MITIGATION OF AN EXISTING AND IDENTIFICATION OF AN EMERGING FUNGAL DISEASE OF KOA

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE

UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

TROPICAL PLANT AND SOIL SCIENCES

December 2020

By

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Keywords: breeding, Koa Wilt, Chitinase, Koa Anthracnose

ACKNOWLEDGEMENTS

I am grateful to my thesis advisor, Dr. Michael Kantar, for his everyday guidance and support throughout my thesis research. I would like to thank Dr. Susan Miyasaka for all the academic and financial support she has given me during my masters' studies. I would like to extend my thanks to my committee members, Dr. Michael Shintaku and Dr. James B. Friday, for their thoughts and comments on the thesis. My special appreciation goes to Dr. James Keach for the training on the cross-pollination of koa, Dr. Angel R. Del Valle Echevarria, and Steven Starnes for teaching molecular techniques. I would like to express my gratitude to Tyler Jones for providing control families and pathogen inoculum. I am thankful to Dr. Mike Muszynski, Dr. Dulal Borthakur, Dr. Brent sipes, and Dr. Birendra Mishra for their help in various ways during my project. I would like to thank Brian C Bushe for his guidance in plant pathology. I am thankful to Joyce Lilly (Ama), Kahua Julian, Tazman Shim, David Russel, and Aaron Shipman for their help in the field and lab at Hilo. I would like to thank Mary Winnicki for helping me with RNA extractions. I appreciate the support of friends and fellow graduate students Roshan Paudel, Kauahi Perez, Jaclyn Nicole Uy, Todd Anderson, Peter Toves, Anna McCormick, Nathan Fumia, and James Dorthey.

I am forever indebted to my parents for their continuing support in my academic journey.

ABSTRACT

Koa (*Acacia koa)* is a culturally, ecologically, and economically important forest tree of Hawaii. Koa wilt and dieback disease caused by *Fusarium oxysporum* has been a continuing challenge to the reforestation of former agricultural lands with koa. Koa breeding for disease resistance has identified some individuals useful as parents for disease resistance breeding. Anthracnose disease is a cause for the failure of the production of hybrid seeds in the breeding program. In this study, I have used families derived from five parents (E, F, G, H, and I) that thrived in a lower elevation as parents in diallel crosses and have evaluated the disease severity of the hybrid seedlings under disease treatment. Parent 'I' appeared to be superior in delivering disease resistance to offspring, parents 'E' and 'F' appeared to be intermediate in resistance, and parent 'G' appeared to contribute disease susceptibility to offspring. The expression of three chitinase genes, *Akchit1a, AkchitII, and Akchit III,* was determined using Reverse Transcription PCR. No correlation was found between gene expression and disease severity, indicating that Chitinase is a poor candidate for a disease resistance marker in koa breeding. Finally, both morphological and phylogenetic approaches were used to identify a previously unidentified pathogen which can cause anthracnose on koa. Both morphological and phylogenetic studies showed the fungus as a member of the *Colletotrichum acutatum* complex; this provides information on ways to control the fungus using specific and general fungicides.

TABLE OF CONTENTS

Table of Contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: THE HISTORY OF KOA (ACACIA KOA GRAY)

Introduction

Koa, *Acacia koa* (L.) Gray. is a tropical hardwood tree endemic to the Hawaiian archipelago. This species is a significant component of Hawaiian forests providing an indispensable ecological contribution to the island ecosystems (Baker et al., 2009). Koa occurs on all major islands of the Hawaiian archipelago except Niihau and Kahoolawe and was once abundant on Kauai, Oahu, Maui, and Hawaii (Baker, Scowcroft, and Ewel 2009). It is the second-largest overstory component of Hawaiian forests. It plays a crucial role in watershed management, the soil nitrogen cycle, carbon sequestration, bird habitat, and forest microbial ecology (Allen et al. 1997; Baker et al. 2009; Leary et al. 2004; Scowcroft et al. 2008). Koa can grow from 50m to 2100m in elevation, and it thrives between elevations of 600m to 1200m. The most massive and best-formed koa trees are found in moderate to well-drained medium acidic soils within the rainfall range of 1900 to 5100mm (Allen et al. 1997).

Botanical Description

Acacia koa belongs to the subfamily Mimosoideae of Fabaceae *aka* Leguminosae family. The average height of Koa ranges from 15 to 20m (50-80ft), while some outliers can grow over 35m (115ft). The canopy of the tree spreads from 6 to 12m (20-40ft) in diameters. Trees are typically harvested when trunks are 24 inches or larger in diameter and usually provides 16-foot logs (Skolmen 1974).

Young seedlings of koa have bipinnately compound true leaves consisting of 12-15 pairs of leaflets (Whitesell 1990). With maturity, the rachis of compound leaves dilates into a sickle-shaped, thick, leaf-like structure called a phyllode. It is a modification for xerophytic adaption to mitigate transpiration loss (Baker, Scowcroft, and Ewel 2009). Mature trees have no true leaves except in the areas of new growth. Phyllodes are 7-25cm in length and 0.5-2.5 cm in breadth (Elevitch et al. 2006). The shape and size of phyllodes vary with the island types (Daehler et al. 1999; St. Johns 1979).

Flowers are borne on the crown's outer parts, beginning from 2 to 3 years (Allen et al. 1997). The inflorescence is an axillary raceme made of pale yellow heads of diameter 8 to 10 mm (Hillebrand 1888; Ishihara et al. 2018). A common peduncle holds one to three flower heads. Flowers are hermaphroditic, but they are highly dichogamous, with anthers dehiscing 3-8 days before exertion of stigmas (Brewbaker 1977). Pollen is carried by insects facilitating cross-pollination between different flowers of the same plant or a different plant (Elevitch, Wilkinson, and Friday 2006). The number of flower heads ranges from 24 to 31 depending upon location (Miyasaka lab, unpublished data).

Fruits are produced 3 to 4 months after pollination. Fruits are flattened, have oblong pods about 15 to 20 cm (6 to 8 inches) long and 2.5 to 4 cm (1 to 1.5 in) wide, containing about six to twelve seeds. Seeds are laterally ellipsoid, six to13 mm (0.23 to 0.51 in) long and 3 to 9 mm (0.11 to 0.35 in) wide, shiny and dark brown to black (Boutilier 2002; Daehler et al. 1999; Whitesell 1990; Elevitch et al. 2006).

Koa has a large taproot and strong lateral root system; some of its lateral roots are exposed to the surface (Elevitch, Wilkinson, and Friday 2006; Ishihara et al. 2018). The development of root suckers into a mature tree is widespread in natural forests (Skolmen 1977). The roots can establish a symbiotic relationship with *Bradyrhyzobium sp.*, contributing nitrogen to the nutrient-poor volcanic forest floor (Leary et al. 2004).

The sapwood of koa is pale brown, and it turns into heartwood at the age of 10 to15 years. The color and figure of *A. koa* heartwood are variable, color ranging from blonde to dark chocolate, with reddish-brown being the most common color. The grain of koa wood is commonly straight, wavy, or curly (N. S. Dudley and Yamasaki 2000; Skolmen 1974).

Koa Genetics, Taxonomy, and evolution

Atchison (1948) studied chromosomes on 40 different *Acacia* species collected from Australia, the Pacific Islands, Asia, Africa, South America, and the West Indies. Three distinct geographical groups had similar ploidy levels; however, *Acacia koa* stood out as the only tetraploid among Australian and Pacific island species with chromosome number 2n=4x=52 (Carr 1978). This peculiarity in chromosome numbers prompted studies of the evolutionary history of the species.

Brown et al. (2012) compared 74 accessions of 34 taxa, revealing that *A. koa* shares common ancestry with *Acacia heterophylla*, another phyllodinous tetraploid with the same chromosome number endemic to the Reunion Island, Mascarene island, and southwest Indian ocean. *Acacia koa* and *A. heterophylla* were independently dispersed from East Australia to two island systems with the movement of Austronesian people. Later, Le Roux et al. (2014) showed evidence for the pre-anthropogenic origin of both *Acacia koa*, revealing that *A. heterophylla* was the closest relative by examining pollen records bird fossils and low divergence in genetic distance. Le Roux et al. (2014) further showed that the dispersal of *A. koa* in the Hawaiian Islands predates the dispersion of *A. heterophylla* in Mascarenes islands, claiming that the latter evolves from the former.

It has been suggested that *A. koa* and *A. heterophylla* be considered a single species (St. Johns 1979; Pedley 1975; Le Roux et al. 2014), but this has not been widely adopted. Wagner et al. (1990) defined three subspecies, *A. koa* sensu stricto, *A. kauaiensis* Hillebr, and *A. koaia* Hillebr, under a standard taxonomic unit *Acacia koa* sensu latio. This delineation of species brought all the Hawaiian Koa under the same species.

Nevertheless, there is significant genetic and morphological variation among *A. koa* within and between the islands. Koa in the older islands, Kauai and Oahu, have narrow and slightly curved phyllodes, whereas the ones from geologically younger Island, the Island of Hawaii, have broader phyllodes (St. Johns 1979). Daehler et al. (1999) showed both morphological differences have a genetic basis, and they recommended recognizing *A. koa* from Oahu and Kauai as a distinct variety, *A. koa var. latifolia.* Conkle (1996) found a close relationship between Oahu, Kauai, and Maui by using isozyme analysis. Brown et al. (2012) and Le Roux et al. (2014) also showed considerable diversity in *A. koa* germplasms collected from across Hawaiian islands. The genetic diversity of Koa in Hawaiian Island is a significant asset that can be utilized in many ways to study and improve this tropical hardwood tree.

Use and Market

The strength, color, and texture of *A.koa* wood make it suitable for a comprehensive range of products. Skolmen (1974) reported *A. koa* wood to be identical to walnut (*Juglans nigra* L.*)* in weight and strength with a specific gravity of 0.55. The wood takes a high polish and has good working properties. Koa was used to construct houses, canoes, paddles, surfboards, and weapons by the Native Hawaiians. In modern days, the wood is used for producing fine furniture, veneer, musical instruments, and crafts.

There is a demand for hardwood products and a reasonably good awareness of beauty and potential for Hawaiian grown woods in the consumer marketplace. However, the demand for products cannot be met by current production; thus, rendering its high price in the market. Hawaii had a forest industry worth 30.7 million in the year 2001 (Yanagida et al. 2004), and 90% of its value is contributed by koa (N. Dudley 2006). A standing koa timber is valued between

\$1000 to 1300 US m^{-3}. The price of koa lumber ranges from \$4.50 to over \$65.00US per board feet. The cost varies with the color and figure of the wood (N. Dudley 2006).

Koa Forests in the Hawaiian Islands

Koa is present in all climatic and moisture regimes of Hawaii. The Hawaiian Islands can be divided into roughly five ecological zones. These zones are alpine, subalpine, montane, lowland, and coastal ecological zones. There are three moisture regimes in each ecological zone: dry, mesic, and wet, with an annual rainfall of less than 1200 mm, 1200 to 2500 mm, and more than 2500 mm, respectively. Koa occurs across the ecological and rainfall regime; however, it is found predominantly in subalpine, montane wet, and lowland forest ecoregions across Hawaii, Maui, Oahu, and Kauai. Koa can be located in a variety of physiognomy. Tall, straight trees are common in forests of wet and moist climates. Short trees with multiple branches and a large canopy are common in drier climates with nutrient-poor soil (Baker, Scowcroft, and Ewel 2009).

