THESIS

WHOLE GENOME ANALYSIS OF THE KOA WILT PATHOGEN (FUSARIUM OXYSPORUM F. SP. KOAE) AND DEVELOPMENT OF MOLECULAR TOOLS FOR EARLY DETECTION AND MONITORING

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ABSTRACT

WHOLE GENOME ANALYSIS OF THE KOA WILT PATHOGEN (FUSARIUM OXYSPORUM F. SP. KOAE) AND DEVELOPMENT OF MOLECULAR TOOLS FOR EARLY DETECTION AND MONITORING

Pathogenic and non-pathogenic *Fusarium oxysporum* are morphologically indistinguishable from each other. Pathogenic *F. oxysporum* f. sp. *koae* (*Fo koae*) is a limiting factor for low to mid elevation, below 610m (2000 ft), *Acacia koa* forests and timber stands. These warmer, lower elevation sites are best suited for optimal growth of *Acacia koa*, but *Fo koae* hinders efforts to establish stands at these elevations. Detection of pathogenic isolates is necessary for informing land managers and disease resistance breeding programs. Current methods to distinguish pathogenic isolates are conducted through costly, extensive greenhouse virulence assays. Genomic comparisons of pathogens and non-pathogens such as amplified fragment length polymorphisms (AFLPs), single nucleotide polymorphisms (SNPs), Random Amplification of Polymorphic DNA (RAPDs), pathogenicity-related genes, and microsatellites have been effective for detecting pathogens, and these genomic comparisons are more time and cost effective than traditional greenhouse virulence assays.

Chapter two of this thesis examined whole genomic comparisons of a pathogenic *F. oxysporum* f. sp. *koae* (*Fo koae* 44) and a *F. oxysporum* (*Fo* 170) isolate found to be non-pathogenic to *Acacia koa*, for the identification of genomic features that could be used to distinguish pathogenicity. Genome sizes were comparable at 48Mb and 50Mb, respectfully. *Fo*

koae 44 and *Fo* 170 shared an average nucleotide identity of 96%. Eleven syntenic putative core chromosomes and one unique putative lineage-specific chromosome were identified when compared to a reference strain of *F. oxysporum* f. sp. *lycopersici*. Pathogenicity-related genes, including the secreted in xylem (*SIX*) genes, *Fusarium* transcription factors, and *Fusarium* transporters, and unique sequences were identified as exclusive to *Fo koae* 44 when compared to *Fo* 170. These variants were used to develop pathogen-specific primers. When tested on previously characterized pathogenic (highly and moderate virulence) *Fo koae* and low virulent or non-pathogenic *Fo* isolates, six primers only amplified moderate and highly virulent isolates of *Fo koae*. Haplotype networks were constructed based on sequencing data of previously characterized *Fo koae* and *Fo* isolates and field collected isolates with no pathogenicity data at the translation elongation factor 1-α and RNA polymerase II second largest subunit to determine the genetic relationships of these two groups. Some field collected isolates grouped with highly virulent *Fo koae* isolates. These results suggested that these field collected isolates might be highly virulent and contain the putative lineage-specific chromosome.

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Island endemic plant species are some of the most susceptible to extinction events due to the loss of genetic diversity (Hamabata et al., 2019). Most island species originated from founder populations of a single to a few individuals that arrived on island by wind, sea, or wings creating a genetic bottleneck (reduced genetic diversity) for the population (Ziegler, 2002). Due to the geographic isolation and natural barriers of most islands, these founder events are rare and result in small, inbreeding, largely homozygous populations that require mutations and immigration to contribute new genotypes into the population (Templeton, 2006). The low genetic diversity observed in these populations make them more susceptible to introduced pests and pathogens due to the lack of gene flow that is necessary for evolution of resistance to introduced diseases (Templeton, 2006). The spread of disease, in turn, results in further loss in genetic diversity often associated with decreased fitness in individuals and populations (Hamabata et al., 2019), thereby increasing the chances of local extirpation due to increased homozygosity and inbreeding. This loss of diversity also hinders island plant populations from being able to respond to abiotic and biotic challenges, particularly during a period of rapid ecological/climatic changes (Hamabata et al., 2019). For this reason, many endemic species on islands, such as the Hawaiian Islands, are threatened, endangered, or already extinct.

