by necrotic lesions, irregular, initially grayish on young leaves and later reddish on older leaves. Young leaves become blackened, twisted and then they fall down when attack is severe. On flowers, anthracnose causes damages by inducing wilt and fall of many flowers, prejudicing fruit set. On this, cashew apples become black and, sometimes, cracked longitudinally, and can fall down or mummify before maturation.

Survival of *Colletotrichum* species can be on various substrates, either infected crop residues in soil or plant itself (UACIQUETE; KORSTEN; VAN DER WAALS, 2013a). Conidia are produced into gelatinous substance which serves to keep them joined and protected, conferring self-inhibition of germination, a strategy ecologically important to their survival. However, there is no known alternative hosts of *Colletotrichum* species pathogenic toward cashew trees (FREIRE et al., 2002; CARDOSO; VIANA, 2011).

Dissemination of *Colletotrichum* species in the field can be through rainwater and/or through wind but, it is still considering as the primary and most important way of dissemination, the auto-infection. Then, presence of free water is important to dilute protector gelatinous substances, enabling conidia dilution, and also removal of auto-inhibitor effect. Because of that, relative humidity and dew become the most important climatic factors (CARDOSO; VIANA, 2011). Once onto shoots and young inflorescences, conidia germinate producing a small mycelium in whose tip is formed an apressorium in response to the physical and chemical stimulus from host-plant (FREIRE et al., 2002).

Apressoria is only formed on a primary germinative tube and, frequently, through conidium, whose function is direct penetration, breaking off cuticle and epidermic cell wall. Penetration occurs mechanically with help of macerating enzymes of cell wall components, regardless of presence of cuticle injury, stomata opening or other natural openings. Therefore, anthracnose is most severe in young tissues, from the flux that occur during or immediately after rainy period (FREIRE et al., 2002; LOPEZ; LUCAS, 2010; CARDOSO; VIANA, 2011; UACIQUETE; KORSTEN; VAN DER WAALS, 2013a). During period of anthracnose severity in the field, the infection index is low in driest period and high during rainy season (MEDEIROS; BARRETO; FREIRE, 2011).

2.3 *Colletotrichum* as a plant pathogenic genus

Colletotrichum is one of the most important genera of phytopathogenic fungi, the causal agent of anthracnose, that affects a high number of host-plants. It can attack plants in any phase of their development, either leaves, flowers or fruits (HYDE et al., 2009b), and is distributed worldwide.

Since its original description as *Vermicularia gloeosporioides* Penz. (HYDE et al., 2009a; WEIR; JOHNSTON; DAMM, 2012), *C. gloeosporioides* was reported from hundreds of host-plant genera as primary agent of plant diseases or as isolated from deteriorated plants. It is prevalent in tropical regions, but it was also already reported in sub-tropical and temperated regions (CAI et al., 2009; CHOI et al., 2012; DAMM et al., 2010; HYDE et al., 2009b).

Colletotrichum species are known as latent pathogens (Dean *et al.,* 2012) and, causal agents of postharvest diseases (PHOULIVONG et al., 2010). Indeed, endophytes and saprophytic isolates are commonly obtained from assymptomatic plant parts and, traditionally, many *Colletotrichum* species have

been named after their host suggesting host-specificity amongst species (CAI et al., 2009). Then, *C. gloeosporioides* is considered economically important toward a wide range of host-plants such as cashew, mango, citrus, avocado, coffee, papaya, strawberry, taro, beans (HYDE et al., 2009a), and many others (WEIR; JOHNSTON; DAMM, 2012).

However, *C. gloeosporioides* is nowadays described as aspecies complex that aggregates various sub-groups, which show different variation degrees among them in respect to their pathogenicity, host-specificity and genetic homogeneity (PHOULIVONG et al., 2010; WEIR; JOHNSTON; DAMM, 2012); complexity that was already suggested by Sutton (1980) based on the analysis of morphological markers such as conidial characteristics. The *C. gloeosporioides* species complex is, therefore, object of every phylogenetic studies because many monophyletic clades, well supported, were already described and identified (WEIR; JOHNSTON; DAMM, 2012; CANNON et al., 2012; LIMA et al., 2013). Effectively, the biggest issue within this species complex is that many of lineages previously described as *C. gloeosporioides* remain undescribed, according to current level of knowledge; what challenges every mycologist to confidently compare species in all related aspects.

2.4 Characterization methods for *Colletotrichum* species

In this section it describes the general aspects of *Colletotrichum* species characterization, morphological characterization, molecular phylogeny, and pathogenic characterization of *Colletotrichum* species, trying to summarize the current status of study of this important genus of phytopathogenic fungi in terms of characterization methods.

2.4.1 General aspects of *Colletotrichum* species characterization

Colletotrichum is considered as the eighth most important genus of phytopathogenic fungi, whose taxonomy continues in a state of flux, with a number of proposed species ranging from 29 to over 700, depending on taxonomic interpretation effected (DEAN et al., 2012; CAI et al., 2009). *Colletotrichum* species have been serving as model system to hemibiotrophic pathogens, which present a short biotrophic phase followed by changes into vegetable infected tissue and development of necrotrophic phase (DEAN et al., 2012).

The need for correct characterization and identification of *Colletotrichum* species is, therefore, considered of significant practical concern (CAIet al., 2009). At the same time the existence of difficulties concerning systematics of *Colletotrichum* species is denoted, due to the lack of reliable morphological markers, which can make species delimitation within the genus somewhat ambiguous and confusing (CROUCH; CLARKE; HILLMAN, 2009; HYDEet al., 2009b).

Traditionally, it is know that *Colletotrichum* species have a suggested host-specificity amongst species but, even though, difficulties in recognizing them prevail, resulting from: (i) reduced and variable number of morphological characteristics; (ii) wide range of pathogenic variability; and (iii) fact of type-specimens are frequently absent and/or in bad conditions, so that they cannot be used to molecular studies (CAI et al., 2009; CROUCH; CLARKE; HILLMAN, 2009). Thus, to overtake the issue of reduced number of informative morphological charactes, Crouch, Clarke and Hillman (2009) refer that other characters have been used, such as data of nucleic acid sequences, physiology, secundary metabolites and pathogenicity, as part of a polyphasic approach, which reflects natural classification of species within the genus.

While identity of many important species of *Colletotrichum* still need revision (HYDE et al., 2009a; DAMM et al., 2010), molecular techniques currently used in characterization improve their specific delimitation, that is almost impossible through morphology, besides the possibility of revealing its phylogenetic relationships (PHOULIVONG et al., 2010; WEIR; JOHNSTON; DAMM, 2012; LIMA et al., 2013). Thus, phylogenetic analysis based on acid nucleic sequences has been successfully used to differenciate species in various genera or groups (DEAN et al., 2012; LIMA et al., 2013) and, the generated information is considered especially important to understand their specific relationships (PHOULIVONG et al., 2010).

2.4.2 Morphological characterization of *Colletotrichum* species

Morphological characterization of *Colletotrichum* species is from years ago, and this classification system combines morphological characters with cuturals (CAI et al., 2009) but, times after, it was observed that morphology itself does not provide sufficient information to a accurate identifying of target organism, especially to *C. gloeosporioides* and *C. dematium* species complexes (CROUCH; CLARKE; HILLMAN, 2009; PHOULIVONG et al., 2010; WEIR; JOHNSTON; DAMM, 2012).

Crouch, Clarke and Hillman (2009) consider that, size and shape of conidia and types of conidial apressoria are taxonomically noninformative and insufficiently usefull for diagnose of *Colletotrichum* species. Thus, species with similar morphological characteristics may significantly vary in physiology and pathogenicity. Because of these, taxonomy based in morphology might result in ambiguity, and recent studies have been showing that morphological characters must be used with other characters to establish species relationships within the genus *Colletotrichum* (PRIHASTUTI et al., 2009; PHOULIVONG et al., 2010).

Morphological characteristics of *Colletotrichum* species already assessed include: (i) characters observed on natural substrates, it means, size and shape of acervuli, conidia, conidiophores and setae; (ii) size and shape of conidia, conidiophores and setae on culture medium; and (iii) size and shape of apressoria; which may vary because of environmental and incubation factors, such as culture medium and cultivation temperature that, unfortunately, were not standarized yet to cultivate and compare species (CAI et al., 2009).

Acervulus, and shape and dimension of conidia on natural substrates may vary due environmental factors, and conidia may be absent in tissues of infected hosts (CAI et al., 2009; DAMM et al., 2010). Some *Colletotrichum* species, such as *C. musae* e *C. gossypii*, consistently fail to produce setae in conidiomata, and their presence on natural hosts is often inconsistent for species diagnosis (CAI et al., 2009). Therefore, shape and dimension of conidia and apressoria, as well as cultural characteristics, must be warily assessed and used, once these characters are highly dependent of growth conditions. Incorrect diagnose may be easily avoided if morphology is used along to other characteristics, such as sequence data of molecular characteristics, biochemical, physiology and host-range tests (HYDE et al., 2009b; DAMM et al., 2010; PHOULIVONG et al., 2010).

2.4.3 Molecular phylogeny of *Colletotrichum* species

Due insufficiency and plasticity of morphological characteristics, analysis of nucleic acid sequences has been considered reliable to classify *Colletotrichum* species (CAI et al., 2009; PHOULIVONG et al., 2010). However, a disadvantage in dependency on a short portion of genome to understand phylogenetic relationships amongst *Colletotrichum* isolates has been the risk of recreating gene trees rather than species trees (CAI et al., 2009).

Molecular phylogeny is used to characterize and describe evolutive relations of *Colletotrichum* species in order to provide a basis for diagnosing species (CAI et al., 2009). Various genes are considered to study *Colletotrichum* species strictly related, identify species, and resolve relationships among species (PRIHASTUTI et al., 2009). These genetic regions are, namely, internal transcribed spacer (ITS region), partial actin (ACT), β-tubulin (TUB2), calmodulin (CAL), glutamine synthetase (GS), glyceraldehyde 3-phosphate dehydrogenase (GPDH), chitin synthase (CHS-1), manganese-superoxide dismutase (SOD2), DNA lyase (APN2), the large subunit of RNA polymerase II (RPB1), translation elongation factor 1-α (EF1α), intergenic region of APN2 and MAT1-2-1 genes (ApMAT), just to name some examples (PRIHASTUTI et al., 2009; PHOULIVONG et al., 2010; WEIR; JOHNSTON; DAMM, 2012; CANNON et al., 2012; LIMA et al., 2013; SHARMA et al., 2013).

Multigene-based phylogenetic analysis is also successfully used to resolve relationships among *Colletotrichum* species with certain characteristics in some host-plants. Indeed, this is a precise and reliably manner for diagnosis of *Colletotrichum* species, though not economic (DAMM et al., 2010). Currently, it is not practical using multigene phylogeny for every *Colletotrichum* species, as different researchers have been using different gene regions; but it is essential an international collaborative effort in order to standardize a research that has been performed to this species group, as previously published in Cai et al. (2009).

It may be noted, however, that Hyde et al. (2009a) have listed sequences of various genes of type and epitype *Colletotrichum* cultures, what provides an excellent platform to data analysis that aim to study evolutionary relationships among *Colletotrichum* species, according to Weir, Johnston and Damm (2012). Therefore, although ITS region is the widely sequenced, there are some concerns from which, its sequence data might provide sufficient resolution to determine

and differentiate *Colletotrichum* species (CAI et al., 2009; CROUCH; CLARKE; HILLMAN,2009; WEIR; JOHNSTON; DAMM, 2012).