Hawaiian forests have been impacted since ancient Polynesians arrived in the islands. These forests suffered further shrinkage after the Europeans arrived in 1798 (Cuddihy and Stone 1990). Forests of predominantly ōhia, koa, and their understory vegetation were cleared for agriculture in lower windward plains and pastures in dry uplands (Tummons 2002). The forest clearing in agricultural land pushed native forests into mesic uplands not favorable for agriculture. The decline of the ranching industry since 1980 has opened up the possibility of restoring native forests and, subsequently, the underlying ecosystem services (Cuddihy and Stone 1990). However, there are several challenges, old and new, which need to be strategically addressed to maintain a sustainable native forest system in Hawaii.

Invasive feral mammals, plants, insects, and microbial pathogens are well-known challenges of maintaining and expanding koa forests. Koa did not evolve with wild pigs, goats, cattle, deer, and rats. Thus, they cause considerable damage to the plant's seedlings, juveniles, and root suckers (Whitesell 1990). Plants like *Passiflora parmigiana, Clidemia hirta and, Rubus argutus* are considered the most aggressive, invasive plants, increasing competition for koa in nutrition, light, and space. Most of the invasive plant species that became established in the islands are found in koa forests (Baker, Scowcroft, and Ewel 2009; Scowcroft 1991; Smith and Tunison 1992).

Koa provides shelter and food to approximately 101 insect species (John D Stein 1983). There are examples of up to five different outbreak events of Koa moth *Scotorythra paludicola* Butler on Maui forests, and Two outbreaks of koa moth were recorded in the island of Hawaii including one in 1950s and another in 2013 (Scowcroft 1984 and Friday 2013). Koa moth outbreak has also been associated with the vascular wilt-causing fungus *Fusarium oxysporum* (Baker, Scowcroft, and Ewel 2009). Similarly, sporadic damage is reported from Koa seed worm *Cryptophlebia illepida* and Black twig borer *Xylosandrus compactus.* Acacia psyllids *Acizzia uncatoides* cause forking by feeding on new terminal growth (Baker, Scowcroft, and Ewel 2009).

Minor pathogens without severe outbreak include fungi and oomycetes such as *Calonectria crotalaria* (seedling blight), *Phytophthora cinnamomi* (root infecting oomycete), and *Atelocauda digital* (Koa rust). The vascular wilt-causing fungus *Fusarium oxysporum* is perhaps the most significant threat in general to koa (Baker, Scowcroft, and Ewel 2009).

In collaboration with different stakeholders, the state of Hawaii has initiated a long-term plan to conserve, restore and improve Koa in the islands. Koa has been researched for disease and drought tolerance, improved wood quality, improvement of nursery and silvicultural practices, and tree physiology. Koa's selective breeding for survival, growth, form, disease resistance and wood quality across its environmental range has been outlined in the Koa action plan under a broader program of establishing Koa for conservation and forest product (Inman-narahari et al. n.d.).

Genetic Improvement in Koa

In 1997 Dr. James Brewbaker initiated the evaluation of over one thousand koa families across the islands and selection of superior families based on survivability, form, disease resistance, and abiotic stresses. Multiple collection programs were carried out, followed by the establishment of nurseries in Hamakua Research Station on Hawaii. Selection of seeds from parents with traits of interest, controlled crosses, screening progenies for disease tolerance and resistance are major activities under the genetic improvement program. The University of Hawaii and Hawaii Agriculture Research Center (HARC) are the two organizations continuing with these genetic improvement projects.

Conventional breeding approaches involve selection from base populations to use as parents for a breeding population. The breeding population is either multiplied directly, or mating is done among the breeding population to create the population for the next cycle (White, 1987). Early tree breeding techniques involved collecting open-pollinated seeds from parents with desired phenotypes. The collected seeds were then subjected to a progeny test (Namkoong et al., 2012).

Breeding trees, as opposed to breeding annuals, presents different challenges. Trees take many years to manifest their genetic attributes, especially for traits like the quality and quantity of woods and fruits. This challenge of long cycle time has been helped by advent of marker-based selection as tree breeders can now screen and select genotypes superior in the trait of interest just by using the marker genotype.

The second chapter aims to screen progenies that gives us the information useful for selection of parents based on the performance of their offspring (retrospective selection) and also for the selection of individuals based on their own performance (prospective selection). This study utilizes the diallel cross technique to measure the hybrid vigor in offspring from different Koa families. It also explores the potential of using gene expression as a marker of koa wilt resistance. The third chapter explores an emerging pathogen in koa, its phylogenic placement, and how it infects koa.

CHAPTER 2

SCREENING OF ACACIA KOA GERMPLASM FOR RESISTANCE TO FUSARIUM WILT BY QUANTIFYING EXPRESSION OF CHITINASE GENES

Abstract

Acacia koa is a nitrogen-fixing, overstory tree with ecological, economic, and cultural significance in the Hawaiian Islands. *Fusarium oxysporum* causes wilt and dieback in young and old koa trees. The Koa plant could utilize the chitinase enzyme to degrade fungal cell walls during pathogenesis and disease development. Five individuals of *A. koa* were cross-pollinated with pedigreed offspring obtained in 2019. These families were screened for expression differences in the Chitinase IA, II, and III genes by using quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR). Differences were observed during disease progression; however, no differences were observed at baseline (no-infection) conditions, indicating that expression of chitinase is not a useful marker for disease tolerance in the absence of infection. Despite the lack of correlation between chitinase expression and disease resistance, there was differential disease resistance among breeding families, where parents ‘I’ and ‘E’ showed tolerance and families derived from these individuals having the most promise for future breeding.

Introduction

Acacia koa, an endemic Hawaiian leguminous tree, is the second most abundant overstory tree species in Hawai`i, after ʻōhiʼa (*Metrosideros polymorpha Gaud*) (Elevitch et al. 2006). As an endemic species, koa is essential to Hawaiian ecosystems contributing to watershed management. The koa forests are home to at least 101 different insects, 128 identified microbes (Jones, 1991), and 87 species of endangered plants (Baker et al. 2009). As a nitrogen-fixing leguminous tree, koa forms both root and canopy nodules in association with *Bradyrhizobium* (Leary et al. 2004). A six-year-old koa stand was found to fix nitrogen at the rate of 23 kg per hectare per year (Pearson, H.L. 1997).

Since 1963, the area of the Koa forest has been reduced by more than 90% (Shi and Brewbaker 2004). A large part of this loss was related to increased ranching and sugar plantations (Brewbaker et al., 1991). However, recently, there has been an increase in attempts to re-establish the Hawaiian forest on previous agricultural lands (Pejchar and Press, 2006). The effort of reforestation has been successful in the higher altitudes of the islands but, Koa wilt and dieback is a significant limitation to increased cultivation of koa in the lower elevation (Shi and Brewbaker 2004)

Koa is complex genetically and has a long generation time, complicating breeding efforts. Koa is an autotetraploid (2n= 4x= 52) with limited genetic resources (Atchison, 1948). One beneficial aspect for breeding is that in contrast to many other polyploid legumes, koa is self-incompatible. Flowers generally start as males with pistil emergence occurring late in development; however, pollen becomes inviable before the emergence of the pistil(Brewbaker, 1997).

Despite the limitation to breeding, koa is a high-value timber tree that accounts for 75% of the 30 million USD Hawaii-grown wood industry (Yanagida et al., 2004). Koa has deep, golden-colored hardwood suitable for construction materials, furniture, surfboards, and musical instruments. In addition to its current value, koa has tremendous historical significance in Hawaii due to its traditional use, particularly in outrigger canoe construction. There is an increasing demand for koa wood in the Hawaiian Islands (Dudley and Quinn, 2004).

Koa wilt and dieback are major limitations to increased koa cultivation, especially in areas with an altitude below 600 meters (Gardner 1980; Shiraishi et al. 2012). Koa wilt is caused by *Fusarium oxysporum formae specialis koae*. It was first reported by Gardner (1980) in Hawai`i Volcanoes National Park. Later, Shiraishi et al. (2012) isolated and identified another highly virulent strain and named *F. oxysporum f.sp. Acaciae*, along with another species called *Fusarium pseudocircinatum.*

The soil-borne pathogen enters the plant through the root system, where it colonizes vascular tissues, thus disrupting the uptake of water and minerals and transportation throughout the plant. Disease symptoms include wilting, foliar chlorosis, and shoot necrosis (Gardner, 1980). Occasionally, infected branches show splitting due to sap accumulation in the region of fungal colonization. This splitting leads to secondary infections of other fungi and bacteria. Shi and Brewbaker (2004) reported 30-90% mortality rates of Koa in lower altitudes under ambient growth conditions for the pathogenesis.

Fusarium oxysporum Schlechten Emendend, Snyder & Hansen is a soil-dwelling fungus. It belongs to the family Nectriaceae of phylum Ascomycota. *Fusarium oxysporum* has a wide occurrence throughout the world. Most strains live near root systems of higher plantsas saprotrophs and sometimes as endophytes in plants. However, some strains have evolved the ability to grow beyond the cortex into the xylem (Gordon and Martyn 1997). Most plant-parasitic *Fusarium* cause vascular wilts, cortical rot, head blight, leaf spot, root rot, fruit rot, cankers, and dieback. *Fusarium oxysporum* is mostly associated with vascular wilts, and specific formae speciales infect particular plant species. There are more than 120 formae speciales and races of *Fusarium oxysporum* (Armstrong and Armstrong 1981). *Fusarium oxysporum f.sp cubense* causes Panama wilt disease in banana and has been highly studied, *F. oxysporum f.sp lycopersici* causes tomato wilt and *F. oxysporum f.sp. vasinfectum* causes cotton wilt. The sexual stage of *F. oxysporum* has not been found in nature yet. Hence the variation among the formae specialis and pathotypes are entirely due to clonal variation among the existing strains (Gordon and Martyn, 1997).

Breeding trees, as opposed to breeding annual plants, constitutes different challenges. Trees take a long time to manifest their genetic attributes, especially for traits such as the quality and quantity of wood or fruits. The current study aims to screen progenies so that the results will guide in future selection of parents during while making crosses. Differential resistance/tolerance to koa wilt has been observed in full and half-sib *koa* families (Dudley et al., 2016; Shiraishi et al., 2012). This variation can be further examined for usefulness to the development of *Fusarium* wilt resistant genetic material. Currently, breeding programs select individuals (known maternal lines) that are deemed tolerant to koa wilt based on the visual symptom assessment after fungal spore inoculation. Controlled crosses between known parents provide the opportunity to quantify the genetic component of disease resistance.

Conventional approaches involve selection from wild stands to make a breeding population. The breeding population is either multiplied directly or crossed to create a base population for the next cycle (White, 1987). Early tree breeding techniques constitute open-pollinated seeds from parents of desired phenotypes. The collected sources are then subjected to a progeny test (Namkoong, Kang, et al., 2012). The advent of marker-based selection has made it easier for tree breeders to screen and select genotypes superior in the trait of interest.