1.2 *Acacia koa* distribution and phenotypic variation

Acacia koa Gray (koa) is a tropical hardwood tree endemic to the Hawaiian Islands and is found on all major islands except Ni'ihau and Kaho'olawe. The distribution of koa is driven by

temperature and moisture, occupying wetter and cooler environments (Little & Skolmen, 1989). Koa is morphologically similar to the diploid *A. melanoxylon* from Australia that is thought to be the ancestral taxon of both tetraploid species, *A. koa* of Hawai'i and *A. heterophylla* of Réunion Island, Africa. Recent evidence from Le Roux et al. (2014) has suggested that *A. heterophylla* dispersed from the Hawaiian Islands to Réunion Island, as the two species are morphologically indistinguishable. *Acacia koaia* is another very closely related species that is also endemic to Hawai'i. However, *A. koa* and *A. koaia* are morphologically distinct with differences including, seed arrangement in pods, growth habit, and their respective ecological niches (Adamski et al., 2012). *Acacia koaia* is found in lowland, dry, and open woodland environments on the western slopes (leeward side) of the Hawaiian Islands. Where the dry open woodland and the wet mesic forests meet, hybridization of *A. koa* and *A. koaia* has occurred to form intermediates. All are susceptible to the same pests and pathogens including the fungal pathogen *Fusarium oxysporum,* but it has been suggested that *A. koaia* is more resistant to the Chinese rose beetle and more suited for drier climates (Elevitch et al., 2006).

1.3 *Acacia koa*'s ecological, economic, and cultural importance

Koa is of great importance to Hawai'i's forests and its people. Koa is the second most abundant tree in Hawai'i's forests growing up to 35 meters tall and serving as the dominant canopy species in over 80,000 acres (32,375 ha.) of forest (Staples & Herbst, 2005; Baker et al., 2009). Many of Hawai'i's native birds [(including the endangered Hawaiian honeycreeper, 'akiapōlā'au (*Hemignathus munroi*)], insects, and understory plants rely on koa for food and shelter (Elevitch et al., 2006). Koa is a legumous tree that forms an association with nitrogen-fixing bacteria in the genus *Bradyrhizobium* in both its roots and phyllodes (Leary et al., 2004).

This increases available soil nitrogen that contributes to the forest's overall health. Koa is considered a pioneer species because of its ability to grow quickly after a disturbance (Elevitch et al., 2006; Staples & Herbst, 2005). This adaptation makes it an ideal candidate for cultivation in Hawai'i's timber industry.

Koa was once used as firewood until its timber value was recognized and since has been used to produce high-end furniture, surfboards, jewelry, guitars, and ukuleles (Elevitch et al., 2006). The wood can be blonde to dark reddish-brown and some variants have curly figure (Dudley & Yamasaki, 2000). Koa wood has been valued as high as $150 per board foot (Baker et al., 2009). Koa wood harvesting contributed $30 million to Hawai'i's forestry industry in 2001 (Yanagida et al., 2004).

Historically, native Hawaiians used koa leaves and ashes medicinally and for the construction of spears, tools, paddles (hoe), ceremonial poles (hulumanu), religious ceremonies, single-hulled outrigger canoes (kaukahi), and their double hulled outrigger canoes (kaulua) (Ziegler, 2002; Elevitch et al., 2006). To make the outrigger canoes, single-trunked, straight trees were required. This artificial selection caused the morphological change from these tall, straight, single-trunked trees to the multi-trunked, spindly trees that are observed today (Ziegler, 2002). This has increased interest in selective breeding programs to develop good form koa for timber and restoration efforts, but diseases and insects have impeded this process.

1.4 *Acacia koa* diseases and pests

Anthropogenic threats in combination with diseases and pests have been important factors that have reduced koa populations. Many fungal diseases have been described on koa. Soil borne fungi have primarily been described as the major contributors to mortality in older

stands either as primary or secondary pathogens (Gardner, 1996). Some noted root pathogens that have been identified in koa stands are *Armillaria gallica* Marxmüller & Romagnesi, *Phytophthora cinnamomi* Rands, *Calonectria crotalariae* (Loos) Bell & Sobers, and *Diatrype princeps* Penz. & Sacc., (Kim et al., 2017; Gardner, 1996). *Fusarium* spp., *Ganoderma* spp. and *Pythium* spp. have also been observed on koa (James et al., 2007; Gardner, 1996). Koa leaf rust, caused by five fungal species, *Atleocauda koae* (Arth.) Cumm. & Y. Hiratsuka, *Atleocauda digitata* (Wint.) Cumm. & Y. Hiratsuka, *Ateleocauda angustiphylloda* Gardner, *Endoraecium acaciae* Hodges & Gardner, and *Endoraecium hawaiiense* Hodges & Gardner, cause distinct symptoms on either the phyllodes or new leaf and branch tissue but are not considered major threats (Nelson, 2009; Scholler & Aime, 2006; Gardner, 1996). Some other fungi that have been observed in koa stands are *Phellinus kawakamii* Larsen, Lombard, & Hodges, *Phaeolus schweinitzii* Fr., *Laetiporus sulphureus* Bull. ex Fr., and *Pleurotus ostreatus* (Jacq.: Fr.) Kumm. (Gardner,1996). There have also been several insects identified as contributing to koa mortality.