In addition, Crouch, Clarke and Hillman (2009) have revealed a high rate of error and error frequency over than 86% in species identification based in ITS data sequence, comparing similarity into the *Colletotrichum graminicola* species complex. Because of this, they have considered that data sequence of ITS region may cause confusion to the final user, because most of those sequences were recorded under incorrect specific name, what means that many identical species were registered under different names.

Wherefore, due ease of acquiring and a wide library of existent sequences, ITS region remains useful in same cases for reconstruction of interspecific relations, but it is not an ideal marker to infer about variations among species within a genus, although nowadays it is a gene region available in almost every type and epitype cultures of *Colletotrichum* species (CAI et al., 2009; WEIR; JOHNSTON; DAMM, 2012).

2.4.4 Pathogenic characterization of *Colletotrichum* species

Pathogenicity tests are important to determinate if a particular *Colletotrichum* isolate is or not pathogenic toward a specific host. Besides, determine if a plant pathogenic fungus has a wide range of host-plants or not. If the taxonomic group of phytopathogenic fungus (taxon) has a wide range of host-plants, the specie is likely to be cosmopolitan, and a possibly an opportunistic phytopathogenic agent (CAI et al., 2009; HYDE et al., 2009b). Recognition of this is particularly important for biosecurity, plant breeding and integrated disease management, according to Cai et al. (2009).

Studies about range of hosts may provide useful data to classification and future species delimitation (CAI et al., 2009). Because of this, Koch’s

Postulates must be perform to confirm pathogenicity of various *Colletotrichum* isolates in a certain study (DAMM et al., 2010). However, many researchs already performed need replicates in order to verify consistence of experiments (LIMA et al., 2013), although in many works this may be neglected (CAI et al., 2009). In addition, even description of a novel species, some times, includes data about pathogenicity tests.

3 MATERIALS AND METHODS

3.1 Sampling and fungal isolation

A total of 61*Colletotrichum* isolates were obtained from cashew in Brazil and Mozambique. From Brazil, 18 isolates were kindly provided by EMBRAPA Tropical Agroindustry in Fortaleza CE, obtained from some Brazilian States with potential to cashew production, namely, Pernambuco, Paraíba, Rio Grande do Norte, Ceará, São Paulo and Maranhão. Other 12 isolates were obtained from cashew leaf samples collected in a germplasm bank in Pacajus CE and in orchards in Beberibe CE and Fortaleza CE, with typical symptoms of cashew anthracnose. Collections were also performed in Mozambique, Southeast of Africa, specifically in Maputo and Gaza provinces, from where we obtained 31 cashew *Colletotrichum* isolates. In Maputo, collections were performed in the Marracuene germplasm bank and, in Gaza, in districts of Xai-Xai, Bilene, Mandlakazi and Chókwe. Actually, collections were performed in 11 locations, from which 6 in Brazil and 5 in Mozambique, in commercial and familiar orchards, and in germplasm banks.

The isolation methods were as outlined in Cai et al. (2009) in which were used MA2% Malt Extract Agar 2% medium, comprised for 20 g/liter malt extract and 20 g/liter agar in distilled water, prepared in flasks, which were plugged and placed in an autoclave. After that, culture medium was poured from flasks into sterilized petri dishes, and soon solidified. Every procedure was carried out as aseptically as possible, in a separate culture room free from drafts and dust. In either case, the work table was wiped with a 70% alcohol, hands were cleaned, and tools such as scalpels, forceps, and needles were dipped in alcohol and flamed to prevent introduction of contaminating microorganisms.

From each sample of leaves that represents a collection point, in an aseptic chamber, using a scalpel previously flamed, were cut small square tissue sections from the margin of lesions, so that they contain both diseased and healthy looking tissue. These pieces were sterilized in 70% alcohol for around one minute. Then, leaf pieces disinfected into alcohol were transferred to a disinfectant solution of 2% sodium hypochlorite, also during one minute, to eliminate or markedly reduce surface contaminants that could interfere with pathogen isolation.

Using flamed forceps, leaf pieces were washed through two rinses into sterile distilled water (SDW) to remove excessive disinfectant, and placed on sterile filter paper to dry them before being placed three to five per dish containing culture medium, for incubation into a growth chamber (BOD) under constant fluorescent light at $23\pm1^{o}C$. These plates were incubated for around 7 days and observed every day to keep up with development of fungal colonies. Around 5 days after, isolated colonies of mycelium appeared as a result of their germination, being transferred to separate plates, and cultivated in same conditions, thus assuring that they will contain desired pathogen, in pure colony, free of contaminants.

After obtaining pure colonies, each *Colletotrichum* isolate was transferred to another petri dish containing culture medium and cultivated for around 7 days until sporulation. From this sporulation, we transferred some spore mass through a needle to a 1.5ml sterile microtube containing 500µl of sterile distilled water. We vortex to obtain homogeneous suspension of fungal spore and then, spotted around 50µL from that suspension onto petri dish containing Synthetic Nutrient-poor Agar (SNA) medium prepared the same way it was prepared MA2%, and spread through Drigalski handle.

The SNA medium was comprised for 1.0 g/liter KH_2PO_4, 1.0g/liter KNO_3, 0.5g/liter $MgSO_4.7H_2O$, 0.5g/liter KCl, 0.2g/liter glucose, 0.2g/liter

sucrose and 20 g/liter agar in distilled water. Around 12 hours after incubation, under stereoscopic microscope (binoculars) we transferred only one spore to a new sterile petri dish containing MA2% and incubated into a BOD under constant fluorescent light at 23±1°C, thus obtaining a single spore culture for each one of all 61 isolates (APPENDIX A). Therefore, the obtained single spore *Colletotrichum* isolates were stored in small discs into 1.5ml sterile microtubes under 10°C for further studies, and are going to be deposited in the Coleção Micológica de Lavras (CML) at Universidade Federal de Lavras, Brazil.

3.2 DNA extraction, amplification and sequencing

Single spore cultures of all 61 cashew *Colletotrichum* isolates were grown in culture medium at 23±1°C, under constant fluorescent light for 7 days. Using a sterile 200µL pippete tip, a small amount of aerial mycelia was scraped from colony and genomic DNA was extracted using Wizard Genomic DNA Purification Kit® (Promega, Madison, USA), according to manufacturer's instructions. After extraction, DNA concentration for each sample was measured through NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, USA) and all DNA samples were stored into 1.5 microtube for further PCR amplifications.

PCR reactions were carried out using Master Mix GoTaq® Incolor Kit (Promega) in a My Cycler™ thermocycler (Bio-Rad, Hercules, USA). DNA of all 61 isolates of this study were amplified and sequenced for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, according to Prihastuti et al. (2009), as an initial measure of phylogenetic relationships (APPENDIX B), which permited selecting a subset of 24 isolates to represent the groups (Table 1). Thus, from those 24 selected isolates were amplified by PCR, partial DNA sequences of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), partial

actin (ACT), intergenic region of APN2 and MAT1-2-1 genes (ApMAT) and internal transcribed spacer (ITS gene region).

Table 1 *Colletotrichum* isolates from cashew selected through GAPDH-based phylogenetic analysis and used to PCR-amplify ITS, ACT and ApMAT gene regions. This subset was also used for morphological study and pathogenicity testing

N/O	Colletion code	Location	Host	Substrate
01	MT07*	MZ, Xai-Xai	*A. occidentale*	Cashew leaf
02	MT09	MZ, Xai-Xai	*A. occidentale*	Cashew leaf
03	MT14	MZ, Xai-Xai	*A. occidentale*	Cashew leaf
04	MT25*	MZ, Bilene	*A. occidentale*	Cashew leaf
05	MT31*	MZ, Mandlakazi	*A. occidentale*	Cashew leaf
06	MT41	MZ, Mandlakazi	*A. occidentale*	Cashew leaf
07	MT46	MZ, Chókwe	*A. occidentale*	Cashew leaf
08	MT47*	MZ, Chókwe	*A. occidentale*	Cashew leaf
09	MT53	MZ, Maputo	*A. occidentale*	Cashew leaf
10	MT66	BR, Pacajus-CE	*A. occidentale*	Cashew leaf
11	MT68*	BR, Pacajus-CE	*A. occidentale*	Cashew leaf
12	MT71	BR, Beberibe-CE	*A. occidentale*	Cashew leaf
13	MT74*	BR, Fortaleza-CE	*A. occidentale*	Cashew leaf
14	CCJ001*	BR, Goiana-PE	*A. occidentale*	Cashew leaf
15	CCJ032	BR, Camocim-PE	*A. occidentale*	Cashew leaf
16	CCJ073*	BR, Camp. Grande-PB	*A. occidentale*	Cashew leaf
17	CCJ077	BR, Camp. Grande-PB	*A. occidentale*	Cashew leaf
18	CCJ082	BR, Mossoró-RN	*A. occidentale*	Cashew leaf
19	CCJ105	BR, Fortaleza-CE	*A. occidentale*	Cashew leaf
20	CCJ195	BR, Taquaritinga-SP	*A. occidentale*	Cashew leaf
21	CCJ201	BR, Pacajus-CE	*A. occidentale*	Cashew leaf
22	CCJ204*	BR, São Luíz-MA	*A. occidentale*	Cashew leaf
23	CCJ215*	BR, Recife-PE	*A. occidentale*	Cashew leaf
24	CCJ216	BR, Colombo-PR	*A. occidentale*	Cashew leaf

* Cashew *Colletotrichum* isolates also used to perform pathogenicity testing. Collection codes were given by the collectors to differentiate isolates, representing the initial letters of their names: CCJ = Chaves, Cardoso and Joilson & MT = Mateus; MZ = Mozambique & BR = Brazil.

The primer-pairs for carrying out PCR amplifications of GAPDH, ACT, ITS and ApMAT regions were GDF and GDR (Templeton *et al.,* 1992), ACT-

512F and ACT-783R (CARBONE; KOHN, 1999), ITS5 and ITS4 (WHITE et al.,1990) and CgDL_F6 and CgMAT1_F2 (ROJAS et al., 2010), respectively. For a 25µL PCR reaction volume we included 12.5µL of GoTaq®Colorless Master Mix 2X, 0.7µL of each 10µM upstream and downstream primers, 1.0µL of DNA template and 10.1µL of Nuclease-Free water.

The cycling parameters for GAPDH region consisted of initial denaturation step at 94°C for 4 minutes, followed by 34 cycles at 94°C for 45 seconds (denaturation), 60°C for 45 seconds (anneling), 72°C for 1 minute (initial extension) and a final extension at 72°C for 10 minutes. Then, cycling parameters for ACT were initiated at 95°C for 5 minutes, for denaturation, followed by 35 cycles at 94°C for 30 seconds (denaturation), 58°C for 30 seconds (anneling), 72°C for 45 seconds (initial extension), and a final extension at 72°C for 7 minutes; according to Weir, Johnston and Damm (2012). The PCR conditions for ITS region were 5 minutes at 95°C, then 35cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes, and then 7 minutes at 72°C, according to Talhinhas et al. (2002). Then, according to Silva et al. (2012), these conditions were used for PCR amplification of ApMAT region, namely, 3 mininutes at 94°C followed by 30 cycles of 45 seconds at 94°C, 45 seconds at 62°C and 1 minute at 72°C, with a final extension of 7 minutes at 72°C.

PCR amplification products were separated by electrophoresis in 1.5% agarose gels in 1.0× TAE buffer (which contains mixture of Tris base, acetic acid and EDTA) and photographed under Ultra Violet (UV) light. These PCR products were purified using Wizard®SV Gel and PCR Clean-Up System kit (Promega) following manufacturer's instructions. All of amplified DNA samples for GAPDH, ACT, ITS and ApMAT regions were sent to Macrogen Corporation in United States of America (USA) for sequencing.