Chitin is a polymer of N-acetylglucosamine and is a primary component of the fungal cell wall. Chitinase enzymes break down the chitin in the fungal hyphal tips and germinating spores in the plant, and therefore, chitinases are reported to play an essential role in plant defense (Kumar et al., 2018). Rushanaedy et al. (2012) isolated four chitinase genes from Koa with putative roles in plant defense: *AKchit1a, Akchit1b, Akchit2, and Akchit3*. Rushanaedy et al. (2012) found increased expression of AKchit1 and AKchit4 among resistant seedlings at 48, 72, and 96 hours post-inoculation. However, they found no difference between Akchit2 and Akchit3. This study introduced the potential for marker-assisted selection for disease resistance in *A. koa* by observing the gene expression in breeding populations.

DNA markers are commonly used to identify species and interspecific hybrids. (Pijut et al., 2007). The DNA marker for disease resistance has not been identified yet in koa; however, four genes associated with chitinase were found upregulated in koa seedlings upon inoculation with *F. oxysporum.* Such upregulation was significantly higher in seedlings of a known resistant family than in seedlings of another known susceptible family (Rushanaedy et al. 2012). The potential of chitinase genes to be used as a marker will be studied by combining both molecular and conventional observations. This study aims to identify whether any progeny obtained from diallel crosses show resistance to *Fusarium oxysporum* and if the half-sib families express high levels of chitinase during disease development. The hypothesis is that there are differences in the resistance of the progenies of parents ‘E,’ ‘F,’ ‘G,’ and ‘I’, and that there are associated differences in *AkChitIA*, *AkChitII*, and *AkchitIII*.

Materials and Methods

During the 1960s, ~500 accessions of koa were collected from all major Hawaiian Islands by Brewbaker(Shi and Brewbaker 2004) . They were planted in a common garden at the University of Hawai’i Hamakua Research Station. This 500-accession represented a wide variation of Hawaiian Koa genotypes (Gugger et al., 2018). Up to 10% of mortality was observed annually in the common garden plantation. In 1999, seeds were collected from the standing healthy and sturdy trees and planted in a new site in the station (Shi and Brewbaker 2004; Shiraishi et al. 2012). We selected five individual tree families from the new plantation and used them for full diallel crosses. Pollination was controlled by restricting the entry of pollinator insects into the flowers by completely covering the twigs containing selected flowers with meshed bag (fig 2B)

Hand pollination was done by brushing the male flowers of one tree with viable pollen against another tree's yellowish colored female flowers. Altogether, 384 pollinations were made. Natural pollination was monitored by tagging 54 open-pollinated flowers. Cross-pollination was carried during the flowering season (February to May 2019). A total of 11 combinations were successfully established, and seed pods were obtained with hybrid seeds. A family of koa is the progeny grown from the seeds from a single mother tree. Half sib family has only one parent in common while full sib families have both parents in common. Seven families produced enough seeds to be used in a replicated trial. Among them, six families had successful germination (Table 1). Resistant 08 and Big Island susceptible obtained from HARC were used as resistant and susceptible checks, respectively. These families were selected based on a 90 day trial done by Tyler Johns (unpublished), where significant variation was observed in disease susceptibility.

Seedpods were harvested after they turned brown and were relatively dry. Pods were sun-dried, and seeds were further dried after removal from pods. The seeds were surface sterilized with 10% bleach for 10 minutes and scarified by treating with water at 90^0C for 30 seconds and Soaking the seed in cold water for 24 hours. (Elevitch et al., 2006). Sixteen seeds from six different families from the available F_1 generation were germinated in seedling growing flats. After a month, seedlings were transferred to dibble tubes. Four-month-old seedlings were inoculated with the *Fusarium oxysporum* inoculum, and four seedlings were sampled from each genotype for RNA extractions. Plants were grown inside the greenhouse under mist irrigation, maintaining the average daily temperature at 25°C. The mist setting was set at 20 seconds of misting every 10 minutes throughout the experiment.

A mixture of different isolates of *F. oxysporum* was obtained from HARC. The isolates 1701, 1405, 1613A, 1617, 166, 1613B, 79, 1417, and 77 were obtained in a dry corn perlite media dominated by the chlamydospores of the *F. oxysporum* . The inocula had been prepared at HARC by growing each isolate in Komoda agar media (Komada 1975) for seven days and separately transferring approximately $10mm^2$ piece of agar into a spawn bag containing cornmeal-perlite-Potato Dextrose Agar. All strains were allowed to colonize the cornmeal-perlite-PDA entirely in 15 days, ensuring there were no nutrients for other microorganisms. The inoculums were obtained in the latter form. They were crushed into powder form by using a mortar and pestle. The powdered media was mixed with autoclaved growing medium (Pro-mix double purpose, Premier Tech Horticulture, Quebec Canada) in the ratio 1:50 (W/W). Plants were transplanted into the new mixed media and watered.

Extraction of RNA was done by using an RNA isolation system for plant tissues rich in secondary metabolites (Ghawana et al. 2011). RNA extraction was done at four different time points: Before inoculation (baseline), 48, 72, and 96 hours after inoculation (HPI) by using the protocol described by (Ghawana et al. 2011). The mRNA from the total RNA obtained was reverse transcribed into cDNA by using a cDNA Synthesis kit (Sensifast™, Meridian Bioscience® New Jersey). Quantitative PCR was carried out using the 1 ul cDNA of concentration 200ng, 5ul PCR mix [SYBR™ Green, ThermoFisher Scientific, USA] was used, 0.5ul of 10um forward primer, 0.5ul of 10 um reverse primer and 3ul nuclease-free water per individual well.

Quantification of gene expression provides important information related to a particular event in a physiological process. Quantitative reverse transcription Polymerase Chain Reaction (qRT-PCR) is a method of quantifying transcript levels of a specific gene. Messenger RNA (mRNA) synthesis is an indicator of gene expression. The magnitude of synthesis of mRNA of a particular protein is valuable to measure the effect of a treatment on a particular gene. Reverse transcriptase enzyme catalyzes the process of reverse transcription of an RNA into its complementary DNA.

Quantitative PCR provides the value of threshold cycle (C_t) in the PCR cycle where a particular gene amplifies. Quantification of transcript levels can be accomplished by either determining the amount of amplified region of cDNA (amplicon) using a standard curve or normalizing the C_t value of the gene of interest with a reference gene. In this study, 18S RNA was used as a reference gene. (Schmittgen and Livak 2008). Primers optimized for qRT-PCR were obtained from Rushanaedy et al. (2012) and were used to quantify the chitinases by

Reverse Transcription PCR (Table 2). Three technical replications were maintained for each sample.

Disease symptoms were evaluated for one month after inoculation. Symptoms were scored in the order of 7 for dead, 6 for wilting & dying, 5 for heavy defoliation, 4 for the dead tip, 3 for the small root system, 2 for yellow leaves or small yellow leaf freckling, and 1, no symptoms (Shiraishi et al. 2012)(Fig. 1). The scoring of disease was done three times throughout the experiment, except for the root phenotype. The average of three scores was calculated and used for further analysis.

The experiment was set up in a completely randomized design. Each genotype was a treatment, and there were altogether 11 experimental units for each treatment. An ANOVA was made using statistical software R (R Core Team, 2018) to determine whether there is significant variation among hybrids on disease resistance. The mean disease scores were compared using Dunnett's Multiple Range Test, where the mean disease score from each family was compared to Big Island Susceptible check at the alpha = 0.05 level using R-studio (R Core Team, 2018). The data were subjected to the Shapiro Wilk normality test to verify the assumptions of ANOVA were fulfilled before moving into further analysis. Gene expression data were analyzed using a relative quantification method called comparative C_t method where expression fold change of chitinase genes was normalized with the housekeeping gene 18S RNA. The average difference in fold change in expression with 18S RNA for each gene was calculated, and standard errors were computed using the Students t-test (Schmittgen and Livak 2008).

Results

Significant differences in disease symptoms were found between and within families ($p < 0.00001$), indicating that selection was effective and that parents had different impacts on disease resistance of progeny. Families FI, IE, IF had lower disease scores while compared to Big Island Susceptible. While families EG, GE, and GF were not found different from susceptible check (Fig 2). Parent ‘I’ is common to all three resistant families, while it is never a parent in any susceptible family. In resistant families, IE parent E was used as a pollen source and parent ‘I’ was a maternal line, while in susceptible families EG and GE, in these ‘E’ is a paternal line and maternal line, respectively. This shows parent ‘E’ had less effect in passing on disease resistance to progeny, whereas, parent ‘I’ was shown to have a consequential impact on resistance of progeny. This effect also suggests that the maternal parent, in particular, may enhance disease tolerance in progeny. Parent ‘G’ is always found in combinations that are susceptible to the disease, which indicates that it transfers susceptibility to disease in progeny.

In this experiment, there was no evidence of differential gene expression at the baseline of no disease presence (Fig. 3).

Discussion

Previous work suggested that chitinase expression was a sign of disease progression due to *Fusarium oxysporum* in Koa (Rushanaedy et al., 2012). It was suggested that expression in the chitinase gene family could be used in marker-assisted selection. To test the utility of gene expression, qRT-PCR was used to quantify expression levels at a single time point before inoculation and three-time periods across a time series after the pathogen treatment. This experimental design had two distinct hypotheses for this gene family's efficacy as a potential marker for breeding. The first was to look at baseline expression; if expression levels were to be useful for selection prior to disease, there must be a differential expression between susceptible and resistant checks, as well as differential response in experimental families. The second hypothesis that would indicate the utility of gene expression as a molecular marker was to assess the correlation between gene expression and disease score. In this experiment, there was no relationship, indicating that while gene expression of the chitinase family does occur during disease progression, it is not a useful marker for breeding.

There was considerable variation in the families assessed. Three families showed superior disease resistance compared to the susceptible check variety. The families IE, IF, and FI can be further used in the breeding program as a parent material or multiplied by vegetative propagation. Parent ‘I’ showed superior characteristics as a parent, while Parent ‘G’ showed poor characteristics. Parents ‘E’ and ‘F’ showed intermediate characteristics, depending upon the material line it was crossed with. This indicated that families with Parent I' should be moved forward for the next round of recurrent selection for disease resistance, and families with Parent ‘G’ should be removed. Parent ‘I’ is also useful in selecting the maternal line based on its performance in this study.

The use of gene expression as a marker for breeding is dependent on baseline conditions. In the case of chitinase genes, a significant difference was not observed between families Resistant/susceptible checks. Neither was expression correlated with disease susceptibility symptoms during disease progression. While differential expression patterns were observed among the three genes, none displayed patterns consistent with the hypothesis that expression was a good predictor of disease progression or resistance. While previous work had shown that there were apparent expression differences, these differences could not be exploited for selection in the absence of the pathogen. Apart from disease resistance, chitinase genes have also shown to have different physiological functions such as signal transduction, or hydrolyzing nod factors produced by nitrogen-fixing bacteria (Xie et al. 1999).

Conclusion

Half of the families evaluated here show excellent disease resistance. Parent ‘I’ shows particularly good characteristics. This indicates the parents that can be used for the next cycle of recurrent selection and shows the potential for continued genetic improvement for disease resistance. To incorporate marker-assisted selection, the next step should be to develop larger mapping populations and used DNA rather than expression-based markers to find QTL. Currently, the families ‘IE’, ‘FI’ and ‘IF’ show resistance similar to the resistant check cultivar, indicating that these families may be appropriate for release as resistant cultivars.