The black twig borer (*Xylosandrus compactus* Eichoff) is an ambrosia beetle that has been associated with *Fusarium solani* (Mart.) Sacc. infection in branches and seedlings (Daehler & Dudley, 2002). They have been observed contributing to a loss of vigor in adult trees and killing seedlings that were hollowed out by the beetle's construction of a maturation chamber for their larvae (Burbano et al., 2012). The koa seed worm (*Cryptophlebia illepida* Butler) and koa seed weevils (*Aracerus levipennis* & *Stator* spp.) can limit regeneration potential through seed predation (Ishihara et al., 2017). The koa looper (*Scotorythra paludicola* Butler) has the potential to defoliate and even kill trees during outbreaks such as the outbreaks described in Haines et al. (2009). The acacia psyllid (*Acizzia uncatoides* Ferris & Klyver) can infest new growth and

reduce vigor and alter tree morphology (Friday, 2010). Some invasive animals have also been identified as contributors to koa mortality.

On land where sugarcane was previously planted, root-knot nematodes (*Meloidogyne* spp.) often cause growth issues in the rhizome of koa (Baker et al., 2009; Friday, 2010). Wild boars (*Sus scrofa*) uproot seedlings and damage roots (Baker et al., 2009; Friday, 2010). Rats (*Rattus rattus*) strip the bark from branches and cause girdling leading to partial dieback of the trees (Baker et al., 2009; Friday, 2010). Cattle, goats, sheep, and deer trample small seedlings and browse on low hanging branches and strip the bark of young trees and saplings (Baker et al., 2009; Friday, 2010).

Overharvesting, land clearing, and introduced diseases and pests have caused lowland forests to become almost devoid of these iconic trees. There has been increased interest in protecting koa from invasive pests and pathogens for restoration and timber. *Fusarium oxysporum* f. sp. *koae* was identified as one of these potentially invasive pathogens that is a threat to koa populations (Gardner, 1980).

1.5 Koa Wilt Disease

Koa wilt, caused by *Fusarium oxysporum* f. sp. *koae* (*Fo koae*) (Gardner, 1980), is a vascular wilt disease isolated to the Hawaiian archipelago due to the endemism of its plant host, *Acacia koa*. The symptomology, host resistance, and stand management practices have been studied but knowledge gaps exist in understanding the mechanisms of disease development (Anderson et al., 2002; James, 2005; Dudley et al., 2007; Baker et al., 2009). *Fo koae* causes disease symptoms similarly as documented in other *Fusarium oxysporum* in that it causes blockages in the xylem and prevents water transport from the roots to the leaves of the trees. *Fo*

koae affects all age classes from seedlings to adult trees. In adult trees, this results in an observed dieback that can start in segments of the crown and as the pathogen transports throughout the tree, total crown dieback can occur in susceptible trees (Anderson et al., 2002). In susceptible seedlings, rapid wilting symptoms are observed including leaflet drop, early loss of lower leaves, and shepherd's crook (Dudley et al., 2015). *Fo koae* can devastate susceptible stands due to the loss of dominant canopy trees as well as regeneration. Other *Fusarium* species have been tested for pathogenicity on koa.

Twelve *Fusarium* species have been screened for pathogenicity on koa to determine if the causal agent of the disease could be associated with a disease complex (James, 2005; Dudley et al., 2007). *Fusarium subglutinans* and *F. solani* were found to be weakly virulent to koa primarily as root rots (James, 2005). *Fusarium semitectum* was found to be moderately virulent to koa seedlings (Dudley et al., 2007). These results emphasized that *F. oxysporum* was the causal agent of koa wilt.

The Hawai'i Agriculture Research Center (HARC) and the University of Hawai'i at Mānoa (UH) have been developing disease resistant koa seedlings through greenhouse screening trials confirmed through field validation (Dudley et al., 2015; Shi & Brewbaker, 2004). HARC and UH have collected germplasm from healthy trees in high and low disease pressure sites from across the Hawaiian Islands. These seeds from natural koa stands have shown a spectrum of resistance to koa wilt. HARC, College of Tropical Agriculture and Human Resources at the University of Hawai'i at Mānoa, USDA and Hawai'i DOFAW have used the information from these disease screening and validation trials and site surveys to provide management guidelines to mitigate loss of koa from timber and reforestation sites (Friday, 2010; Baker, Scowcroft, & Ewel, 2009; Dudley & Yamasaki, 2000; Baker & Scowcroft, 2005; Dudley et al., 2010). Some

recent molecular analyses have been conducted to better understand koa wilt resistance and the pathogen populations.