3.3 Phylogenetic analyses

For phylogenetic analyses, sequences of isolates were edited to obtain consensus sequences in SeqAssem software (SequentiX, Klein Raden, Germany), and aligned in MEGA 5.2 software (TAMURA et al., 2011) for each sequenced gene region. Thus, we searched for related sequences, or sequences that are sufficiently similar to sequences of interest that likely share a common ancestor, through National Center for Biotechnology Information's BLAST (Basic Local Alignment Search Tool). Having chosen related sequences to include on the trees (Table 2), we downloaded each of those sequences to MEGA's Alignment Explorer.

Sequences in the Alignment Explorer were optimized manually to assure positional homology, and aligned with MUSCLE (Multiple Sequence Comparison by Log-Expectation) by being slightly more accurate and 2-3 times faster in typical-size data sets (EDGAR, 2004; NUIN; WANG; TILLIER, 2006). Besides, we treated gaps as pairwise deletion data and, when we encountered sequences that were much longer than the majority, after alignment, we removed the excess through MEGA 5.2.

Table 2 List of *Colletotrichum* strains included in the multi-gene sequence analysis with information of taxon, host, geographic location and GenBank accession number for ITS, ACT, GAPDH and ApMAT gene sequences

Taxon	Isolate designation	Host	Georgraphic location	GenBank accession number			
				ITS	ACT	GAPDH	ApMAT
C. asianum	ICMP 18580 = CBS 130418*	*Coffea arabica*	Thailand	FJ972612	JX009584	JX010053	FR718814
C. asianum	ICMP 18696 = IMI 313839	*Mangifera indica*	Australia	JX010192	JX009576	JX009915	
C. asianum	ICMP 18605 = HKUCC 10862	*Mangifera indica*	Thailand	JX010194	JX009465	JX010021	
C. asianum	ICMP 18648 = CBS 124960	*Mangifera indica*	Panama	JX010193	JX009546	JX010017	
C. asianum	GM595 = MTCC 11680	*Mangifera indica*	India				JQ894554
C. asianum	GM021 = MTCC 11676	*Mangifera indica*	India				JQ894557
C. asianum	GM414 = MTCC 11678	*Mangifera indica*	India				JQ894573
C. boninense	ICMP 17904 = CBS 123755*	*Crinum asiaticum*	Japan	JX010292	JX009583	JX009905	
C. fructicola	ICMP 18581 = CBS 130416 *	*Coffea arabica*	Thailand	JX010165	FJ907426	JX010033	JQ807838
C. fructicola	ICMP 18615	*Limonium sp.*	Israel	JX010170	JX009511	JX010016	
C. fructicola	ICMP 18120	*Dioscorea alata*	Nigeria	JX010182	JX009436	JX010041	

“Table 2, continued”

Taxon	Isolate designation	Host	Georgraphic location	GenBank accession number			
				ITS	**ACT**	**GAPDH**	**ApMAT**
C. fructicola	ICMP 18645 = CBS 125395	*Theobroma cacao*	Panama	JX010172	JX009543	JX009992	
C. fructicola	ICMP 18646 = CBS 125397(*)	*Tetragastris panamensis*	Panama	JX010173	JQ071914	JQ071918	JQ807839
C. fructicola	GM567, MTCC 11679	*Mangifera indica*	India				JQ894576
C. gloeosporioides	ICMP 17821 = CBS 112999*	*Citrus sinensis*	Italy	JX010152	JX009531	JX010056	JQ807843
C. gloeosporioides	ICMP 18694	*Mangifera indica*	South Africa	JX010155	JX009481	JX009980	
C. ignotum	1087	*Theobroma cacao*	Panama	GU994377			GU994438
C. ignotum	3679	*Theobroma cacao*	Panama	GU994375			GU994439
C. ignotum	3589	*Theobroma cacao*	Panama	GU994376			GU994440
C. ignotum	E886*	*Tetragastris panamensis*	Panama	GU994372			GU994441
C. ignotum	CBS 119195 = 7574 = AR 4040	*Theobroma cacao*	Panama	GU994374			GU994442
C. ignotum	E183	*Genipa americana*	Panama	GU994367			GU994443
C. ignotum	8395	*Theobroma cacao*	Panama	GU994370			GU994444
C. siamense	ICMP 18578 = CBS 130417*	*Coffea arabica*	Thailand	JX010171	FJ907423	JX009924	JQ899289
C. siamense	ICMP 12565	*Persea americana*	Australia	JX010249	JX009571	JX009937	
C. siamense	ICMP 18572	*Vitis vinifera*	USA	JX010160	JX009487	JX010061	
C. siamense	ICMP 18571	*Fragaria* × *ananassa*	USA	JX010159	JX009482	JX009922	
C. siamense	ICMP 18642 = CBS 125378*	*Hymenocallis americana*	China				JQ807842

"Table 2, continued"

Taxon	Isolate designation	Host	Georgraphic location	GenBank accession number			
				ITS	ACT	GAPDH	ApMAT
C. siamense	GM057 = MTCC 11590	*Mangifera indica*	India				JQ894551
C. siamense	GM172 = MTCC 11591	*Mangifera indica*	India				JQ894562
C. siamense	GM385	*Mangifera indica*	India				JQ894568
C. siamense	ICMP 19118 = MTCC 10990*	*Jasminum sambac*	Vietnam				JQ807841
C. siamense	MTCC 9660	*Mangifera indica*	India				JQ894548
C. siamense	NK24 = MTCC 11599	*Mangifera indica*	India				JQ894582
C. siamense	GM473 = MTCC 11589	*Mangifera indica*	India				JQ894553
C. siamense	GM529 = MTCC 11592	*Mangifera indica*	India				JQ894575
C. siamense	NK28 = MTCC 11593	*Mangifera indica*	India				JQ894588
C. siamense	GM390 = MTCC 11677	*Mangifera indica*	India				JQ894570
C. siamense	GM018 = MTCC 11672	*Mangifera indica*	India				JQ894556
C. theobromicola	ICMP 18649 = CBS 124945*	*Theobroma cacao*	Panama	JX010294	JX009444	JX010006	
C. theobromicola	ICMP 17099	*Fragaria × ananassa*	USA	JX010285	JX009493	JX009957	
C. theobromicola	ICMP 18650 = CBS 125393	*Theobroma cacao*	Panama	JX010280	JX009503	JX009982	
C. theobromicola	ICMP 18566	*Olea europaea*	Australia	JX010282	JX009496	JX009953	
C. theobromicola	ICMP 17927 = CBS 142.31(*)	*Fragaria × ananassa*	USA				JQ807844

"Table 2, conclusion"

Taxon	Isolate designation	Host	Georgraphic location	GenBank accession number			
				ITS	ACT	GAPDH	ApMAT
C. theobromicola	GJS 08-48	*Theobroma cacao*	Panama				GU994446
C. theobromicola	CBS 124944 = GJS 08-43	*Theobroma cacao*	Panama				GU994447
C. theobromicola	CBS 124945 = GJS 08-50	*Theobroma cacao*	Panama				GU994448
C. theobromicola	GM592 = MTCC 11673	*Mangifera indica*	India				JQ894578
C. tropicale	ICMP 18653 = CBS 124949*	*Theobroma cacao*	Panama	JX010264	JX009489	JX010007	GU994425
C. tropicale	ICMP 18672 = MAFF 239933	*Litchi chinensis*	Japan	JX010275	JX009480	JX010020	
C. tropicale	ICMP 18651 = CBS 124943	*Annona muricata*	Panama	JX010277	JX009570	JX010014	GU994431
C. tropicale	7423 = AR 4041	*Theobroma cacao*	Panama				GU994423
C. tropicale	CBS 124952 = E406	*P. macrophylla*	Panama				GU994424
C. tropicale	E1164	*T. tuberculata*	Panama				GU994426
C. tropicale	CBS 124954 = Q633	*Cordia alliodora*	Panama				GU994427
C. tropicale	E2303	*V. surinamensis*	Panama				GU994428
C. tropicale	8401	*Theobroma cacao*	Panama				GU994429
C. tropicale	4861	*Theobroma cacao*	Panama				GU994430

* = type specimen or authentic culture; (*) = ex-type or authentic culture of synonymised taxon. Sequences were downloaded from GenBank. ICMP: International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; CBS: Centraalbureau voor Schimmel cultures, Utrecht, The Netherlands; MTCC: Microbial Type Culture Collection and Gene Bank, Chandigarh, India; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.; MAFF: Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan; and HKUCC: University of Hong Kong Culture Collection, China.

Through MEGA 5.2, a Neighbor-Joining tree based on GAPDH dataset was obtained, from the alignment, for all cashew *Colletotrichum* isolates with other 45 members in the *C. gloeosporioides* species complex (WEIR; JOHNSTON; DAMM, 2012) from GenBank. Clade stability of the tree resulting from maximum composite likelihood model was assessed by bootstrap analysis with 1000 replicates, and bootstrap values over than 50% were shown in each node (APPENDIX B). The resulting tree showed different groups from which we selected their representatives - a subset of 24 samples - for the following studies.

ApMAT-based phylogeny analysis was carried out for the subset of 24 cashew *Colletotrichum* isolates with other 42 members in the *C. gloeosporioides* species complex obtained from the GenBank. Clade stability of the tree also resulted from Neighbor-Joining analysis by bootstrap analysis with 1000 replicates.

Bayesian inference (BI) was used to reconstruct phylogenetic trees through MrBayers 3.2 software, based on the notion of posterior probabilities or, probabilities that are estimated based on some model after learning something about the data (RONQUIST; HUELSENBECK, 2003). It was chosen because has significant advantages over other methods such as maximum likelihood and maximum parsimony, according to Archibald et al. (2003), and provides measures of clade support as posterior probabilities rather than random resampling bootstraps (WEIR; JOHNSTON; DAMM, 2012; RONQUIST; HUELSENBECK, 2003).

Twenty-three sequences of *Colletotrichum* species obtained from GenBank (Table 2) were included in BI analysis. The nucleotide multiple sequence alignments of all genes were concatenated for three GAPDH, ACT and ITS gene regions, with *Colletotrichum boninense* (ICMP 17904 = CBS 123755) as a outgroup species. Then, following some templates MrBayes blocks, that

were suitable to most of situations we were likely to encountered, we suited our situation to create an execution file, so that after aligning a set of sequences in MEGA and saved them in the FASTA format, we exported them in PAUP 3.0 format (***.nxs*** extention).

jModelTest software v0.1.1 (POSADA, 2008) was used to carry out statistical selection of the best suited models of nucleotide substitution. For ITS it resolved K2P (or K80) + I + G (Kimura 2-parameters model plus a percentage of invariable sites and gamma-shaped rate variation among sites), for ACT: HKY + G, and GAPDH: HKY. Analysis on full data set were run twice, first for $1\times10^6$and secondly for1.5×10^6 generations. Samples were taken from every posterior 500 generations, and the first 25% of generations were discarded as burn-in data. Sequences derived from this study are going to be deposited in the GenBank.

3.4 Morphological study

A subset of 24 single spore cashew *Colletotrichum* isolates (Table 1), already selected through Neighbor-Joining inference phylogenetic for GAPDH gene region, as previously described, was characterized by colony morphology and conidial characteristics. We used 90mm petri dishes containing Potato Dextrose Agar (PDA) medium for 7 days under constant fluorescent light at $23\pm1^{o}C$, in a biochemical oxygen demand (BOD).