Figure 1 Visual guide to disease scoring of fusarium wilt on koa used as per Shiraishi et al. (2012). Symptoms were scored in the order of 7 for dead, 6 for Wilting & dying, 5 for heavy defoliation, 4 for the dead tip, 3 for the small root system, 2 for yellow leaves or small yellow leaf freckling, and 1, no symptoms

Figure 2 Controlled pollination of A. koa flower by A. Brushing male flower to female flower and B covering the flower head with a meshed bag to prevent pollination by insects.

Table 1 List of numbers of seeds obtained from each diallel cross to be used in chitinase expression assay.

Family	Maternal family	Paternal Family	No of seeds
EG	E	G	36
FI	F	I	46
GE	G	E	60
GF	G	F	42
IE	I	E	114
IF	I	F	79

***Resistant Check**— Resistant08
†**Susceptible check**—Big Island Susceptible

Table 2. List of primers to be used for qRT-PCR analysis, the primers were designed and used in the chitinase expression study in resistant and susceptible family by (Rushanaedy et al. 2012).

Primer type	**Forward primer (5'->3')**	**Reverse Primer (5'->3')**
18s RNA (HKG)	TTA ACG AGG ATC CAT TGG AGG GCA	TCA ACC CAA CCC AAG GTC CAA CTA
Akchit IA	CCC GGT GCT GAT TAT TAT GG	GTG TGC CGA AGG TTG TTT TT
Akchit II	AGA CGG CAA TAT GGT TCT GG	GCA CTC CAA CCC TCC ATT TA
Akchit III	GTG CGG CCA GAC TTA TGA TT	GGC GTC GTA GGT GTA GAA GC

*Figure 3. Box-plot showing mean (black solid line) with 25th percentile (lower bound) and 75th percentile (upper bound) for disease severity of fusarium wilt of koa as scored by the protocol from Shiraishi et al. (2012). *indicates significantly different from susceptible control at alpha = 0.05, ** indicates significantly different from susceptible control at alpha = 0.01, *** indicates significantly different from susceptible control at alpha = 0.001*

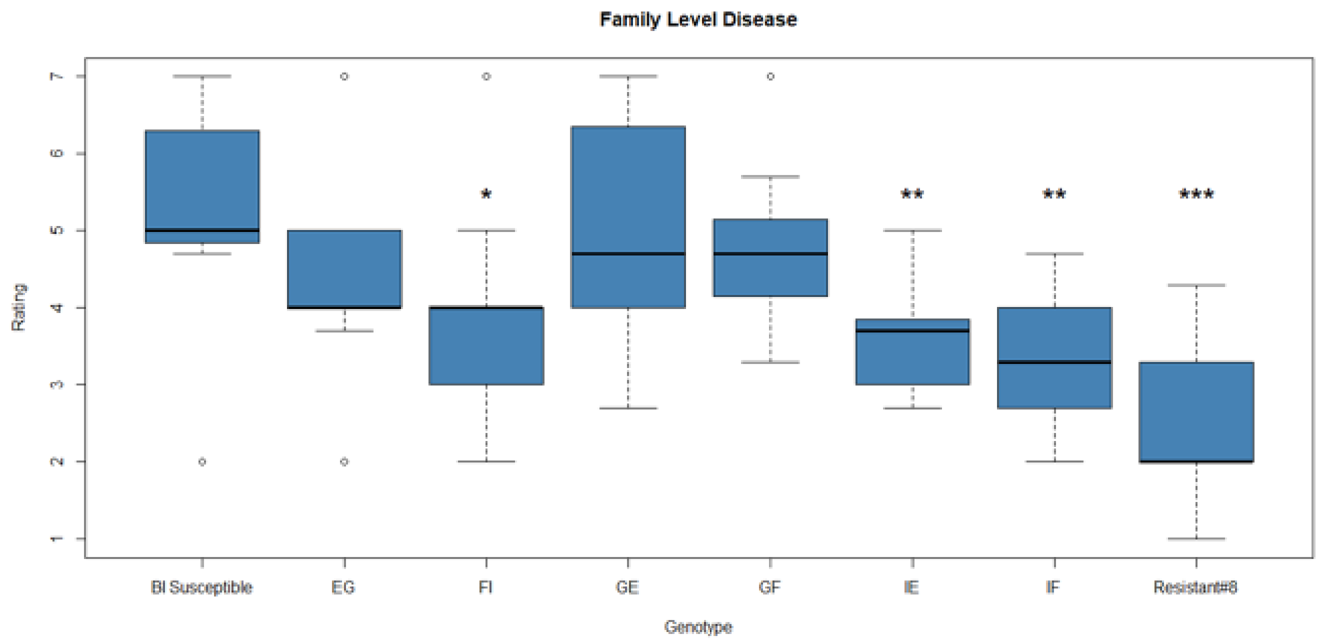
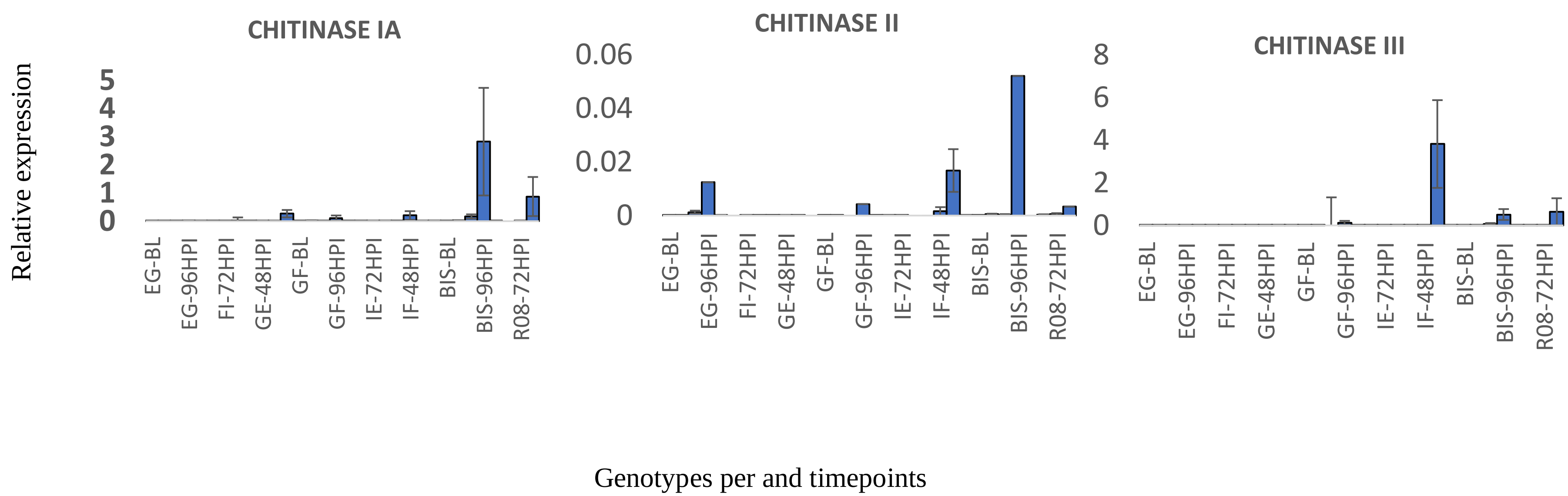


Figure 4. Relative expression levels of three types of chitinase genes (y-axis), within Acacia koa. Gene expression is derived from qRT-PCR using 18S RNA as a control gene at four timepoints, baseline(BL), 48 hours post inoculation(48hpi), 72 hours post inoculation(72hpi) and 96 hours post inoculation(96hpi). Each bar represents gene expression of each genotype represented by EG, FI, GE, GF,IE, IF at particular time point A) Chitinase IA B) Chitinase II and C) Chitinase III as expressed by tested genotypes, Resistant, and Susceptible controls.



CHAPTER 3: IDENTIFICATION OF ANTHRACNOSE CAUSING FUNGUS OF ACACIA KOA SEED PODS IN HAWAII

Abstract

A new disease has emerged, anthracnose (*Colletotrichum sp.*) in *Acacia koa*, a tropical hardwood plant endemic to Hawai'i. Infected pods were collected from the Hamakua coast, on the island of Hawai'i, the fungus was cultured andshowed distinctive, salmon-colored mycelium, grey rice grain-shaped conidia, and acervuli suggesting the genus *Colletotrichum*. DNA from five loci ACT, CHS-1, ITS, GAPDH, TUB-2, was sequenced. These sequences were aligned with 98 taxa representing different clades of the genus *Colletotrichum.* Phylogenetic relationships were explored using Bayesian methods. The newly identified strain was found to be nested among the clade *acutatum* as part of a large polytomy containing *C. chrysanthemi, C. paxtonii, C. simmondsii, C. sloanei, C. brisbnense, C. nymphaeae, C. laticiphilum, C. guajavae, C. scovillei, C. cosmi, and C. walleri*. Conidial shape had characteristic acute ends with a dimension similar to the *Colletotrichum acutatum complex.*

Introduction

With more than a 500 species, the genus *Acacia* covers a large portion of the tropical and subtropical world *Acacia koa* is an endemic leguminous tree in Hawai`i. Koa is a large contributor to carbon sequestration (Scowcroft, 1986), nitrogen fixation (Whiteshell 1990), watershed management (Jones, 1991; Baker et al. 2009) and the local economy (Yanagida *et al*., 2004). Koa is used for construction of canoes, houses, boards, artifacts and musical instruments, and is intricately tied down with Hawaiian heritage. There is a need to restore and protect the native forest; however, increasing demand of koa wood, disease pressure and limited land availability has created significant pressure on koa stands (Brewbaker, Glover, and Moore 1991).

Superior genotype selection and breeding for economic traits was initiated by Brewbaker et al. (1991). Currently, there are koa breeding programs at the University of Hawai`i and Hawai`i Agriculture Research Center. Success in cross pollination is rare, however, there are additional problems that koa breeders face once seed pods starts developing. Along with Koa moth, feral cattle, and boring beetles, anthracnose is emerging as a new problem in koa seed development. Anthracnose in young and developing green pods was observed in two consecutive years of 2018 and 2019 in the common garden nursery at the Hamakua Research Station, Paauilo, Hawaii (Lat: 20.00 N, Lon: 155.39W, elevation 770.76m). The nursery is used for a koa breeding program and such damage in seed pods posed a serious threat to the program. Therefore, the present study was conducted with the objective of isolating and identifying the causal agent of anthracnose in *Acacia koa*.

The major candidate for the causal agent is *Colletotrichum*, a genus within the class Ascomycetes in the kingdom Fungi. This fungal genus causes disease in a wide range of plant species both pre- and post-harvest (Dean et al. 2012). The genus *Colletotrichum* encompasses diverse species that are endophytic, hemi-biotrophic, to completely parasitic. The name *Colletotrichum* was introduced by Corda in 1831(Cannon et al. 2012). Today there are more than 170 species reported, which are roughly divided into nine major clades and two undefined clades. The goal of this study is to identify the genus of the emerging anthracnose pathogen using: 1) phylogenetic characterization; and 2) morphological characterization.