Microsatellite markers and resistance genes have been used to differentiate disease resistant koa populations without the need for lengthy greenhouse screening and validation trials (Fredua-Agyeman et al., 2008; Rushanaedy et al., 2012). Microsatellite markers were developed that could identify populations of koa on four of the major Hawaiian Islands. They identified that the koa populations in plantations on Kauai were genetically distinct when compare to populations on Oahu, Maui, and Hawai'i. The within island variation was attributed to artificial seed selection within islands. This information could be useful in identifying resistant populations and for diversifying genotypes in plantations (Fredua-Agyeman et al., 2008). Chitinase genes *Akchit 1a* and *1b* were found to be indicators of resistance because they were upregulated in response to the presence of the pathogen in resistant seedlings (Rushanaedy et al., 2012). Amplified fragment length polymorphism (AFLP) and vegetative compatibility group (VCG) analysis found the *Fo koae* pathogen were all in the same VCG group with support from the AFLP phylogenetic analysis (Shiraishi et al., 2012). Kim et al. (2015) conducted phylogenetic analysis on known pathogenic *Fo koae* and non-pathogenic *F. oxysporum* isolates and found that the pathogenic isolates of *Fo koae* clustered into a well-supported clade. This genetic clustering suggests that unique genomic features may exist among pathogenic *Fo koae* that could be used to develop molecular tools to distinguish morphologically indistinguishable pathogenic *Fo koae* from non-pathogenic *F. oxysporum* isolates.

1.6 *Fusaria*

Fusarium is a fungal genus of worldwide economic and ecological importance due to the broad host range and the diversity of environments that the various species of this genus can inhabit (Ma et al., 2013). The observed plasticity of this genus has been attributed to variation in its morphological, cultural, and physiological characteristics (Nelson et al., 1994). Members of this genus have been found living endophytically, epiphytically, or in the soil around almost every plant on the planet (Leslie & Summerell, 2006). *Fusarium* species have been described as plant pathogens, saprophytes, and even biocontrols (Fravel et al., 2002; Panina et al., 2007; Thongkamngam & Jaenaksorn, 2017). There are more than 300 species of phylogenetically distinct *Fusarium*, and this number continues to increase as advances in molecular diagnostic tools identify new host-specific species (Balajee et al., 2009; O'Donnell et al., 2015). *Fusaria* are noted plant pathogens and can cause root or stem rots, cankers, wilts, fruit or seed rots, and foliar diseases (Rana et al., 2017). Some noted species are *F. graminearum* Schwabe, *F. verticillioides* (Sacc.) Nirenberg, *F. solani* (Mart.) Appel & Wollenw. emend. Snyder & Hansen, and *F. oxysporum* (Schlechtend, Fr.) emend. Snyder & Hansen.

The most important of these species, in terms of impact and abundance, is *F. oxysporum* (*Fo*) (Mehrabi et al., 2011). *Fo* is a cosmopolitan fungus that can exist as both an innocuous soil saprophyte as well as an important vascular wilt pathogen in agriculture fields, nurseries, and forests infecting major crops and some valuable tree species (James, 2004; Ma et al., 2010; Ma et al., 2013). There have been over 100 described formae speciales that are putatively host-specific (Baayan et al., 2000; O'Donnell et al., 2009; Ma et al., 2013; Gordon, 2017). The observed host-specificity suggests there are significant genetic differences among formae speciales.

1.7 *Fusaria* phylogenetics

Morphology has proven unreliable to characterize *Fusarium* species due to diagnostic features. For example, polyphialides, main morphological feature used to distinguish *Fo* from another closely related species, *F. commune*, are rarely observed in culture making pathogenic and non-pathogenic isolates morphologically indistinguishable (Stewart et al., 2006; Stewart et al., 2012). Molecular phylogenetic analysis has shown to be a much more reliable method of classification of *Fusarium* spp. Single- and multi-locus phylogenies have been used to analyze evolutionary origins of pathogenicity factors and classify species, formae speciales, and races (Suga & Hyakumachi, 2004). Multiple conserved regions of the genome, primarily ribosomal genes, have been used to achieve this, such as loci including the translation elongation factor 1-α, RNA polymerase II second largest subunit, mitochondrial small subunit, large subunit ribosomal ribonucleic acid, 18S ribosomal RNA, internal transcribed spacer, and the ribosomal intergenic spacer region have been commonly used (White et al., 1990; O'Donnell et al., 1998; O'Donnell et al., 2004; O'Donnell et al., 2009). Shared virulence-associated genes have also been used to characterize the evolutionary history of this group of pathogens (Taylor et al., 2016; Sperschneider et al., 2015; van Dam et al., 2018).