After 6 days were, then, assessed the colonies colour, relative abundance of mycelium, and mycelial grow rate (mm day^{-1}). Mycelial grow rate was obtained measuring colony diameter in two diametrically opposite directions. Was also assessed sporulation in PDA medium and conidial size. After 7 days, size and shape from 30 arbitrary conidia were measured under Olympus CX40

microscope for each isolate cultured on PDA under conditions previously described.

The experiment was performed in triplicate, in completely randomized design with three replicates and each three plates represented the experimental parcel. One-way analysis of variance (ANOVA) was conducted to determine significance of differences in conidia dimensions and grow rates (BANZATTO; KRONKA, 2006). The R statistical package fBasics was used for graphical plots. The box plots show the median, upper and lower quartiles, and the whisker extends to the outlying data or to a maximum of 1.5× the interquartile range, individual outliers outside this range are shown as dots (R CORE TEAM, 2013).

3.5 Pathogenicity testing

To establish whether isolates of *Colletotrichum* obtained from symptomatic tissues can cause anthracnose toward cashew seedlings or they are just endophyte isolates, pathogenicity tests were performed in a greenhouse using cashew seedlings approximately 45 days old. Seedlings used to perform pathogenicity testing were locally produced from cashew seeds kindly provided by EMBRAPA Tropical Agroindustry in Fortaleza CE. Seeds were adequately selected, of medium size, as outlined in Cavalcanti Júnior and Chaves (2001). After selecting, seeds were put into water in a bucket for 24 hours, and seeded burying them up to half on a tray containing substrate prepared mixing 1:1 sand and organic manure. Trays were covered using sisal sack, and irrigated almost everyday and/or when necessary. Just after starting germination, around 8 days after seeding, seeds were transferred to pots, with a capacity of 3 litres, containing the same substrate used to fill trays.

We planted one seed for each of 150 pots, and watered them twice a week. Percentage of germination was around 92.8%, which started 9 days after planting in the pots, and took 5 days. A month and a half after planting, seedlings were about 30 cm high. Then, we selected 104 seedlings to compose the pathogenicity experiment, which was performed in completely randomized design with 13 treatments represented by cashew isolates selected from the 24 isolates used to perform morphological studies, and the control.

The experiment was performed in duplicate for each isolate, each treatment consisted in eight replicates and each seedling represented a experimental parcel. Before performing the experiment itself, we tested the inoculation method using only 6 seedlings for each spraying method - with and without injury - with fungal suspension containing 1×10^6 spores/ml. As for both two tested inoculation methods it was possible to trigger anthracnose symptoms on leaves of inoculated seedlings, we choose inoculation method with no injuries to perform the rest of experiments.

To obtain fungal suspension, single spore cultures of 10 *Colletotrichum* isolates (Table 1), one *Colletotrichum* isolate from *Coffea arabica* (CML 1590) and one *Colletotrichum* isolate from *Gossypium* sp. (CML 2391) were placed on petri dishes containing PDA medium and grown under constant fluorescent light at 23 ± 1^oC. Cashew *Colletotrichum* isolates used in pathogenicity testing were selected as representatives of each ApMAT-based phylogenetic lineage. Around 7 days after, when every isolate had already sporulated, we prepared fungal suspension according to Cai et al. (2009) protocols. Then, calibration of fungal suspension was carried out through Neubauer haemocytometer, adjusting final concentration to around 1×10^6 spores/ml.

Inoculation was performed by spraying fungal suspension on seedling leaves without injury, until we could observe fungal suspension almost runoff. The control treatment consisted in seedlings sprayed with sterile distilled water,

and inoculated seedlings underwent a pre- and post-treatment in a moist chamber for 24 hours before and 48 hours after inoculation to allow conidia germination and penetration into plant tissues under suitable conditions. Plants were evaluated daily for 30 days and, seven days after inoculation, it was possible visualising the first symptoms. Then, 15 days after, we evaluated the disease incidence, which is defined here as percentage of plants with anthracnose symptoms for each treatment. Pathogen was reisolated from lesions of cashew anthracnose 30 days after inoculation, thus completing Koch's postulates.

We did not assess severity of cashew anthracnose on leaves because anthracnose leaf incidence is consistently associated with leaf severity (UACIQUETE; KORSTEN; VAN DER WAALS, 2013a). Besides, these authors, by analysing relationships between incidence and severity of anthracnose on cashew genotypes in Mozambique, they revealed that, for epidemic comparisons, it may be used incidence data.

4 RESULTS

4.1 Phylogenetic analyses

The phylogenetic analysis based on GAPDH, ACT, ITS and ApMAT gene regions involved 262 sequences, from which 133 were got from GenBank database. Initial analysis of the GAPDH partial sequence was carried out for all 61 isolates, which revealed a total of seven groups of cashew isolates, four of them with bootstrap support over than 69% (Figure 1). This result also revealed that these groups belong to the *Colletotrichum gloeosporioides* species complex, as outlined in Weir, Johnston and Damm (2012).

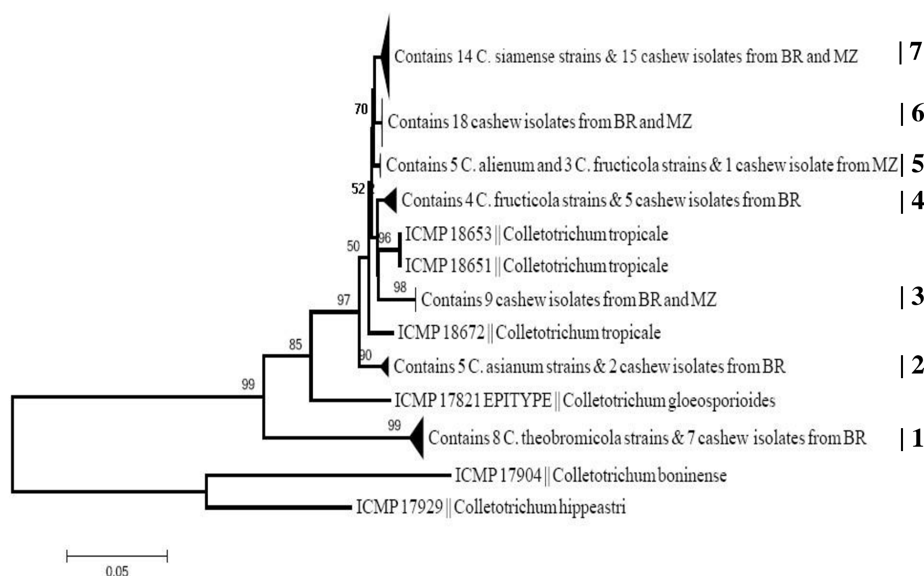

Figure 1 Neighbor-Joining phylogenetic tree for GAPDH gene region of 61 strains of *Colletotrichum* from cashew and additional members of the *C. gloeosporioides* species complex. Bootstrap values over than 50% are shown in each node. The scale bar indicates the number of expected changes per site. Details are shown in APPENDIX B, parts A-D

According to Figure 1, the clade 1 comprises 7 cashew *Colletotrichum* isolates with representatives of *Colletotrichum theobromicola*; the clade 2 comprises two cashew *Colletotrichum* isolates with representatives of *Colletotrichum asianum*; the clade 3 comprises 9 cashew *Colletotrichum* isolates; the clade 4 comprises two sister taxa, one of them constituted with 5 cashew *Colletotrichum* isolates and the other with representatives of *Colletotrichum fructicola* (details in APPENDIX B, Part C). The clade 5 comprises one cashew *Colletotrichum* isolate with representatives of *Colletotrichum alienum* and *fructicola*; the clade 6 comprises 18 cashew *Colletotrichum* isolates; and the clade 7 comprises 15 cashew *Colletotrichum* isolates with representatives of *Colletotrichum siamense*.

Based on this preliminary grouping, 24 representative isolates were selected for sequencing of partial ACT, ITS-5.8S and ApMAT gene regions. Sequences of partial GAPDH gene ranged from 254 to 280 bp, partial ACT gene ranged from 230 to 299 bp, ITS-5.8S gene region ranged from 577 to 608 bp and ApMAT intergenic region ranged from 715 to 955 bp.

The Neighbor-Joining (NJ) ApMAT-base analysis of the 24 selected isolates resolved 6 different groups in the *Colletotrichum gloeosporioides* species complex, grouping two isolates in the *C. theobromicola* clade, one isolate in the *C. fructicola* clade, four isolates in the *C. tropicale* clade. Other five cashew isolates formed a distinct lineage with 100% bootstrap support. Beside this, one isolate grouped in the *C. asianum* clade with 99% bootstrap support, and eleven isolates in *Colletotrichum siamense sensu lato,* with 74% bootstrap support (Figure 2).

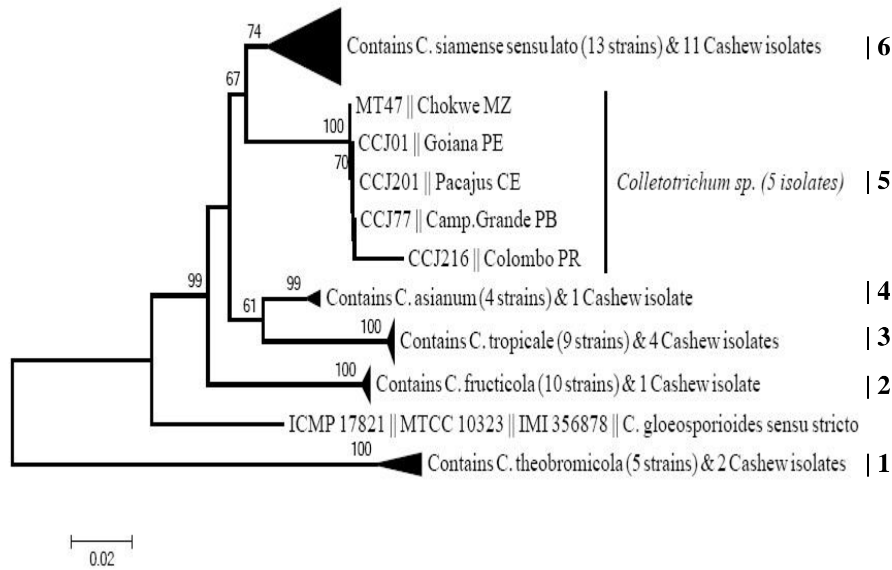

Figure 2 Neighbor-Joining tree based on ApMAT dataset inferred for 24 cashew *Colletotrichum* isolates associated with *Anacardium occidentale* L. from Brazil and Mozambique with other 42 members in the *C. gloeosporioides* species complex. Bootstrap values over than 50% are shown in each node. The scale bar indicates the number of expected changes per site. Details are shown in figure 2, parts A and B