Materials and Methods

Koa pods containing anthracnose lesions were collected from the common garden koa orchards at the Hamakua research station on the island of Hawaii (Lat: 20.00 N, Lon: 155.39W, elevation 770.76m). The structure of the asexual fruiting body was closely observed under a dissecting microscope, and small pieces of tissue were cut from diseased pods having the following composition one-third diseased and two-thirds green. To remove saprophytes and bacteria, tissue was immersed in 1% NaOCl for 30 seconds, followed by 70% alcohol for 10 seconds, and three rinses with DI water. Sterilized tissues were transferred onto 10% water agar after drying under sterile conditions. Thus, established cultures were grown under 12 hours light and 12 hours dark at 25°C for two days. After growth, the edges of hyphal tips were excised and transferred to potato dextrose agar with 1% lactic acid (PDLA) in order to promote fungal growth and to discourage the development of bacteria. Colonies with salmon-colored spores and were sub-cultured using the quadrant streak method (Korhonen and Hintikka 1980). Twenty-four hours later, individual germinating conidia were located and transferred to fresh sterile Potato Dextrose Agar plates.

In order to observe mycelium color, spore color, shape and size, cultures were grown on potato dextrose agar and synthetic nutrient poor agar (SNA) in 50 mm petri-dishes (Nirenberg 1976). Spores from the isolated culture were teased and planted at the center of each SNA plate.

The prepared cultures were observed over a period of 15 days. Length and breadth of conidia was measured by using AM 720p C-mount Microscope Digital Camera (AmScope, USA). The images taken at 25X and 40X magnification from a microscope (Nikon E200 LED Laboratory Microscope,) which was calibrated using an objective micrometer. The measurement was done originally in Pixel units and later converted to the micrometer based on the calibration.

DNA extraction, PCR amplification and Sanger sequencing

Fungal genomic DNA was extracted using a plant DNA extraction kit (Qiagen,Germantown, MD) with the following modifications, briefly, ~100mg of fungal mycelium were collected from 5-day old cultures making sure to not include any media in the sample. Fungal cells were disrupted in mortar and pestle using liquid nitrogen and the powdered sample was transferred to the 1.5ml tube with AP1 buffer provided by Qiagen. The remainder of the procedure were carried out following the manufactures recommendations. PCR primers were designed for four genes, actin (ACT), beta tubulin (TUB2), chitin synthase (CHS), and the ribosomal internal transcribed spacer (ITS) . (Wu 2019.; Weir, Johnston, and Damm 2012), that have shown to be useful in identifying species within the *Colletotrichum* genus (Table 1). Genes were amplified using the following PCR conditions, 4 minutes at 95°C as cycle 1, 35 cycles of 95°C for denaturation, followed by 35 cycles of primer specific temperatures of annealing for different primers and 72°C for 45 seconds as cycle 2, and the final cycle a cycle of 72°C for 7 minutes. The annealing temperatures for each primer were ACT: 60°C, TUB2: 60°C, CHS1:55.7°C, ITS:52.6°C. Annealing temperatures were determined by gradient PCR. The amplified gene regions were sent for sanger sequencing to Genewiz (San Francisco Lab) after cleaning the PCR product using ExoCip™ (New England BioLabs, Boston, MA**).** The sequences were examined and edited by comparing the reverse and forward sequences using a computer software Geneious 8.1 (Kearse et al. 2012). Consensus sequences were prepared for each locus and Basic Local Alignment Search Tool (BLAST) was done using National Center for Biotechnology Information (NCBI) (Johnson et al. 2008).

Review of public data and phylogenetic analysis

Based on the genus wide phylogeny developed by Cannon et al. (2012), gene sequences for Actin (ACT), β-tubulin 2 (TUB2), chitin synthase (CHS-1), the ribosomal internal transcribed spacer (ITS) and 98 different species of *Colletotrichum* were obtained from the National Center for Biotechnology Information (NCBI) (Table1). The sequences were sorted separately for each locus, aligned in Geneious 8.1 (Kearse et al. 2012) using MUSCLE (Edgar 2004), followed by manual editing. The sequences of all the loci for taxa were concatenated and partitioned. The gene boundaries in the alignment were Actin: 1-283, TUB2: 284-806, CHS: 807-1069, and ITS: 1070-1956. Bayesian analysis of partitioned, concatenated alignment of selected genes was carried out using Mr. Bayes 3.2.6 (Ronquist et al. 2012) as implemented in Geneious 8.1 (Kearse et al. 2012). The GTR substitution model was used with gamma rate variation using 4 gamma categories. Markov Chain Monte Carlo (Odencrantz et al. 2000) was used using chain length of 1,100,000 with 4 heated chain. Sub sampling frequency was 1000 and burn-in length was 50,000. *Monilochaetes infuscans* a member of same order Glomerellales was used as the outgroup to provide direction to the evolutionary tree (Cannon et al. 2012). Individual genes were also subjected to Bayesian analysis using same parameters.

Results

Identification of Colletotrichum sequences

BLAST searches of the sequences obtained from the PCR products of four genes ACT, TUB2, CHS, and ITS revealed matches with the species within the *Colletotrichum acutatum* species complex. Actin matched with 93 isolates of *Colletotrichum nympheaeae,* 6 isolates of the strains named *Colletotrichum sp. I*, and 1 individual isolate of *Colletotrichum simmondsii.* Beta tubulin matched with 54 isolates of *C. nympheaeae,* 14 isolates of *C. nymphaeae*, 9 isolates of *C. acutatum*, 7 isolates of *C. carathami*, 3 isolates of *C. scovillei*, and there was 1 hit for *C. laticiphilum and C. fiorinae.* Chitin synthase sequence matched with 153 individuals from *C. acutatum* species complex comprising 80 *C. nymphaeae*, 49 *C. lupini*, 3 *C. chrysanthemi*. Similarly, ITS matched with 153 individuals from the *C. acutatum* species complex that included 42 individuals from *C. nymphaeae*, 33 isolates of *C. acutatum*, 22 isolates of *C. simmondsii*, and 16 isolates of *C. chrysanthemi.*

Phylogenetic analysis

Bayesian analysis of concatenated sequences of the Actin, Beta Tubulin, Chitin Synthase, and ITS sequences from 99 individual species of *Colletotrichum* including the unknown isolate from koa provided a partially resolved phylogenetic tree. The tree rooted to *Monilochaetes infuscans* clearly defined nine clades and a few, undefined individual species and groups. The branch support provided by posterior probability was above 0.9 for each clade. The unknown isolate clustered in the acutatum clade sharing a common node with *C. chrysanthemi* with a posterior probability of 0.9. However, the immediate internal node was not resolved, and it appeared as a polytomy with a posterior probability of 0.55. This soft polytomy comprised of *C. paxtonii, C. simmondsii, C. sloanei, C. brisbense, C. nymphaeae. C. laticiphilum, C. guajavae, C. scovillei, C. indonesiense, C. cosmi,* and *C. walleri.* (Fig. 4). The phylogenetic tree obtained from individual gene trees did not significantly differ from the tree obtained from concatenated gene sequences. In each gene tree, the *A. koa* isolate grouped with the actuatum clade (Fig. 5). The phylogenetic tree from the chitin synthase was more resolved compared to other gene trees and a concatenated tree with posterior probability higher than 0.65 for all outer and inner nodes was found (Fig 5C). Similarly, the branch support in the actin gene tree were greater than 0.85 (Fig 5A).

Morphological analysis

Cultures in potato dextrose agar (PDA) were initially white, then turned cottony after 3 days with grey spores appearing over concentric fungal rings after 5 days. Conidia formed at the tip of salmon-colored sporodochia; thus, the culture showed alternate grey and salmon-colored rings. Much older cultures started showing darker grey rings often covering salmon-colored parts (Fig 6). Cultures grown on synthetic nutrient agar (SNA) did not show any salmon-colored regions, and there were concentric rings with lighter grey and darker grey regions. The conidia observed from both cultures were smooth walled, single celled, colorless, cylindrical with both ends acute resembling the shape of rice grains (Fig-6). Measurements were taken from the culture in SNA. Conidia measurements in length× breadth ranged from 7.12 × 2.94μm to 17.38 × 5.01μm (12.76 ± 0.30 × 4.01± 0.05, n=62). Mycelium were septate and were hyaline (Fig. 3b). No chlamydospores were observed in the cultures.

Discussion

The phylogenetic analysis of 99 strains of *Colletotrichum* including the unknown pathogen, had a similar topology to Cannon et al. (2012) who used 119 species to resolve species relationships in *Colletotrichum*. Here, nine clades were resolved, with the unknown isolate being nested in the actuatum clade. Of the loci tested, the highest resolution was obtained using the chitin synthase locus, where it appeared in a single branch as a sister taxon to the subclade containing *C. chrysanthemi, C. cuscutae, C. lupini, C. tamarilloi, C. melonis, C. limetticola and C. costariscense.* However, on the gene trees from ACT, TUB2 and ITS, our isolate joined a large polytomy containing the species *Simmondsii, paxtonii, brisbanse, nymphaeae, chrysamthemi, guajavae, scovillei, indonesiense, laticiphilum, sloanei, limetticola, lupini, tamarilloi, melonis, cuscutae, costaricense, cosmi, walleri, fiorinae.* Acutatum clade is a monophyletic group containing 29 species closely related to *C. acutatum* (Damm *et* al., 2012). The phylogeny obtained from Bayesian analysis showed similar polytomy that is in monophyly with *C. acutatum.* This suggests that the unknown pathogen should be either one of the species that is present in the polytomy or a new species that shares same ancestor with *C. acutatum.* Either way, it is safe to say the isolate of *Colletotrichum* causing anthracnose in koa in Hamakua Hawaii is a member of acutatum clade in the genus wide phylogeny.

Damm *et* al. (2012) had outlined the internal structure within the subclades by using 331 strains of acutatum complex with the detail of morphological feature of major species. The conidia of our isolate resemble the species *acutatum, australe, brisbanse, cosmi, costaricense, fironiae, cuscutae, guajavae, indonesiense, and johnstonii.* However, the length and breadth of conidia of our isolate was closer to 1 strain of *C. costariscense,* 4 strains of *C. acutatum,* 2 strains of *C. fiorinae,* 4 strains of *C.godetiae, C. guajavae, C. indonesiense, C. laticiphilum , C. lupini, C. simmondsii* and 3 strains of *C. nymphaeae. Colletotrichum chrysanthemi* had resolved as a sister taxon to our isolate in the concatenated tree however, the conidial characteristics are visibly different between two taxa. *Colletotrichum chrysanthemi* is responsible for causing leaf spot and vascular discoloration on *Chrysanthemum coronarium, and* twisted stem in *Carthamus sp.*, have much more ovoid conidia with length by width ratio of 1.7 while the ratio is 3.11 in our strain (Damm et al. 2012). *Colletotrichum simmondsii* overlapped both phylogenetic and morphological parameters used in this study.

Colletotrichum acutatum is a frequently reported pathogen complex across the world. It was first reported in *Carica papaya*, *Capsicum frutesens, and Delphinium ajacis* in Australia by Simmonds (1965). The species complex has prevailed across climatic zones and across different families. It is a major postharvest problem in strawberry by causing anthracnose on fruit (Garrido et al. 2009). *Colletotrichum acutatum* causing anthracnose has been reported on leather fern (Schiller et al. 2006) in Costa Rica. It is also damaging to Pine, citrus, olive and apple. Similarly, one isolate of *C. acutatum* from *Leucaena sp.* was recorded in Damm et al. (2012). The isolate was nested in sub-clade2 among the 5 subclades of *C. acutatum* as a sister taxon to *C. nymphaeae* in the complex. Based on the evidence, the isolate can should be considered a member of the *C. acutatum* complex. Further studies should be done comparing with the taxa studied by (Damm et al. 2012). However, the present study should provide enough information useful for the management of disease.