1.8 *Fusarium oxysporum* biology and distribution

Fusarium oxysporum is found on every continent and across the Pacific Islands and in every climate from the equator to the Arctic (Rana et al., 2017). *Fo* has travelled across the globe easily due to its spores in soil or plant debris that can easily disperse through wind, water, and transportation via equipment, vehicles, animals, and people (Summerell et al., 2010). Most *Fo* formae speciales can exist as soil saprophytes feeding on organic matter in the soil. In their native environments, many *Fo* formae speciales have not been observed as being pathogenic on

native plant communities (Gordon & Martyn, 1997; Gordon, 2017). Changing climates that are more favorable to the pathogen and stress the host can influence the susceptibility of that host to disease (Summerell et al., 2010; Gautam et al., 2013).

Fo is a soil-borne pathogen that infects its plant host through the roots and transports through the plant through the xylem (Agrios, 2005; Pietro et al., 2003; Olivain & Alabouvette, 1999). Once in the main stem, it elicits defense responses in the host that ultimately cause blockages in the xylem. These blockages, caused by tyloses and the mycelium or spores from the fungus itself, prevent water transport from the roots to the leaves and cause the plant host to exhibit wilting symptoms (Pietro et al., 2003; Olivain & Alabouvette, 1999). *Fo* is a necrotrophic pathogen that actively kills its host to feed on the dead tissue (Gordon, 2017). Initial infection is usually asymptomatic but later staining from necrosis of the xylem vessels is observed (Ma et al., 2013).

Unlike other *Fusaria*, *Fo* does not have an observed sexual or teleomorph stage (Baayan et al., 2000). *Fo* exists as the asexual, anamorph stage and the observed genetic diversity between formae speciales has been attributed to horizontal gene and chromosome transfer during anastomoses (fusion of hyphae between itself or other individuals) and heterokaryon formation (multinucleate cells formed by the fusion of two genetically different cells) between compatible formae speciales (Mehrabi et al., 2011; Ma et al., 2010). Compatibility between formae speciales has been described as vegetative compatibility groups (VCGs), in which individuals can form anastomoses with others that are compatible with that VCG (Edel-Hermann & Lecomte, 2019). Horizontal gene and chromosome transfer are the predominant methods for the observed genetic diversity between different strains of *Fo* (Mehrabi et al., 2011; Ma et al., 2010). Transposons and

repetitive elements have also been described as possible mechanisms of creating genetic diversity (Rep & Kistler, 2010; Daboussi & Langin, 1994).

1.9 *Fusarium oxysporum* host specificity and genetic diversity

There have been 106 well-characterized *Fo* formae speciales (Edel-Hermann & Lecomte, 2019). It has been well documented that strains of *Fo* exhibit high host specificity (Edel-Hermann & Lecomte, 2019; van Dam et al., 2017; Ma et al., 2013). The forma speciales designation denotes that that strain causes disease on the specific host it is named for, i.e. banana (*Musa* spp.) is infected by *F. oxysporum* f. sp. *cubense*, gladiolus (*Gladiolus* spp.) is infected by *F. oxysporum* f. sp. *gladioli*, tomato (*Lycopersicon* spp.) is infected by *F. oxysporum* f. sp. *lycopersici*, muskmelon (*Cucumis spp.*) is infected by *F. oxysporum* f. sp. *melonis*, and mimosa (*Albizia julibrissin*) is infected by *F. oxysporum* f. sp. *perniciosum*, etc. (Edel-Hermann & Lecomte, 2019). *Fo* formae speciales with a prefix of radicis- indicates that that *Fo* produces a form of rot rather than wilting symptoms (Edel-Hermann & Lecomte, 2019). Unique sets of virulence genes have been found among different formae speciales and have been attributed to the observed host specificity (van Dam et al., 2016). These virulence factors have been identified on a varied number of conditionally dispensable, accessory chromosomes (Han et al., 2001; Ma et al., 2010; van der Does et al., 2016).