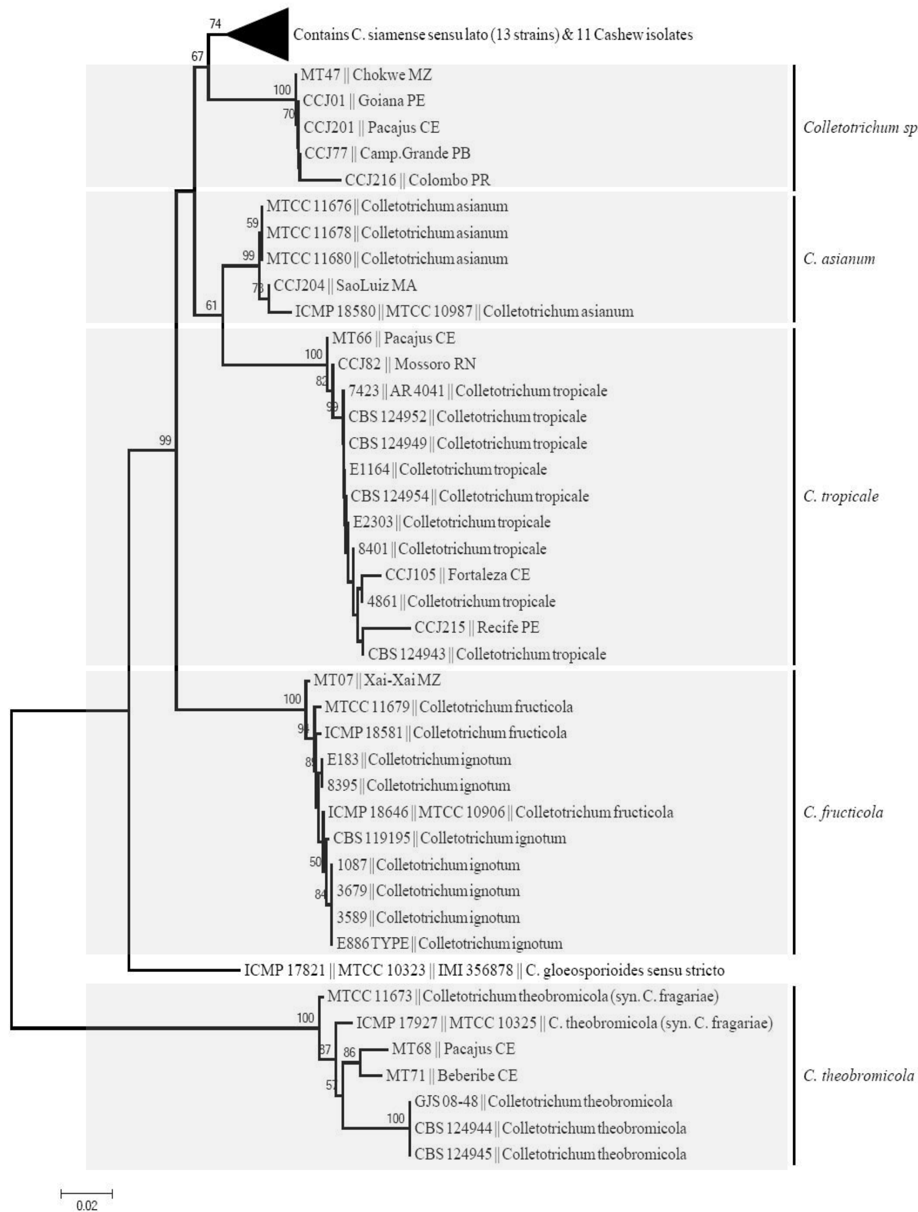

Figure 2 (continued), Part A. Shows 5 clades expanded, namely, *Colletotrichum theobromicola* which represents clade 1, *Colletotrichum fructicola* which represents clade 2, *Colletotrichum tropicale* which represents clade 3, *Colletotrichum asianum* which represents clade 4, and *Colletotrichum* sp. which represents clade 5

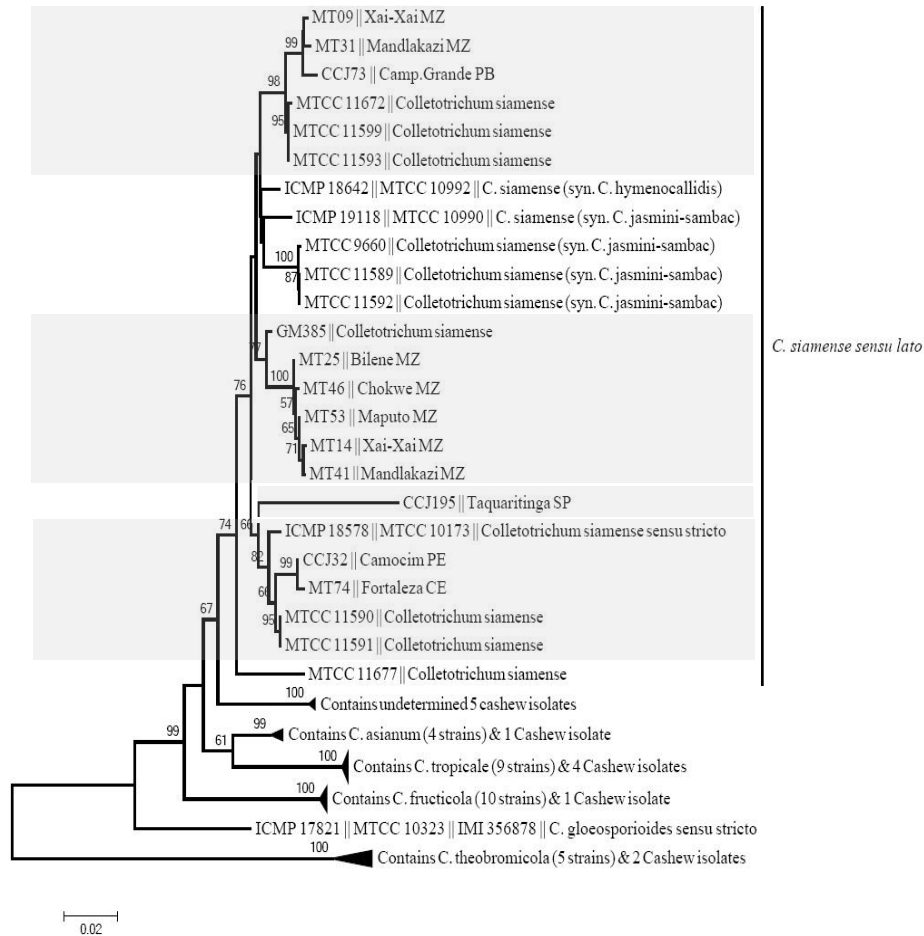

Figure 2 (continued), Part B. Shows *Colletotrichum siamense sensu lato* which represents clade 6 that contains four sub-clades with cashew *Colletotrichum* isolates (on shaded branches)

The *C. theobromicola* clade was composed by representatives of *C. theobromicola*, *C. fragarie* (Table 2), and two cashew isolates that formed a monophyletic group with 86% of bootstrap support. The isolate MT07 formed a sister relationship with *Colletotrichum fructicola* taxa in the species complex (Part A, Figure 2). These taxa comprise *Colletotrichum fructicola* and their

synonyms, *Colletotrichum ignotum*, according to Weir, Johnston and Damm (2012).

Among *Colletotrichum tropicale* taxa there are four cashew isolates that are likely to be *Colletotrichum tropicale* by sharing close relationships into the clade, with good bootstrap support (100%). The cashew isolate CCJ204 was found to belong to the well supported *C. asianum* clade (Part A, Figure 2).

A group of five isolates (*Colletotrichum* sp.) formed a well supported clade (100% bootstrap), that did not contain any representatives of known species included in the analysis, and may represent a novel lineage inside the *Colletotrichum gloeosporioides* species complex (Part A, Figure 2).

Colletotrichum siamense sensu lato constitutes the largest clade in the tree, containing 11 cashew isolates. It comprises representatives of *Colletotrichum siamense sensu stricto, C. hymenocallidis* and *C. jasmini-sambac* (Part B, Figure 2). Therefore, this clade can be split in four well supported subtaxa, three of them with bootstrap over than 95%, and a single strain originated from a node with 66% of boostratp, represented by the isolate CCJ195.

The Bayesian inference phylogenetic analysis of 24 cashew *Colletotrichum* isolates, and other members in the *C. gloeosporioides* species complex was performed with the combined datasets of ITS-5.8S region, partial ACT and GAPDH and comprised 1.219 aligned characters. The gene boundaries in the alignment were: ITS-5.8S: 1-613, ACT: 614-927, GAPDH: 928-1219. Sequences of reference *Colletotrichum* strains from the GenBank were included in the analysis (Table 2).

The phylogenetic tree based on combined dataset resolved four clades related to those obtained through Neighbor-Joining inference phylogenetic tree for ApMAT dataset, namely *C. theobromicola, C. fructicola, C. asianum and Colletotrichum* sp. (Table 3). All four *Colletotrichum* isolates from cashew that

represented the taxon *C. tropicale* in the phylogenetic tree based on ApMAT dataset (MT66, CCJ82, CCJ105 and CCJ215), through phylogenetic analysis based on combined dataset they were resolved in a well supported monophyletic clade (100% of posterior probability support). Other well supported monophyletic group of *Colletotrichum* isolates from cashew resolved in combined dataset was also composed by four isolates (MT25, MT41, MT53 and MT73), which had been resolved in the taxon *C. siamense sensu lato* through ApMAT-based phylogenetic analysis. Through phylogenetic analysis based on combined dataset, this two monophyletic groups were resolved with none of reference strains obtained in the GenBank (APPENDIX C).

In the taxon *C. siamense sensu lato,* only one *Colletotrichum* isolate from cashew (CCJ32) was resolved through phylogenetic analysis based on combined dataset. The remaining *Colletotrichum* isolates that had been resolved in this taxon through phylogenetic analysis based on ApMAT dataset, one of them (MT74) was resolved alone and the others (MT09, MT14, MT31, MT46 and CCJ195) formed a polytomy with some strains of *Colletotrichum siamense* obtained in the GenBank, both through phylogenetic analysis based on combined dataset (APPENDIX C).

Table 3 Cashew isolates and statistical support for each dataset-based analysis

Taxon*	**Statistical support (%)**		**Cashew isolates**
	ApMAT-based analysis	**Combined dataset-based analysis**	
C. asianum	99	100	CCJ204
C. fructicola	100	100	MT07
C. siamense sensu lato	74	-	MT09, MT14, MT25, MT31, MT41, MT46, MT53, MT74, CCJ32, CCJ73, and CCJ195
C. theobromicola	100	100	MT68 and MT71
C. tropicale	100	-	MT66, CCJ82, CCJ105 and CCJ215
Colletotrichum sp.	100	100	MT47, CCJ01, CCJ77, CCJ201 and CCJ216

* Taxa obtained from Neighbor-Joining (N-J) tree based on ApMAT dataset analysis.

About geographical location of cashew *Colletotrichum* isolates we realized, through this study, that most of taxa were composed by cashew isolates obtained in Brazil, namely *C. asianum, C. theobromicola,* and *C. tropicale*. Only *C. fructicola* was composed by a single isolate from Mozambique. The two remaining taxa, *C. siamense sensu lato* and the *Colletotrichum* sp., were both composed by isolates from Mozambique and Brazil.

4.2 Morphological study

Investigating morphological characteristics for a subset of 24 representative isolates previously selected through Neighbor-Joining inference phylogenetic for GAPDH gene region (Table 1) it was found that isolates could be grouped into six different morphotypes, according to Lacap, Liew and Hyde (2003), with some variation in colony color, relative abundance of mycelium, mycelial grow rate (mm day^{-1}) and sporulation in PDA medium (Figure 3).

Morphotype 1 is comprised of colonies fast growing, white greyish mycelium, with little conidia mass, but dense aerial mycelium. Morphotype 2 comprises colonies that do not grow perfectly in circle and have pale greyish mycelium, in reverse buff colonies. Morphotype 3 comprises the slowest growing colonies, salmon-coloured, and dark in the center. Morphotype 4 comprises pale greyish mycelium colonies, fast growing and sporulating. Morphotype 5 comprises white greyish mycelium, with little conidial mass, and easily sectorized. Morphotype 6 comprises white greyish mycelium from above, with little conidia mass, and dark from below other than the edges.