Conclusion

The position of the newly collected strain in the phylogeny of *Colletotrichum* provides an opportunity to understand the possible causal agent and to prepare a management strategy. Future studies can use this information to understand the mechanism of pathogenesis, the amount of risk posed by the fungus and possible control measures. Further study of morphological features will provide more evidence to refine the identification of the fungus.

The pathogen of strawberry anthracnose is in the same clade as koa anthracnose. Use of Propiconazole, bitertanol, imazalil, and hexaconazole as spray were found effective in controlling strawberry anthracnose; similarly, carbendazim and thiabendazole were also found effective upon dipping (de los Santos García de Paredes et.al, 2002).. Natamycin (Haack et al., 2018) and thymol and carvacrol (Numpaque et al. 2011) were reported effective bio-fungicides for *C. acutatum*. Fungicide use can be effective in the management of anthracnose in koa, since it is unlikely likely to affect human consumers as a food crop. However, careful evaluation of effects of fungicides is necessary in order to prevent collateral damage to the forest microbiota.

Figure 5 Phylogenetic tree derived from a Bayesian analysis of a partitioned, concatenated alignment of ACT (283 bp), TUB2 (523 bp), CHS-1 (263 bp), and ITS (887 bp) sequences, run for 1×107 generations with a GTR model of DNA evolution for each partition

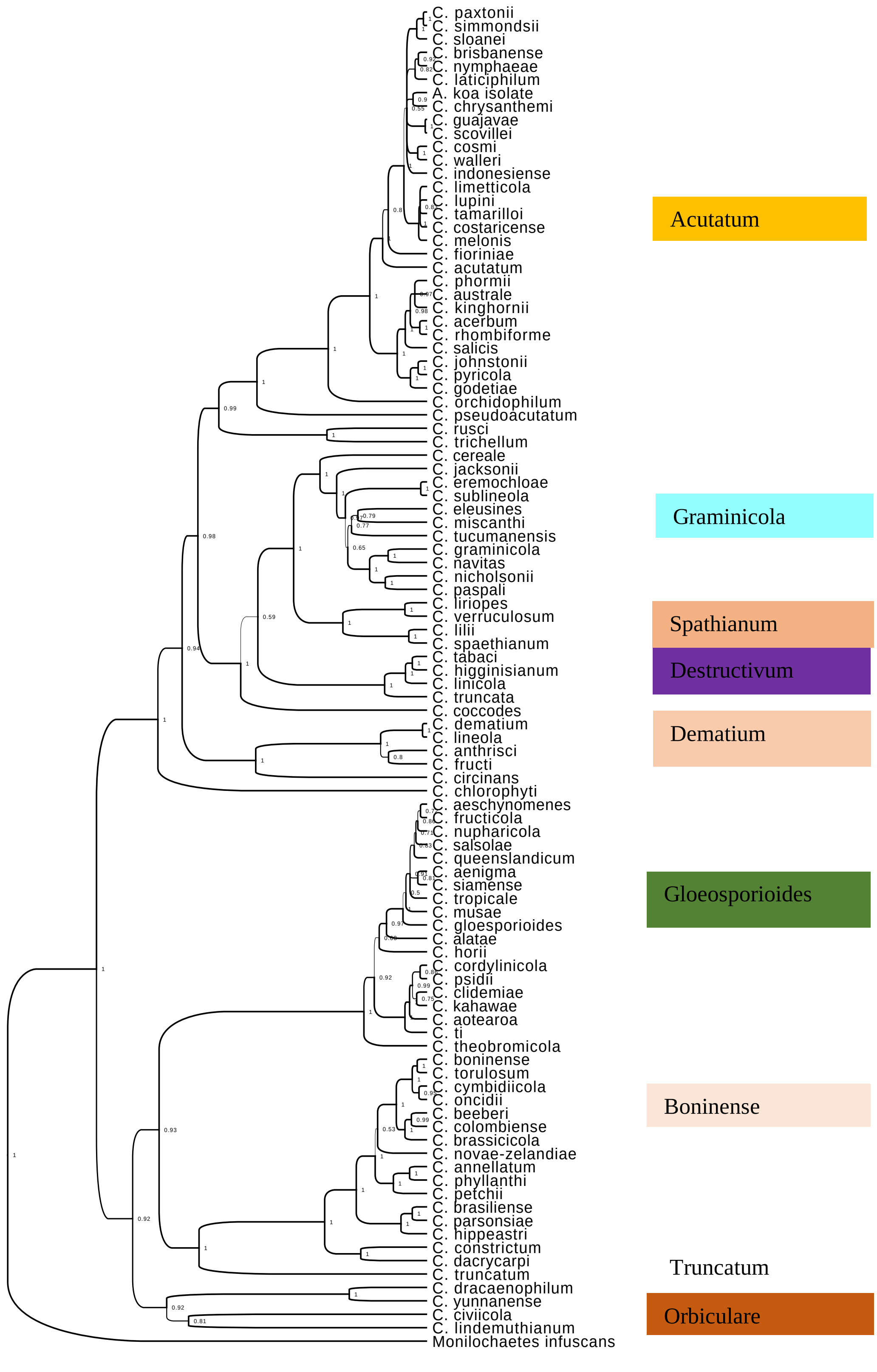


Figure 6. phylogenetic tree derived from consensus obtained from Bayesian analysis of alignments of A. ACT (283 bp), B. TUB2 (523 bp), C. CHS-1 (263 bp), and D. ITS (887 bp) sequences, run for 1×107 generations with a GTR model of DNA evolution for each partition

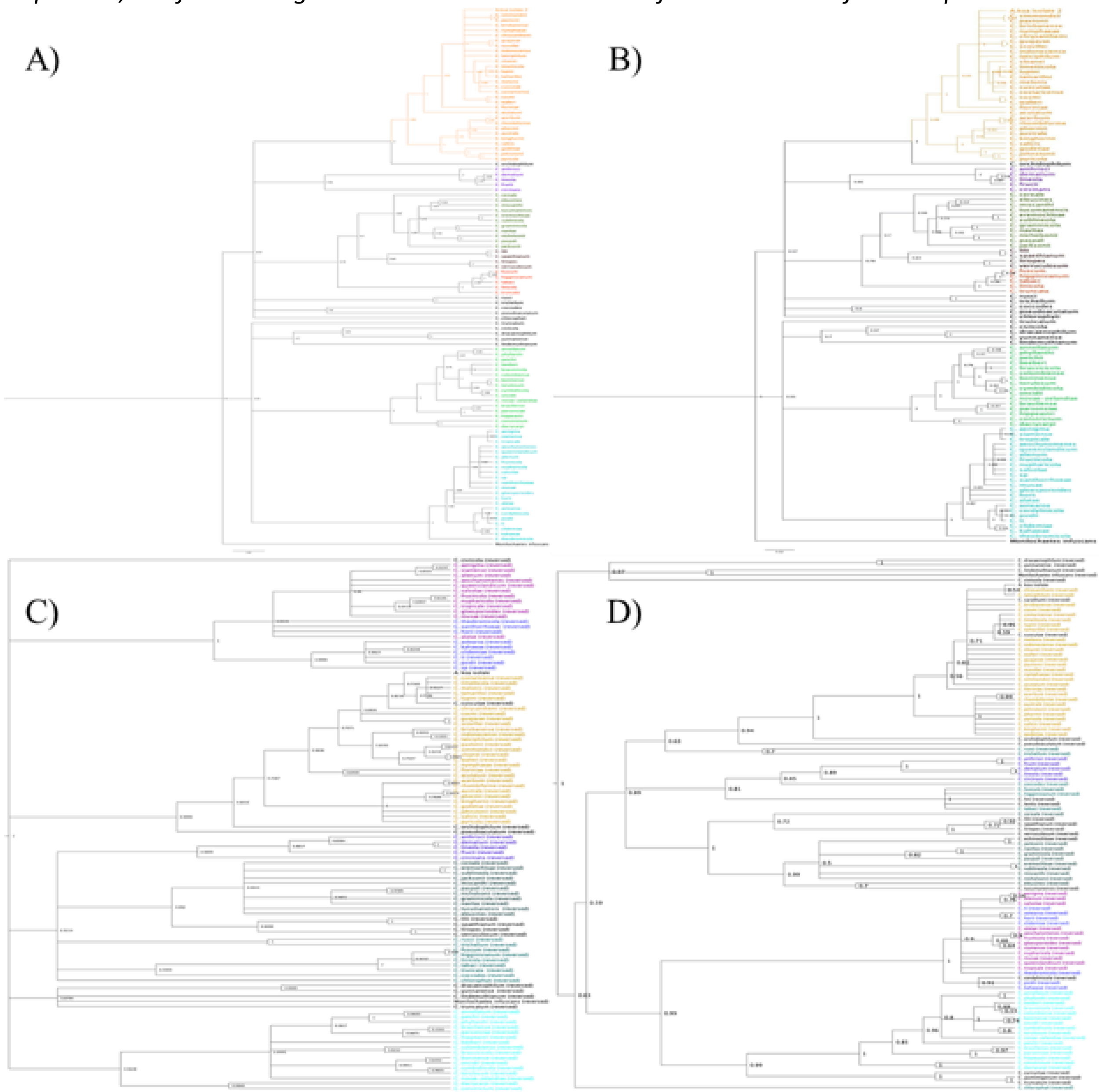


Figure 7 (a)and (c) conidia of fungus obtained from anthracnose symptom in Acacia koa pods under 10X and 25X magnifications respectively ;(b) 2-day old Mycelium of fungus in Acacia koa pods under 10X Magnification; (d) Culture in PDA