Fo exhibits aneuploidy and has been identified as having a wide range of accessory chromosomes that have been found to play a significant role in pathogenicity (Kistler, 1997). The ancestral lineage of *Fusarium* has been theorized to have had 11 chromosomes (van Dam et al., 2017; Ma et al., 2013). Between four and 15 chromosomes have been observed in different *Fusarium* spp. (Ma et al., 2010). The core genomic chromosomes in *F. oxysporum* contain

conserved genes responsible for normal cell function and growth (Ma et al., 2013). Studies have observed the transfer of accessory chromosomes through karyotyping, using contour-clamped homogeneous electric field (CHEF) electrophoretic karyotyping, and southern analysis (van Dam et al., 2017). These accessory chromosomes have been found to be enriched with pathogenicity-related genes (Han et al., 2001).

1.10 *Fusarium oxysporum* pathogenicity-related genes

Fo develops a molecular arsenal to break down host defenses and/or remain undetected by the plant host, while the host develops compounds to detect and defend against the invading pathogen (Takken & Rep, 2011; van der Does et al., 2016; Ma et al., 2013). Current studies have been investigating these gene-for-gene interactions to better inform breeding programs (Takken & Rep, 2011; van der Does et al., 2016). Many *Fo* formae speciales specific proteins and secondary metabolites have been characterized.

Fo formae speciales host-specificity has been attributed to the variation in pathogenicity-related proteins and secondary metabolites (van Dam et al., 2017). The genes involved in production of shared pathogenicity related proteins and secondary metabolites have shown nucleotide variation between formae speciales and have even been used to distinguish pathogens from non-pathogens (van Dam et al., 2018; Suga et al., 2013). In these comparisons, many genes have been found to be exclusive to the pathogen but only a few have been described as important for virulence.

Secondary metabolites are primarily produced from nonribosomal peptide synthetases, polyketide synthases, or terpene synthases in *Fo* (Ma et al., 2013). Carbohydrate active-enzymes (CAZymes) are cell wall degrading enzymes that pathogens use to break down host cells.

Hydrate esterase and pectate lyases have been noted as being important for pathogenicity (Rana, et al., 2017). Oxylipin jasmonic acids are signaling molecules used by the pathogen (that the plant also produces) to manipulate the host lipid metabolism to alter its defense response (Thatcher et al., 2009). Accompanying this pathway is the production of reactive oxygen species by the pathogens nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes that can control transcription factors or aids the pathogen in overcoming host defenses (Rana et al., 2017). Virulence factors such as the secreted in xylem (*SIX*) and host specific mycotoxins play significant roles in the host specificity of the *Fo* forma specialis (Rana et al., 2017). *SIX* genes code for effectors that have been shown through gene knock-out experiments to be involved in functions necessary for disease development or avoiding plant host detection (Meldrum & Fraser-Smith, 2012). There are multiple signaling pathways that are used by the pathogen but mitogen-activated protein kinase (MAPK) cascades have been noted for their importance for virulence (Rana et al., 2017). MAPK have been associated with downstream transcription factors (Rispail & Pietro, 2009). Fusarium transcription factors (*Ftf*) genes are important for vital functions of growth, stress responses, virulence and toxin production (van der Does et al., 2016; Sutherland et al., 2013; Ramos et al., 2007). Cytochrome P450 monooxygenases are involved in the production of host specific toxins (van Bogaert et al., 2010). ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters are important for persisting in the host and transporting toxins produced by the pathogen or host, out of the cell (Deng et al., 2017).

These host-specific pathogenicity-related proteins are important factors that distinguish pathogens from non-pathogens. These unique sequences are prime targets for the development of pathogen-specific probes that allow for rapid detection and monitoring of the pathogen. These

probes are useful in disease resistance breeding programs and informing land managers for timber or reforestation efforts.

1.11 Objectives for this work

The goal of this thesis is to identify genomic features that can be used to distinguish pathogenic from non-pathogenic isolates of *Fusarium* spp. that associated with koa wilt disease on *Acacia koa*. *Fusarium oxysporum* isolates can be pathogenic or non-pathogenic without any discernable morphological differences. Molecular characteristics can be evaluated to identify these unique genes, that may be vital for disease development, that can be used to differentiate pathogens from non-pathogens. This thesis shows that *F. oxysporum* f. sp. *koae* (*Fo koae*) can be differentiated from non-pathogenic *F. oxysporum* (*Fo*) through analysis of whole genomic sequences. We sequenced and annotated the genomes of one pathogenic isolate of *Fo koae* and one non-pathogenic *F. oxysporum* isolate. Comparison of genomic regions identified pathogen-specific gene sequences and a putative lineage-specific chromosome. This comparison led to the development of pathogen-specific primers that distinguish highly virulent pathogens of *Fo koae* from non-pathogens of *Fo*.