Through cross assessment between morphological and ApMAT-based phylogenetic characters we realized that morphological groups are related to the phylogenetic ones, so that morphotype 1 contains isolates belonging to the *C. siamense sensu lato* clade, morphotype 2 to the *Colletotrichum* sp. clade, morphotype 3 to the *C. asianum* clade, morphotype 4 to the *C. tropicale* clade, morphotype 5 to the *C. fructicola* clade, and morphotype 6 to the *C. theobromicola* clade.

The growing rate ranged from 4.98 mm day^{-1} to 7.92 mm day^{-1} and there was no statistical difference among taxa represented by *C. fructicola*, *C. siamense sensu lato, C. theobromicola, C. tropicale* and *Colletotrichum* sp. clades, these which grown faster than *C. asianum* when compared by Tukey test at 5% significance level (Table 4).

Table 4 Morphological characteristics of cashew *Colletotrichum* isolates

Taxon*	**Conidia characteristics**			**Growth rate (mm day^{-1})**
	Length (µm)	**Width (µm)**	**Shape**	
C. asianum	15.64 ab	3.28 c	Cylindrical	4.98b
C. fructicola	15.27 b	4.52 a	Cylindrical	6.98a
C. siamense sensu lato	13.92 c	3.71 b	Fusiform tocylindrical	7.35a
C. theobromicola	16.28 a	3.73 b	Subcylindrical	7.49a
C. tropicale	13.64 c	4.24 a	Cylindrical	7.50a
Colletotrichum sp.	14.32 c	3.67 b	Cylindrical	7.92a

* Taxa obtained from Neighbor-Joining tree based on ApMAT dataset analysis. Averages followed by the same letter in the column do not differ one another by Tukey test at 5% of significance level.

Conidial size, recorded after seven days of growth, showed that cashew *Colletotrichum* taxa, in this study, have differences in conidial size when compared by Tukey test at 5% significance level. The taxon *C. theobromicola* has the longest conidia, although does not have the widest one, when statistically compared among all taxa, so that there were statistically significant differences in length and width of conidia among some taxa in this study. There were three different conidial shapes among studied taxa (Table 4).

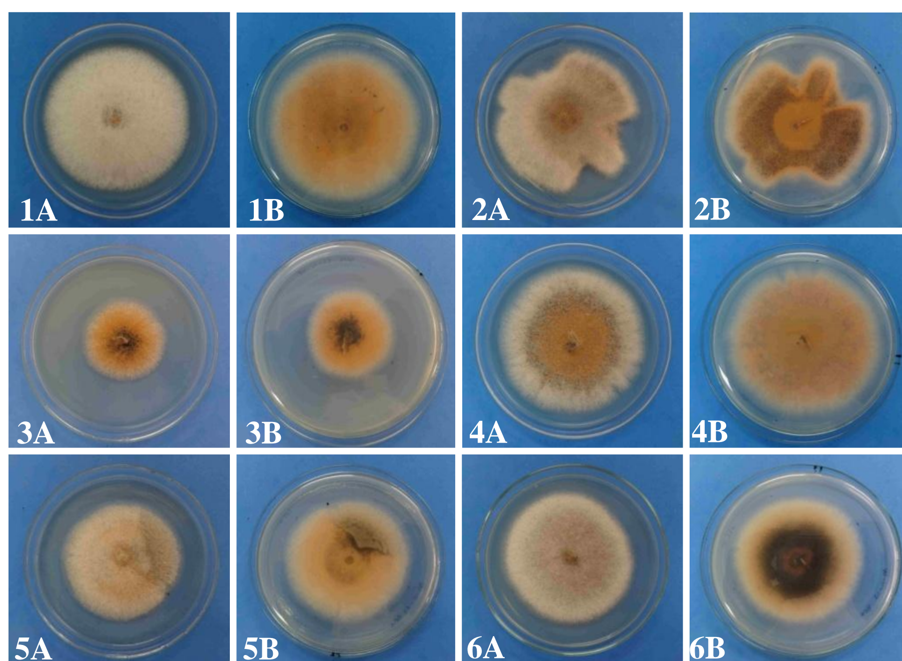

Figure 3 Cashew *Colletotrichum* morphotypes (1 to 6) on PDA, seven days growth from single conidia. (A) view from above, and (B) view from below.

4.3 Pathogenicity testing

Cashew seedlings inoculated with 1×10^6conidial suspension of selected *Colletotrichum* isolates (Table 1) developed typical necrotic lesions, irregular, initially grayish and later reddish on seedling leaves (Figure 4). All *Colletotrichum* isolates were pathogenic toward cashew seedlings, and there was no significant difference in incidence among inoculated isolates ($p<0.05$), although one plant of each isolates CCJ73 and MT31, and two plants of isolate CCJ215 did not develop any symptom. The control did not developany symptoms during experimental period.

The cross inoculation of *Colletotrichum* isolates obtained from *Coffea arabica* (CML 1590) and *Gossypium sp.* (CML 2391), also triggered typical

symptoms of cashew anthracnose on cashew seedlings, suggesting that *Colletotrichum* species are not host-specific. The pathogen was consistently re-isolated from infected cashew leaves on PDA medium, thus completing Koch's postulates.

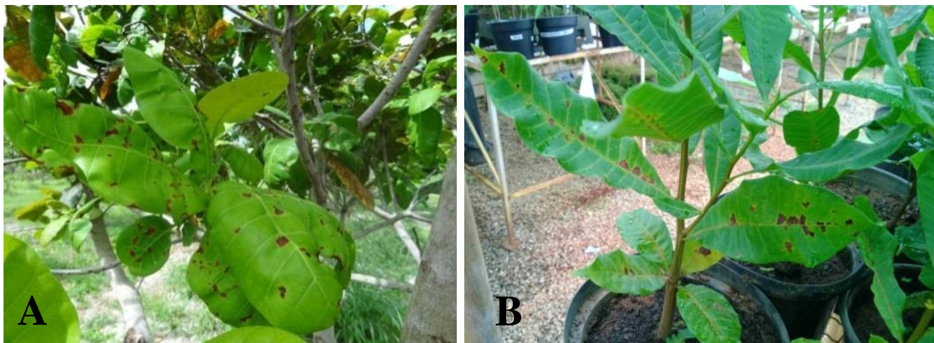

Figure 4 Cashew *Colletotrichum* symptoms on leaves. (A) Symptoms on plants, from where isolates were colected. (B) Symptoms on seedlings, triggered through artificial inoculation.

5 DISCUSSION

Thepurpose of this study was to test the hypothesis that there are several species or forms of *Colletotrichum* associated with the cashew tree, which can be defined as distinct phylogenetic lineages in the *Colletotrichum gloeosporioides* species complex. Through Neighbor-Joining phylogenetic analysis based on ApMAT dataset and phylogenetic analysis based on combined dataset, we characterized six different taxa in the complex. These findings support what was previously referred in Phoulivong et al. (2010) that *Colletotrichum gloeosporioides sensu stricto* is not a common pathogen on tropical fruits. In fact, none of the 61cashew *Colletotrichum* isolates was *C. gloeosporioides sensu stricto*.

Within the *C. gloeosporioides* species complex, the phylogenetically distinct taxa identified were originally described from fruits causing anthracnose or similar symptoms in other tropical fruits (WEIR; JOHNSTON; DAMM, 2012; SHARMA et al., 2013; UDAYANGA et al., 2013). In this study, representatives of *C. asianum, C. tropicale, C. fructicola*, and *C. theobromicola* were less frequently found than representatives of *Colletotrichum siamense sensu lato*, but they may also be considered important species associated with cashew trees. *Colletotrichum siamense sensu lato* is probably a species complex, which could not be resolved in the combined ITS-5.8S-ACT-GAPDH dataset, but represented distinct phylogenetic lineages in the ApMat based phylogeny, in agreement with the results of Sharma et al. (2013) and Udayanga et al. (2013). This finding suggests the need of use of another gene-based sequences, seeking to improve resolution of inferences, in order to well characterize these *Colletotrichum* spp.

The understanding of species characterization and identification in the genus *Colletotrichum* has been revolutionized through molecular-based

phylogenetic studies (PHOULIVONG et al., 2010; WEIR; JOHNSTON; DAMM, 2012; UDAYANGA et al., 2013), but a lot of species previously described as *C. gloeosporioides* remain undescribed, according to current level of knowledge as outlined in Cannon et al. (2012). Through this study, besides species already delimitated, namely *C. asianum, C. tropicale, C. fructicola*, *C. theobromicola* and *Colletotrichum siamense sensu lato*, we also characterized an undesignated species, a potentially novel lineage found to be associated with cashew tissues, and pathogenic toward cashew seedlings.

Morphological, cultural and host-preference criteria have been the primary basis for species identification and delimitation (SILVA et al., 2012). Thus, morphological characteristics have been using along molecular and/or phylogenetic studies (LIMA et al., 2013; SHARMA et al., 2013), demonstrating fulfilment of what was outlined in Cai et al. (2009) protocols, and theoretical background from which cultural, conidial and appressorial characters can be used to differentiate taxa into species complexes, but cannot separate species within a complex (PHOULIVONG et al., 2010). Based on this knowledge, we could identify six morphological groups which have variation in colony color, relative abundance of mycelium, mycelial grow rate and sporulation on PDA, although share some characteristics. Yet, those morphological groups are related to the taxa described throughNeighbor-Joining phylogenetic analysis based on ApMAT dataset, as it has been happening in other cases (ROJAS et al., 2010; Sharma et al., 2013), and also showed that conidial dimensions and growth rate are compatible with that already described in the literature (PHOULIVONG et al., 2010; WEIR; JOHNSTON; DAMM, 2012; LIMA et al.,2013; SHARMA et al., 2013).

Although *Colletotrichum* species encompasses endophytic, epiphytic, saprophytic, and plant pathogenic lifestyles (HYDE et al., 2009b), and all taxa belonging to the *Colletotrichum gloeosporioides* complex comprise species

already described as pathogens associated with tropical fruits, and endophytes in many others (PRIHASTUTI et al., 2009; PHOULIVONG et al., 2010; WEIR; JOHNSTON; DAMM, 2012; LIMA et al., 2013; SHARMA et al., 2013; UDAYANGA et al., 2013), we demonstrated here that all *Colletotrichum* isolates used in this study are plant pathogenic and can trigger cashew anthracnose on leaves, with similar characteristics from those they were obtained. Moreover, *Colletotrichum* isolates used in this study are not host-specific toward cashew seedlings, according to pathogenicity testing, although many *Colletotrichum* species have been named after their host, which suggested erroneously host-specificity amongst species (CAI et al.,2009). Actually, this had been happening because it was done with no phylogenetic basis.

Anthracnose is a very important disease on cashew trees (FREIRE et al., 2002; UACIQUETE; KORSTEN; VAN DER WAALS, 2013a) because, besides damages caused on plants, reducing cashew nut yields in all cashew producing countries, plantations are nowadays obtained from vegetatively produced seedlings. This production and seedling distribution, facilitates cashew anthracnose propagation, yet in latent infections (DEAN et al., 2012; HYDE et al., 2009a) which become active after plantation. Of course, this process may also mediate its spreading toward other plants and producing areas. Then, pathogenicity testing proved that *Colletotrichum* species do not need natural openings to penetrate and colonize plant tissues, because according to Cardoso and Viana (2011) once onto any plant part, under suitable conditions, they emit a germinative tube that can break off cuticle and epidermic cell wall, causing mechanic penetration with help of macerating enzymes of cell wall components. Indeed, a pre- and post-treatment in a moist chamber for 24 hours before and 48 hours after inoculation in this study was important to allow ease conidia germination and penetration into plant tissues.