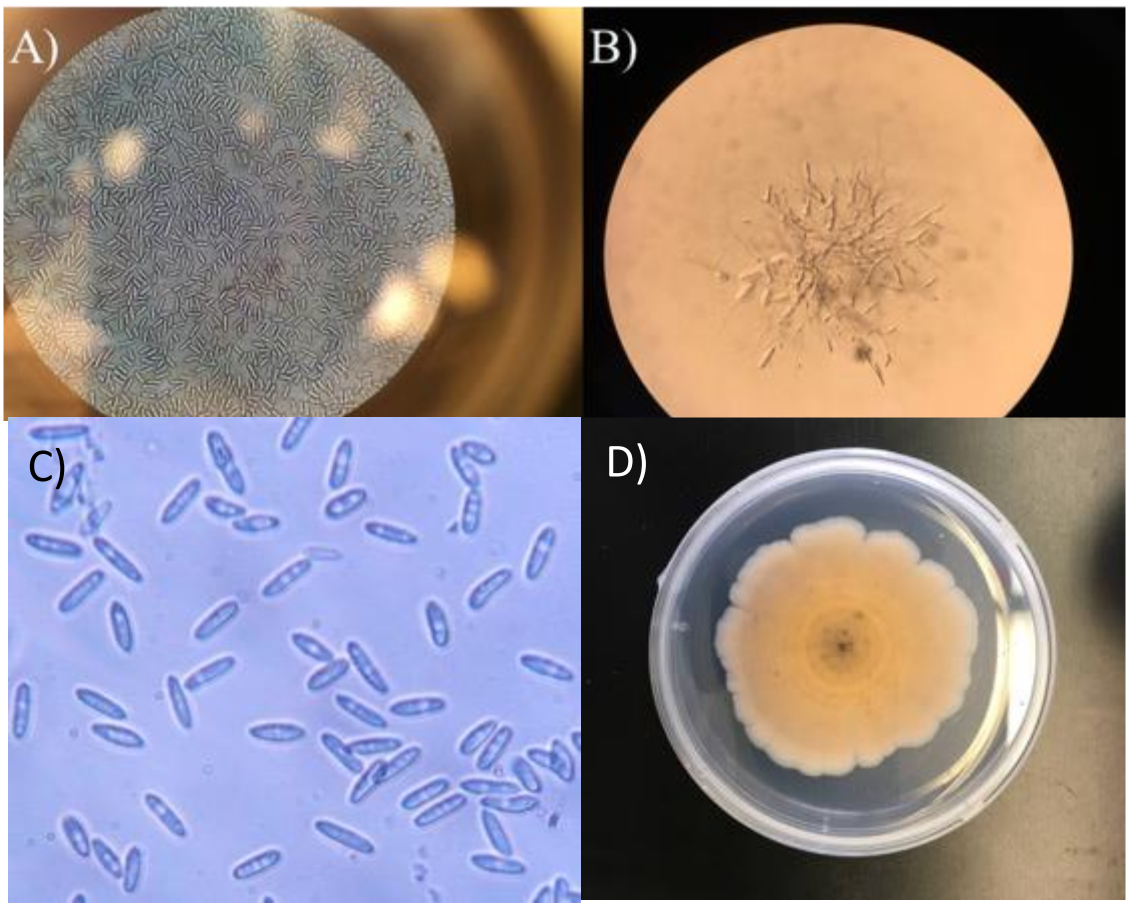


Table 3. Primers used in this study with sequences and sources.

Gene	Product name	Primer	Direction	Sequence (5'-3')	Reference
ACT	Actin	ACT-512F	Forward	ATG TGC AAG GCC GGT TTC GC	Carbone & Kohn 1999
		ACT-783R	Reverse	TAC GAG TCC TTC TGG CCC AT	Carbone & Kohn 1999
TUB2	β-Tubulin 2	T1	Forward	AAC ATG CGT GAG ATT GTA AGT	O'Donnell & Cigelnik 1997
		Bt2b	Reverse	ACC CTC AGT GTA GTG ACC CTT GGC	Glass & Donaldson 1995
CHS	Chitin synthase	CHS-79F	Forward	TGG GGC AAG GAT GCT TGG AAG AAG	Carbone & Kohn 1999
		CHS-345R	Reverse	TGG AAG AAC CAT CTG TGA GAG TTG	Carbone & Kohn 1999
ITS	Internal transcribed spacer	ITS-1F	Forward	CTT GGT CAT TTA GAG GAA GTA A	Gardes & Bruns 1993
		ITS-4	Reverse	TCC TCC GCT TAT TGA TAT GC	Gardes & Bruns 1993

Table 4. Authentic Sequences of Colletotrichum aligned with sequences obtained from the species obtained from koa anthracnose lesions.

Species	Source Material	Status of Source Materials	Gene bank accession number(s)	Reference
C. acerbum	CBS 128530	Culture from holotype(holotype)	ACT: JQ949780ITS: JQ948459; TUB2: JQ950110; CHS-1: JQ949120;	(Damm et al. 2012)
C. acutatum	CBS 112996	Culture from epitype (Epitype)	ITS: JQ005776; TUB2: JQ005860; ACT: JQ005839; CHS-1: JQ005797	(Vinnere et al., 2002)
C. aenigma	ICMP 18608	Holotype	ITS: JX010244; TUB2: JX010389; ACT: JX009443; CHS-1: JX009774;	Weir *et al.* (2012)
C. aeschynomenes	ICMP 17673	Holotype	ITS: JX010176; TUB2: JX010392; ACT: JX009483; CHS-1: JX009799	weir et al. (2012)
C. alatae	CBS 304.67, ICMP 17919	Holotype	ITS: JX010190; TUB2: JX010383; ACT: JX009471; CHS-1: JX009837	Weir et al. (2012)

C. annellatum	CBS 129826	Holotype	ITS: JQ005222; TUB2: JQ005656; ACT: JQ005570; CHS-1: JQ005396	Damm et al. (2012a)
C. anthrisci	CBS 125334	Holotype	ITS: GU227845; TUB2: GU228139; ACT: GU227943; CHS-1: GU228335	Damm et al. (2012a)
C. aotearoa	ICMP 18537	Holotype	ITS: JX010205; TUB2: JX010420; ACT: JX009564; CHS-1: JX009853	Weir et al. (2012)
C. australe	CBS 116478	Holotype	ITS: JQ948455; TUB2: JQ950106; ACT: JQ949776; CHS-1: JQ949116	Damm et al. (2012a)
C. beeveri	CBS 128527	Holotype	ITS: JQ005171; TUB2: JQ005605; ACT: JQ005519; CHS-1: JQ005345	Damm et al. (2012a)
C. boninense	CBS 123755	Holotype	ITS: AB051400, JQ005153; TUB2: JQ005588; ACT:	Moriwaki et al. (2003),

			JQ005501; CHS-1: JQ005327	Damm et al. 2012b
C. brasiliense	CBS 128501	Holotype	ITS: JQ005235; TUB2: JQ005669; ACT: JQ005583; CHS-1: JQ005409	Damm *et al.* (2012b)
C. brassicicola	CBS 101059	Holotype	ITS: JQ005172; TUB2: JQ005606; ACT: JQ005520; CHS-1: JQ005346	Damm *et al.* (2012b)
C. brisbense	CBS 292.67	Holotype	ITS: JQ948291; TUB2: JQ949942; ACT: JQ949612; CHS-1: JQ948952	Damm *et al.* (2012b)
C. cereale	CBS 129663	none	ITS: DQ126177, JQ005774; TUB2: JQ005858; ACT: JQ005837; CHS-1: JQ005795	Crouch *et al.* (2006). O'Connell *et* al. (2012)
C. chlorophyti	IMI 103806	holotype	ITS: GU227894; TUB2: GU228188; ACT: GU227992; CHS-1: GU228384	Damm *et al.* (2009)

C. chrysanthemi	IMI 364540	none	ITS: JQ948273; TUB2: JQ949924; ACT: JQ949594; CHS-1: JQ948934	Damm *et al.* (2012a)
C. circinans	CBS 221.81	epitype	ITS: GU227855; TUB2: GU228149; ACT: GU227953; CHS-1: GU228345	Damm *et al.* (2009), Schoch *et al.* (2012)
C. clidemiae	ICMP 18658	holotype	ITS: JX010265; TUB2: JX010438; ACT: JX009537; CHS-1: JX009877	Weir *et al.* (2012)
C. cliviae	CBS 125375	holotype	ITS: GQ485607, JX519223; TUB2: GQ849440, JX519249; ACT: GQ856777, JX519240; CHS-1: GQ856722	Yang *et al.* (2009), Canon etal
C. coccodes	CBS 369.75	neotype	ITS: HM171679, JQ005775; TUB2: JQ005859; ACT: HM171667, JQ005838; CHS-1: JQ005796	Liu *et al.* (2011), O'Connell *et al.* (2012)

C. colombiense	CBS 129818	holotype	ITS: JQ005174; TUB2: JQ005608; ACT: JQ005522; CHS-1: JQ005348	Damm *et al.* (2012b)
C. constrictum	CBS128504	holotype	ITS: JQ005238; TUB2: JQ005672; ACT: JQ005586; CHS-1: JQ005412	Damm *et al.* (2012b)
C. cordylinicola	ICMP 18579	holotype	ITS: HM470246, JX010226; TUB2: HM470249, JX010440; ACT: HM470234; CHS- 1: JX009864	Phoulivong *et al*. (2010), Weir *et al*. (2012)
C. cosmi	CBS 853.73	holotype	ITS: JQ948274; TUB2: JQ949925; ACT: JQ949595; CHS-1: JQ948935	Damm *et al.* (2012a)
C. costaricense	CBS 330.75	holotype	ITS: JQ948180; TUB2: JQ949831; ACT: JQ949501; CHS-1: JQ948841	Damm *et al.* (2012a)
C. cymbidiicola	IMI 347923	epitype	ITS: JQ005166; TUB2: JQ005600; ACT:	Damm *et al.* (2012b)

			JQ005514; CHS-1: JQ005340	
C. dacrycarpi	CBS 130241	holotype	ITS: JQ005236; TUB2: JQ005670; ACT: JQ005584; CHS-1: JQ005410	Damm *et al.* (2012b)
C. dematium	CBS 125.25	epitype	ITS: GU227819; TUB2: GU228113; ACT: GU227917; CHS-1: GU228309	Damm *et al.* (2009), Schoch *et al.* (2012)
C. dracaenophilum	CBS 118199	holotype	ITS: DQ286209, JX519222; TUB2: JX519247; ACT: JX519238; CHS-1: JX519230	Farr *et al.* (2006), Canon etal
C. elusines	MAFF 511155	epitype	ITS: EU554131, JX519218; TUB2: JX519243; ACT: JX519234; CHS-1: JX519226	Crouch *et al.* (2009c, d), Canon et al
C. eremochloae	CBS 129661	holotype	ITS: JQ478447, JX519220; TUB2: JX519245; ACT:	Moriwaki & Tsukiboshi

			JX519236; CHS-1: JX519228	(2009), Crouch *et al.* (2009c, d)
C. fioriniae	CBS 128517	holotype	ITS: EF464594, JQ948292; TUB2: EF593325, JQ949943; ACT: JQ949613; CHS-1: JQ948953	Marcelino *et al.* (2008), Shivas & Tan (2009), Damm et al. (2012a)
C. fructi	CBS 346.37	epitype	ITS: GU227844; TUB2: GU228138; ACT: GU227942; CHS-1: GU228334	Damm *et al.* (2009)
C. fructicola	CBS 130416	holotype	ITS: FJ972603, JX010165; TUB2: FJ907441, JX010405; ACT: FJ907426; CHS-1: JX009866	Prihastuti *et al.* (2009), Weir *et al.* (2012)

C. gloesporioides	CBS 112999	epitype	ITS: EU371022, JQ005152, JX010152; TUB2: FJ907445, JQ005587, JX010445; ACT: FJ907430, JQ005500, JX009531; CHS-1: JQ005326, JX009818	Damm *et al.* (2012b), Weir et al. (2012)
C. godetiae	CBS 133.44	holotype	ITS: JQ948402; TUB2: JQ950053; ACT: JQ949723; CHS-1: JQ949063	Damm *et al.* (2012a)
C. graminicola	CBS 130836	epitype	ITS: DQ003110, JQ005767; TUB2: JQ005851; ACT: JQ005830; CHS-1: JQ005788	Du *et al.* (2005), Crouch *et al.* (2009 d), O'Connell *et al.* (2012)
C. guajavae	IMI 350839	holotype	ITS: JQ948270; TUB2: JQ949921; ACT:	Damm *et al.* (2012a)