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CHAPTER 2: WHOLE GENOME ANALYSIS OF THE KOA WILT PATHOGEN (FUSARIUM OXYSPORUM F. SP. KOAE) AND DEVELOPMENT OF MOLECULAR TOOLS FOR EARLY DETECTION AND MONITORING

2.1 Preface

This study identified virulence-associated genes that may be used to distinguish highly virulent isolates of *Fusarium oxysporum* f. sp. *koae* (*Fo koae*), which causes koa wilt disease on *Acacia koa* (koa). Whole genome analyses of one highly virulent *Fo koae* isolate and one non-pathogenic *F. oxysporum* (*Fo*) isolate, allowed for the identification of a putative lineage-specific chromosome and predicted genes necessary for disease development on *A. koa*. Using putative chromosome sequences and predicted gene comparisons, *Fo koae* exclusive virulence genes were identified. The identification of a putative lineage-specific chromosome and the secreted in xylem genes (e.g., *SIX1* and *SIX6*) on this chromosome, may be necessary for disease development on *A. koa*. Unique genes from *Fo koae* were used to develop pathogen-specific PCR primers. These diagnostic primers amplified the characterized pathogenic *Fo koae* isolates but did not amplify low virulence or non-pathogenic isolates of *Fo*. The primers developed in this study will be useful for early detection and monitoring of highly virulent strains of *Fo koae*. This is important to disease resistance breeding programs that require highly virulent *Fo koae* isolates for their disease screening assays for the development of resistant *A. koa*.

2.2 Introduction

Genomic comparisons between pathogenic and non-pathogenic fungal isolates can elucidate differences that may be key factors required for disease development in plant hosts

(Rep & Kistler, 2010; Sperschneider et al., 2015). These factors include unique genes that are not only vital to the pathogen but allow for the differentiation of pathogens and non-pathogens for detection and monitoring. For example, pathogenic fungi are classified into formae speciales (forma specialis singular) of *Fusarium oxysporum* that exhibit high host-specificity that has been attributed to effector profiles (van Dam et al., 2017). Variation in genome size, predicted effectors, and unique gene sequences have been used to differentiate plant pathogens from saprophytic non-pathogens (Bao et al., 2002; Brader et al., 2017; Chakrabati et al., 2011; Guo et al., 2014; Laurence et al., 2015). Just as the host exhibits variation in resistance, pathogenic fungi also exhibit variation in degrees of virulence (Bao et al., 2002; Brader et al., 2017; Chakrabati et al., 2011; Guo et al., 2014; Laurence et al., 2015).

Pathogenic isolates of *F. oxysporum* have been divided into a diverse group of formae speciales that exhibit high levels of plasticity (Raffaele & Kamoun, 2012). This characterization has been used to describe differences in host specificity and pathogenicity among *F. oxysporum* pathogens (van Dam et al., 2016). The observed host-specificity within formae speciales of *F. oxysporum* is driven by differences in genes that allow for the progression of disease development or evade plant host detection (Rep & Kistler, 2010; Sperschneider et al., 2015). For *F. oxysporum*, genes involved in virulence, like effectors, that are necessary for disease development are housed on one to many conditionally dispensable, lineage-specific chromosome(s). These chromosome(s) are repeat- and transposon-rich, which aids in the ability to create effector diversity without sexual recombination (Presti et al., 2015). Comparisons of these virulence genes through whole-genome analyses have shown to be useful to distinguish pathogens from non-pathogens on the susceptible host that forma species of *F. oxysporum* infects (Sperschneider et al., 2015).

Gardner (1980) described a disease in low to mid-elevation, below 610m (2000 ft), forests in the Hawaiian archipelago affecting an endemic tree species, *Acacia koa* A. Gray (Anderson et al., 2002). *Acacia koa* (koa) is a valuable tree ecologically, economically, and culturally to Hawai'i. Koa is the second most abundant tree in Hawai'i's forests and many of Hawai'i's indigenous flora and fauna rely on this tree species for food and shelter (Pejchar et al., 2005; Pejchar et al., 2006; Anderson et al., 2002). For this reason, there has been increased reforestation efforts to reincorporate koa to forests that have declining populations (Dudley et al., 2015). Historically, native Hawaiians used koa for the construction of spears, tools, paddles (hoe), ceremonial poles (hulumanu), religious ceremonies, "leaves" and ashes medicinally, single-hulled outrigger canoes (kaukahi), and their double-hulled outrigger canoes (kaulua) (Ziegler, 2002; Elevitch, Wilkinson, & Friday, 2006). To make the outrigger canoes, single-trunked, straight trees were required. This artificial selection caused the morphological change from these tall, straight, single-trunked trees to the multi-trunked, spindly trees that are observed today (Ziegler, 2002). This has increased interest in selective breeding programs to develop koa of good form for timber and restoration efforts, however, diseases and insects have impeded this process. There are numerous diseases described on koa, but the predominant threat to its native range is the pathogen that causes koa wilt disease [caused by *Fusarium oxysporum* f. sp. *koae* (*Fo koae*)] (Dudley et al., 2017).