Cashew *Colletotrichum* isolates from different geographical locations, Brazil and Mozambique, some got together and others got apart among taxa in this study. This finding can be because of two main reasons: (i) cashew was taken from Brazil to India and then to Mozambique in the 16th century and, from these two areas, cashew spread to other countries (PAIVA; BARROS; CAVALCANTI, 2009; ZHENG; LUO, 2013). Of course, it is likely that cashew *Colletotrichum* isolates were spread along, as latent infections, which became active after finding suitable conditions; and (ii) it is likely that *Colletotrichum* species from other host-plants have become opportunistic pathogens toward cashew trees during times because of lack of host-specificity, as suggested in Sharma et al. (2013) and in Álvarez et al.(2014). Then, these reasons make these taxa to have a worldwide geographic distribution, as it has already reported (WEIR; JOHNSTON; DAMM, 2012; LIMA et al., 2013).

Therefore, by characterizing cashew isolates obtained from Brazil and Mozambique we demonstrated that multilocus-based phylogenetic analysis for ITS, ACT and GAPDH gene regions; and the Neighbor-Joining phylogenetic analysis based on ApMAT dataset provide useful information in identification of *Colletotrichum* isolates. But, combined dataset did not resolve clearly all cashew *Colletotrichum* isolates in the complex, which shows the need of sequencing another two or more gene regions, such as calmodulin (CAL) and β-tubulin (TUB2), seeking to improve cross-inference resolution between these two method-based analysis.

6 CONCLUSIONS

According to the results obtained in this study, we conclude that *Colletotrichum* isolates associated with the cashew tree belong to the *Colletotrichum gloeosporioides* species complex. However, cashew anthracnose is not caused by *Colletotrichum gloeosporioides*, but at least six distinct species, namely, *C. siamense sensu lato*, *C. asianum, C. tropicale, C. fructicola*, *C. theobromicola*, and a still undesignated taxon (*Colletotrichum* sp.), which can be defined by multigene-based phylogeny analyses.

Because combined datasets of ITS region, partial ACT and GAPDH regions did not resolve clearly all cashew *Colletotrichum* isolates in the complex, another gene-based sequences are needed to improve resolution of this method-based phylogenetic analysis in order to allow us increasing quality on estimating relationships among isolates.

Six morphotypes were characterized, and are related to the taxa defined through multigene-based phylogeny analyses. And, *Colletotrichum* species, such as from cashew, coffee and cotton, for instance, are not host-specific, being able to trigger typical symptoms of anthracnose toward cashew seedlings.

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APPENDICES

APPENDIX A - *Colletotrichum* isolates obtained from lesions of infected cashew leaves

N/O	Colletion code*	Collection site	Collection date	GPS
01	CCJ001	Goiana-PE, Littoral	April, 2008	07º38’09”S 34º57’15”W, A90
02	CCJ002	Goiana-PE, Littoral	April, 2008	07º38’09”S 34º57’15”W, A90
03	CCJ031	Camocim-PE, Fen-altitude	May, 2008	08º19’16”S 35º45’08”W, A675
04	CCJ032	Camocim-PE, Fen-altitude	May, 2008	08º19’16”S 35º45’08”W, A675
05	CCJ041	Barreiros-PE, Littoral	July, 2008	08º51’45”S 35º09’51”W, A20
06	CCJ073	Camp. Grande-PB, Wasteland	Nov., 2009	07º13’30”S 35º54’17”W, A539
07	CCJ077	Camp. Grande-PB, Wasteland	Nov., 2009	07º13’30”S 35º54’17”W, A539
08	CCJ081	Mossoró-RN, Semiarid	Out., 2009	04º46’49”S 37º28’15”W, A43
09	CCJ082	Mossoró-RN, Semiarid	Out., 2009	04º46’49”S 37º28’15”W, A43
10	CCJ105	Fortaleza-CE, Littoral	Nov., 2008	03º45’06”S 38º34’34”W, A38
11	CCJ106	Fortaleza-CE, Littoral	Nov., 2008	03º45’06”S 38º34’34”W, A38
12	CCJ201	Pacajus-CE, Littoral	Nov., 2008	04º09’48”S 38º26’38”W, A39
13	CCJ195	Taquaritinga-SP	Dez., 2009	21º24’23”S 48º30’20”W, A545
14	CCJ197	Taquaritinga-SP	Dez., 2009	21º24’23”S 48º30’20”W, A545
15	CCJ204	São Luiz-MA, Littoral	-	02º31’51”S 44º18’25”W, A10
16	CCJ206	São Luiz-MA, Littoral	-	02º31’51”S 44º18’25”W, A10
17	CCJ215	Recife-PE, Littoral	July, 2008	08º01’07”S 34º56’39”W, A13
18	CCJ216	Colombo-PR	June, 2004	29º17’35”S 49º13’25”W
19	MT01	MZ, Xai-Xai, Chongoene	April, 2013	
20	MT06	MZ, Xai-Xai, Maciene	April, 2013	
21	MT07	MZ, Xai-Xai, Nhamavila	April, 2013	
22	MT09	MZ, Xai-Xai, Nhamavila	April, 2013	
23	MT11	MZ, Xai-Xai, Conjoene	April, 2013	
24	MT14	MZ, Xai-Xai, Pomelene	April, 2013	
25	MT15	MZ, Xai-Xai, Pomelene	April, 2013	
26	MT20	MZ, Xai-Xai, Cavelene	April, 2013	
27	MT23	MZ, Bilene, BMF1	Marc, 2013	
28	MT25	MZ, Bilene, BMCH1	Marc, 2013	
29	MT30	MZ, Mandlakazi, Chizavane	April, 2013	
30	MT31	MZ, Mandlakazi, Chizavane	April, 2013	

APPENDIX A - continued

N/O	Colletion code*	Collection site	Collection date	GPS
31	MT32	MZ, Mandlakazi, Chizavane	April, 2013	
32	MT33	MZ, Mandlakazi, J. Clonal	April, 2013	
33	MT34	MZ, Mandlakazi, J. Clonal	April, 2013	
34	MT35	MZ, Mandlakazi, Nguzene	April, 2013	
35	MT36	MZ, Mandlakazi, Mazucane	April, 2013	
36	MT37	MZ, Mandlakazi, Mazucane	April, 2013	
37	MT39	MZ, Mandlakazi, Mazucane	April, 2013	
38	MT40	MZ, Mandlakazi, Mangunze	April, 2013	
39	MT41	MZ, Mandlakazi, Mangunze	April, 2013	
40	MT42	MZ, Chókwè, Chiaquelane	Marc, 2013	
41	MT43	MZ, Chókwè, Chiaquelane	Marc, 2013	
42	MT44	MZ, Chókwè, Chiaquelane	Marc, 2013	
43	MT45	MZ, Chókwè, Mapapa	Marc, 2013	
44	MT46	MZ, Chókwè, Mapapa	Marc, 2013	
45	MT47	MZ, Chókwè, Hókwè	Marc, 2013	
46	MT49	MZ, Chókwè, Inchovane	Marc, 2013	
47	MT50	MZ, Chókwè, Inchovane	Marc, 2013	
48	MT53	MZ, Maputo, BG-Ricatla	April, 2013	
49	MT55	MZ, Maputo, BG-Ricatla	April, 2013	
50	MT65	Pacajus-CE, Experim. field	June, 2013	
51	MT66	Pacajus-CE, Experim.field	June, 2013	
52	MT67	Pacajus-CE, Experim.field	June, 2013	
53	MT68	Pacajus-CE, Experim. field	June, 2013	
54	MT69	Pacajus-CE, Experim. field	June, 2013	
55	MT70	Beberibe-CE	June, 2013	
56	MT71	Beberibe-CE	June, 2013	
57	MT72	Beberibe-CE	June, 2013	
58	MT73	Beberibe-CE	June, 2013	

APPENDIX A - conclusion

N/O	Colletion code*	Collection site	Collection date	GPS
59	MT74	Fortaleza-CE, EMBRAPA	June, 2013	
60	MT75	Fortaleza-CE, EMBRAPA	June, 2013	
61	MT76	Fortaleza-CE, EMBRAPA	June, 2013	

* Collection code given by the collectors to differentiate isolates, representing the initial letters of their names: CCJ = Chaves, Cardoso and Joilson & MT = Mateus.

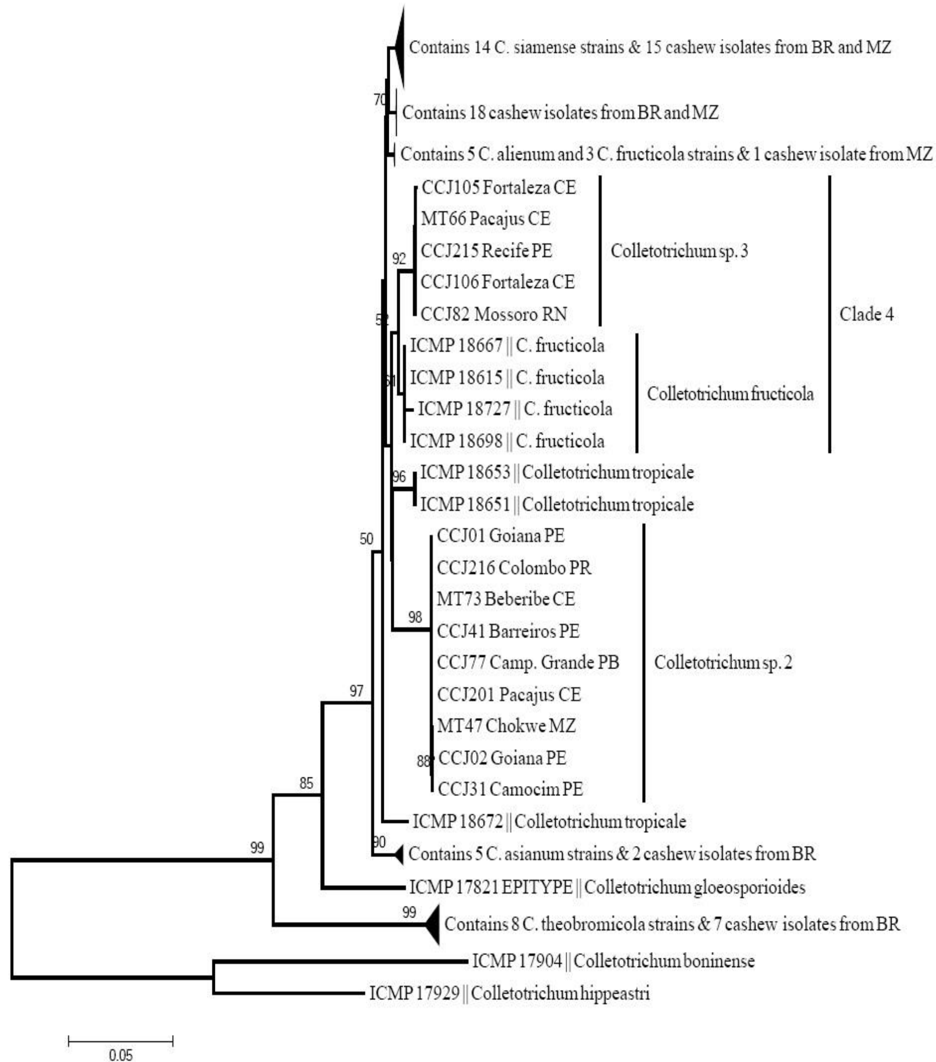
APPENDIX B - Neighbor-Joining phylogenetic tree for GAPDH gene region of 61 strains of *Colletotrichum* from cashew

Part A, shows the *Colletotrichum siamense* taxon expanded in the Neighbor-Joining inference phylogenetic tree for GAPDH gene region used to select 24 representative cashew isolates to perform further studies, such as morphological and multigene-based phylogeny analysis.