			JQ949591; CHS-1: JQ948931	
C. higginisianum	IMI 349063	none	ITS: JQ005760; TUB2: JQ005844; ACT: JQ005823; CHS-1: JQ005781	O'Connell *et al.* (2012)
C. hippeastri	CBS 125376	holotype	ITS: GQ485599, JQ005231; TUB2: GQ849446, JQ005665; ACT: GQ856788, JQ005579; CHS-1: GQ856725, JQ005405	Yang *et al.* (2009), Damm *et al.* (2012b)
C. horii	ICMP 10492	Culture from neotype	ITS: GQ329690; TUB2: JX010450; ACT: JX009438; CHS-1: JX009752	Weir & Johnston (2010), Weir *et al.* (2012)
C. indonesiense	CBS 127551	holotype	ITS: JQ948288; TUB2: JQ949939; ACT: JQ949609; CHS-1: JQ948949	Damm *et al.* (2012a)
C. jacksonii	MAFF 305460	holotype	ITS: EU554108, JX519216; TUB2:	Crouch *et al.* (2009c,

			JX519241; ACT: JX519233; CHS-1: JX519224	d), this study
C. johnstonii	CBS 128532, ICMP 12926	holotype	ITS: JQ948444; TUB2: JQ950095; ACT: JQ949765; CHS-1: JQ949105	Damm *et al.* (2012a)
C. kahawae	ICMP 18539	holotype	ITS: GU174550, JX010231; TUB2: JX010444; ACT: JX009452; CHS-1: JX009813	Weir *et al.* (2012)
C. kinghornii	CBS 198.35	holotype	ITS: JQ948454; TUB2: JQ950105; ACT: JQ949775; CHS-1: JQ949115	Damm *et al.* (2012a)
C. laticiphilum	CBS 112989	holotype	ITS: JQ948289; TUB2: JQ949940; ACT: JQ949610; CHS-1: JQ948950	Damm *et al.* (2012a)
C. lilii	CBS 109214	morphology congruent with	ITS: GU227810; TUB2: GU228104;	Damm *et al.* (2009)

		original description	ACT: GU227908; CHS-1: GU228300	
C. limetticola	CBS 114.14	epitype	ITS: JQ948193; TUB2: JQ949844; ACT: JQ949514; CHS-1: JQ948854	Damm *et al.* (2012a)
C. lindemuthianum	CBS 144.31	none	ITS: JQ005779; TUB2: JQ005863; ACT: JQ005842; CHS-1: JQ005800	O'Connell *et al.* (2012)
C. lineola	CBS 125337	epitype	ITS: GU227829; TUB2: GU228123; ACT: GU227927; CHS-1: GU228319	Damm *et al.* (2009)
C. linicola	CBS 172.51	none	ITS: JQ005765; TUB2: JQ005849; ACT: JQ005828; CHS-1: JQ005786	O'Connell *et al.* (2012)
C. liriopes	CBS 119444	holotype	ITS: GU227804; TUB2: GU228098; ACT: GU227902; CHS-1: GU228294	Damm *et al.* (2009)

C. lupini	CBS 109225	neotype	ITS: DQ286119, JQ948155; TUB2: JQ949806; ACT: JQ949476; CHS-1: JQ948816	Nirenberg *et al.* (2002), Damm *et al.* (2012a)
C. melonis	CBS 159.84	holotype	ITS: JQ948194; TUB2: JQ949845; ACT: JQ949515; CHS-1: JQ948855	Damm *et al.* (2012a)
C. miscanthi	MAFF 510857	holotype	ITS: EU554121, JX519221; TUB2: JX519246; ACT: JX519237; CHS-1: JX519229	Crouch *et al.* (2009c, d), canon et al
C. musae	CBS 116870	epitype	ITS: HQ596292, JX010146; TUB2: HQ596280; ACT: HQ596284, JX009433; CHS-1: JX009896	Su *et al.* (2011), Weir *et al.* (2012)
C. navitas	CBS 125086	holotype	ITS: GQ919067, JQ005769; TUB2: JQ005853; ACT:	Crouch *et al.* (2009a), O'Connell

			JQ005832; CHS-1: JQ005790	*et al*. (2012)
C. nicholsonii	MAFF 511115	holotype	ITS: EU554126, JQ005770; TUB2: JQ005854; ACT: JQ005833; CHS-1: JQ005791	Crouch *et al.* (2009c, d), O'Connell *et al*. (2012)
C. novar-zenlandiae	CBS 128505	holotype	ITS: JQ005228; TUB2: JQ005662; ACT: JQ005576; CHS-1: JQ005402;	Damm *et al.* (2012b)
C. nupharicola	CBS 470.96	holotype	ITS: JX010187; TUB2: JX010398; ACT: JX009437; CHS-1: JX009835	Weir *et al*. (2012)
C. nymphaeae	CBS 515.78	epitype	ITS: JQ948197; TUB2: JQ949848; ACT: JQ949518; CHS-1: JQ948858	Damm *et al.* (2012a)
c. oncidii	CBS 129828	holotype	ITS: JQ005169; TUB2: JQ005603; ACT:	Damm et al. (2012b)

			JQ005517; CHS-1: JQ005343	
C. orchidophilum	CBS 514.97	holotype	ITS: JQ005778; TUB2: JQ005862; ACT: JQ005841; CHS-1: JQ005799	Damm et al. (2012a)
C. parsonsiae	CBS 128525	holotype	ITS: JQ005233; TUB2: JQ005667; ACT: JQ005581; CHS-1: JQ005407	Damm et al. (2012b)
C. paspali	MAFF 305403	holotype	ITS: EU554100, JX519219; TUB2: JX519244; ACT: JX519235; CHS-1: JX519227	Crouch et al. (2009c, d), Canon et al
C. paxtonii	IMI 165753	holotype	ITS: JQ948285; TUB2: JQ949936; ACT: JQ949606; CHS-1: JQ948946	Damm et al. (2012a)
C. petchii	CBS 378.94	epitype	ITS: JQ005223; TUB2: JQ005657; ACT: JQ005571; CHS-1: JQ005397	Damm et al. (2012b)

C. phormii	CBS 118194	epitype	ITS: DQ286136, JQ948446; TUB2: JQ950097; ACT: JQ949767; CHS-1: JQ949107	Farr et al. (2006), Damm etal 2012
C. phyllanthi	CBS 175.67	holotype	ITS: JQ005221; TUB2: JQ005655; ACT: JQ005569; CHS-1: JQ005395	Damm et al. (2012b)
C. pseudoacitatum	CBS 436.77	holotype	ITS: JQ948480; TUB2: JQ950131; ACT: JQ949801; CHS-1: JQ949141	Damm et al. (2012a)
C. psidii	CBS 145.29, ICMP 19120	Authentic strain	ITS: JX010219; TUB2: JX010443; ACT: JX009515; CHS-1: JX009901	Weir et al. (2012)
C. pyricola	CBS 128531	holotype	ITS: JQ948445; TUB2: JQ950096; ACT: JQ949766; CHS-1: JQ949106	Damm et al. (2012a)
C. queenslandicum	ICMP 1778	epitype	ITS: JX010276; TUB2: JX010414; ACT:	Weir et al. (2012)

			JX009447; CHS-1: JX009899	
C. rhombiforme	CBS 129953	holotype	ITS: JQ948457; TUB2: JQ950108; ACT: JQ949778; CHS-1: JQ949118	Damm et al. (2012a)
C. rusci	CBS 119206	holotype	ITS: GU227818; TUB2: GU228112; ACT: GU227916; CHS-1: GU228308	Damm et al. (2009)
C. salicis	CBS 607.94	epitype	ITS: JQ948460; TUB2: JQ950111; ACT: JQ949781; CHS-1: JQ949121	Damm et al. (2012a)
C. salsolae	ICMP 19051	holotype	ITS: JX010242; TUB2: JX010403; ACT: JX009562; CHS-1: JX009863	Weir et al. (2012)
C. scovillei	CBS 126529	holotype	ITS: JQ948267; TUB2: JQ949918; ACT: JQ949588; CHS-1: JQ948928	Damm et al. (2012a)

C. siamense	CBS 130417	holotype	ITS: FJ972613, JX010171; TUB2: FJ907438, JX010404; ACT: FJ907423; CHS-1: JX009865	Prihastuti et al. (2009), Weir et al. (2012)
C. simmondsii	CBS 122122	holotype	ITS: FJ972601, JQ948276; TUB2: FJ907443, JQ949927; ACT: FJ907428, JQ949597; CHS-1: JQ948937	Shivas and Tan (2009), Damm et al. 2012a
C. sloanei	IMI 364297	holotype	ITS: JQ948287; TUB2: JQ949938; ACT: JQ949608; CHS-1: JQ948948	Damm et al. (2012a)
C. spaethianum	CBS 167.49	epitype	ITS: GU227807; TUB2: GU228101; ACT: GU227905; CHS-1: GU228297	Damm et al. (2009) and Schoch et al. 2012
C. sublineola	CBS 131301	lectotype	ITS: DQ003114, JQ005771; TUB2: JQ005855; ACT:	Crouch & Tomaso-

			JQ005834; CHS-1: JQ005792;	Peterson (2012
C. tabaci	CBS 161.53	none	ITS: JQ005763; TUB2: JQ005847; ACT: JQ005826; CHS-1: JQ005784	O'Connell *et al*. (2012)
C. tamarilloi	CBS 129814	holotype	ITS: JQ948184; TUB2: JQ949835; ACT: JQ949505; CHS-1: JQ948845	Damm *et al.* (2012a)
C. theobromicola	CBS 124945	neotype	ITS: GU994360, JX010294; TUB2: GU994477, JX010447; ACT: JX009444; CHS-1: JX009869	Rojas *et al*. (2010), Weir *et al.* (2012)
C. ti	ICMP 4832	holotype	ITS: JX010269; TUB2: JX010442; ACT: JX009520; CHS-1: JX009898	Weir *et al*. (2012)
C. torulosum	CBS 128544	holotype	; HIS3: GU227997; LSU: JN940815	Damm *et al.* (2012b)
C. trichellum	CBS 217.64	Morphology congruent with	ITS: GU227812; TUB2: GU228106;	Damm *et al.* (2009)

		original description	ACT: GU227910; CHS-1: GU228302	
C. tropicale	CBS 124949	holotype	ITS: GU994331, JX010264; TUB2: GU994454, JX010407; ACT: JX009489; CHS-1: JX009870	Rojas *et al*. (2010), Weir *et al*. (2012)
C. truncata	CBS 151.35	unknown		canon etal
C. truncatum	CBS127604	epitype	ITS: GU227862; TUB2: GU228156; ACT: GU227960; CHS-1: GU228352	Damm *et al.* (2009), Schoch *et al.* (2012)
C. tucumanensis	CBS147945	unknown		canon etal
C. verruculosum	IMI4525	holotype	ITS: JQ005164; TUB2: JQ005598; ACT: JQ005512; CHS-1: JQ005338	Damm *et al.* (2009)
C. walleri	CBS1254472	holotype	ITS: JQ948275; TUB2: JQ949926; ACT: JQ949596; CHS-1: JQ948936	Damm *et al.* (2012a)

C. yunnanense	CBS132135	holotype	ITS: EF369490 ; TUB2: JX519248; ACT: JX519239; CHS-1: JX519231	Liu *et al.* (2007b), canon et al.
Monilochaetes infuscans	CBS869.96	unknown		canon et al.

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