APPENDIX B - continued

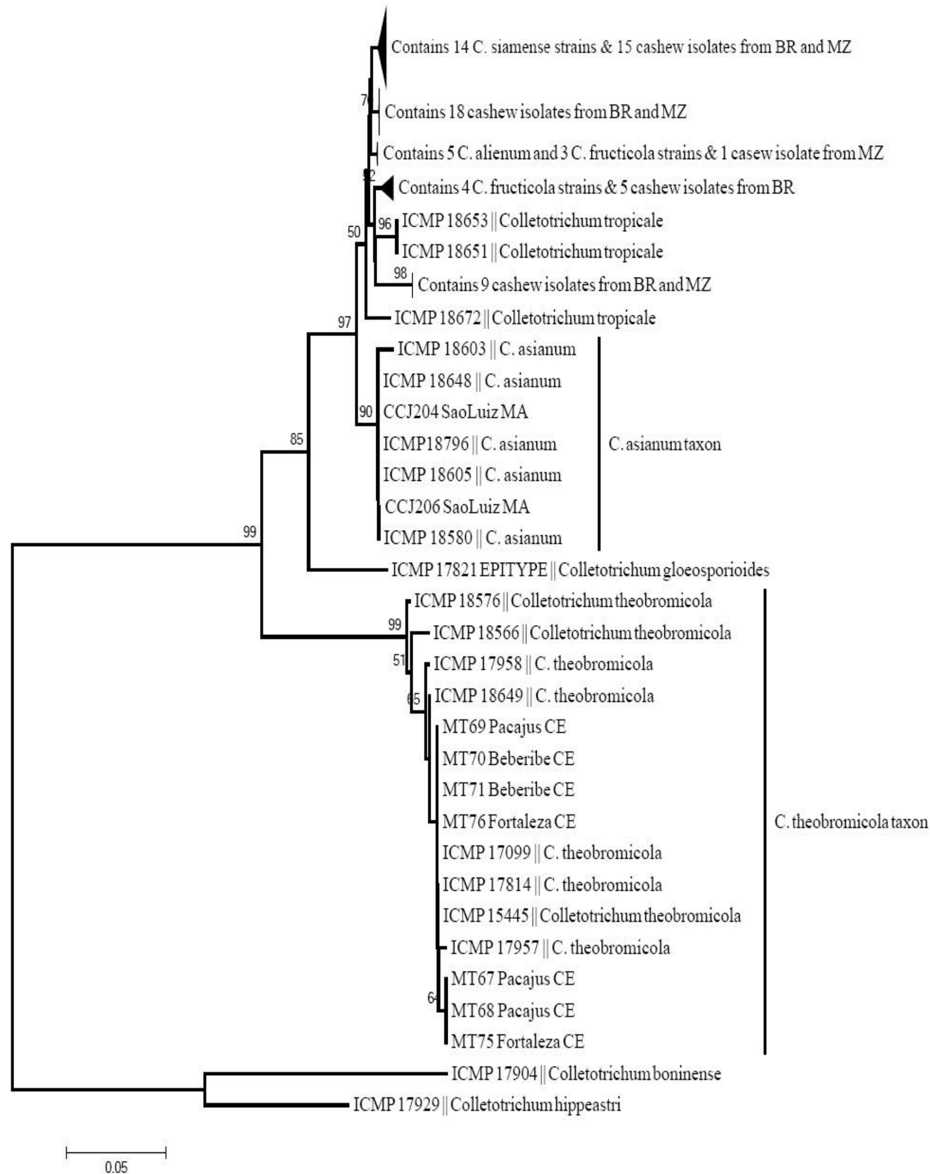
Part B, shows the *Colletotrichum* sp. 1 and *Colletotrichum alienum* with *Colletotrichum fructicola* taxa expanded in the Neighbor-Joining inference phylogenetic tree for GAPDH gene region used to select 24 representative cashew isolates to perform further studies, such as morphological and multigene-based phylogeny analysis.

APPENDIX B - continued



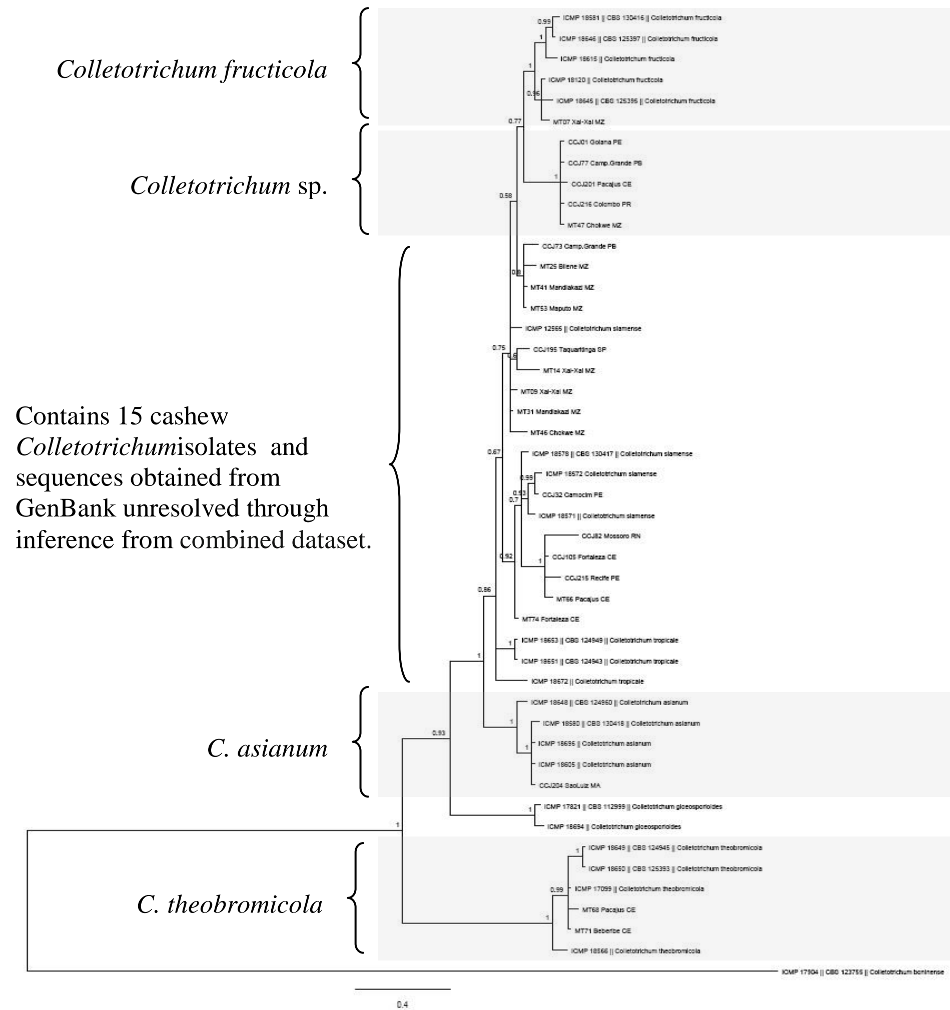
Part C, shows the *Colletotrichum* sp. 2 and *Colletotrichum fructicola* sharing sister relationships with *Colletotrichum* sp. 3, both expanded in the Neighbor-Joining inference phylogenetic tree for GAPDH gene region used to select 24 representative cashew isolates to perform further studies, such as morphological and multigene-based phylogeny analysis.

APPENDIX B - conclusion



Part D, shows the *Colletotrichum theobromicola* and *Colletotrichum asianum* taxa expanded in the Neighbor-Joining inference phylogenetic tree for GAPDH gene region used to select 24 representative cashew isolates to perform further studies, such as morphological and multigene-based phylogeny analysis.

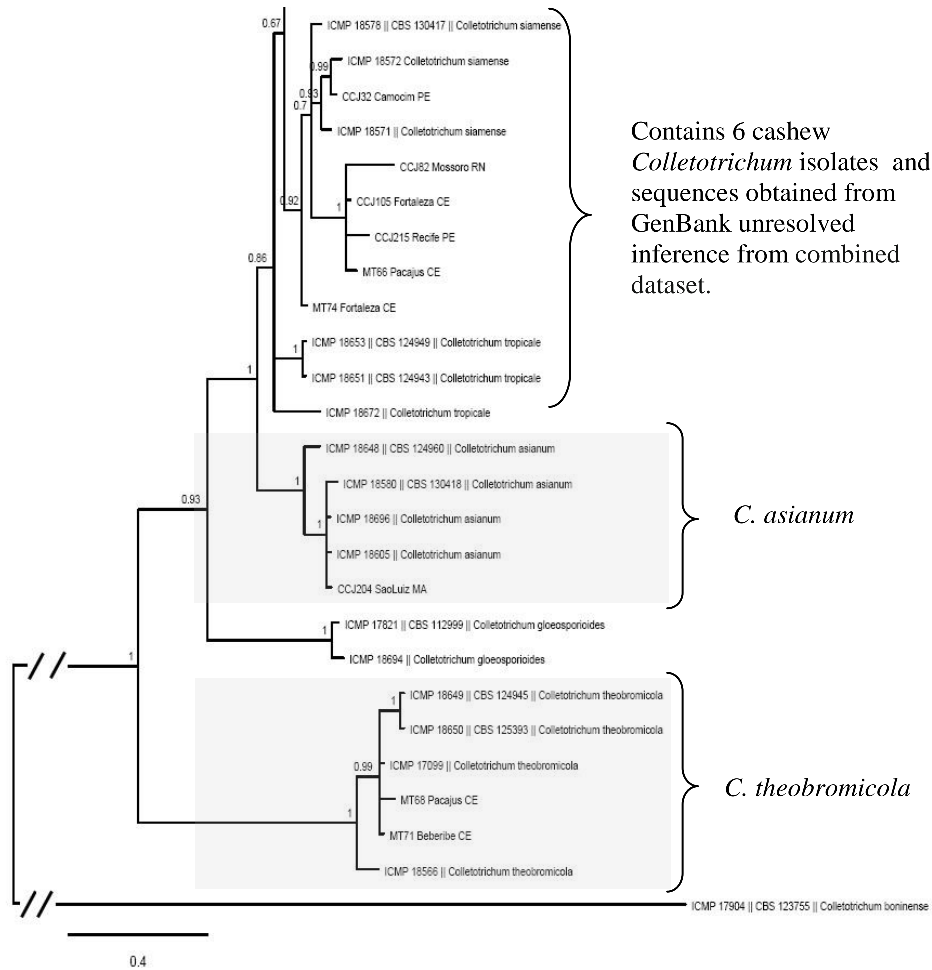
APPENDIX C - Bayesian inference phylogenetic tree for 24 representative cashew *Colletotrichum* isolates



Bayesian inference phylogenetic tree for 24 representative cashew isolates using combined partial sequence data of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), partial actin (ACT) and internal transcribed spacer (ITS gene region). The tree shows phylogenetic relationships of cashew *Colletotrichum* isolates and adictional sequences obtained from GenBank for the *Colletotrichum gloeosporioides* species complex, WEIR *et al.* (2012). *Colletotrichum boninense* was used as outgroup, and Bayesian posterior probability values over than 50% are shown on each node.

* See details in next parts (A and B) enlarged.

APPENDIX C - continued, Part A.



APPENDIX C - continued, Part B.