Currently, methods of control for plant pathogenic *F. oxysporum* include fumigation and pesticides that are harmful to the environment and are not cost-effective methods to control this disease in forests and/or timber stands (Fravel et al., 2002), so efforts have focused primarily on producing disease-resistant seedlings using seed collected from naturally occurring stands (Dudley et al., 2015). These efforts require conducting extensive greenhouse disease resistance

screening assays on wild-collected seed to find naturally occurring resistance to the pathogenic strains of the fungus (Sniezko, 2006; Dudley et al., 2012). Additionally, screening trials are costly and require a variety of pathogenic strains to ensure that the disease resistance is robust. Developing a faster, more cost-effective strategy for screening pathogenic isolates would greatly benefit these programs. It has been well documented that pathogenic fungi can evolve quickly; much faster than their plant hosts (Takken & Rep, 2010; Sperschneider et al., 2015; Ma et al., 2013). For this reason, it is necessary to incorporate new, genetically diverse strains of the pathogen to ensure that disease resistance can be maintained against newly evolved or introduced strains of the pathogen existing in Hawai'i's forest and agricultural lands. Obtaining new pathogenic isolates is an arduous and time-consuming process that requires three-month greenhouse virulence assays, after collection, to confirm pathogenicity on susceptible host seedlings. A pathogen-specific, PCR primer would allow for rapid detection of pathogenic strains of *Fo koae*. This would aid collections of new pathogenic strains for disease resistance breeding programs as well as growers and land managers for restoration and/or timber purposes.

Objectives for this study are to 1) conduct and analyze whole genome sequencing of one highly-virulent *Fo koae* isolate and one non-pathogenic *F. oxysporum* (*Fo*) isolate; 2) identify virulence-associated genes that are putatively important for disease development in the host; and 3) use the identified differences to develop a *Fo koae*-specific PCR primer.

2.3 Materials and Methods

2.3.1 Molecular Characterization of Greenhouse Confirmed Pathogenic and Non-pathogenic *Fusarium* Isolates

Fusarium isolates that were previously screened for pathogenicity through greenhouse trials were provided by the Hawai'i Agriculture Research Center (HARC) (Dudley et al., 2007) for this study (Table 1). HARC screened these isolates through the protocol described in Dudley and others (2007). At the end of the 90 days after inoculation in koa seedlings under greenhouse conditions, isolates were classified as non-pathogenic (<10% mortality) or low (10 – 30% mortality), moderate (30 – 70% mortality), or high virulence (70 – 100% mortality) (Table 1).

Table 1 – Hawai'i Agriculture Research Center *Fusarium oxysporum* f. sp. *koae* and *F. oxyporum* isolates that have been screened for pathogenicity through greenhouse virulence assays.

Isolate ID	**Virulence**	**% Mortality***	**Collection Site (Island)**
44	High	95	Hawai'i
78	High	92	Kauai
77	High	80	Kauai
79	High	72	Kauai
0540K	Moderate	70	Hawai'i
90	Moderate	68	Oahu
20	Moderate	66	Hawai'i
17	Moderate	62	Hawai'i
166	Moderate	60	Hawai'i
76	Moderate	60	Kauai
8	Moderate	58	Hawai'i
34	Low	12	Hawai'i
72	Nonpathogenic	8	Kauai
27	Nonpathogenic	8	Maui
45	Nonpathogenic	6	Hawai'i
53	Nonpathogenic	4	Maui
81	Nonpathogenic	4	Kauai
170	Nonpathogenic	0	Maui

*% mortality of 24 inoculated seedlings per isolate displaying foliar wilt, chlorosis or necrotic symptoms (Dudley et al. 2007; Dudley et al. 2017)

Seven pathogenic *Fusarium* isolates of high (HARC: 44, 77, 78, and 79) and moderate (HRAC: 76, 90, and 190) virulence , that are currently used in HARC's disease resistance

screening trials and three non-pathogenic (HARC: 27, 45, and 170) *Fusarium* isolates were selected for molecular characterization. Isolates were sub-cultured on ¼ strength Potato Dextrose Agar (PDA) in petri dishes and maintained at 25˚C in darkness for three days. DNA from the 10 *Fusarium* isolates were extracted using a Chelex® extraction protocol, modified from Brewer and Milgroom (2010). A mycelial scrape of each isolate was added to tubes with 100µl of a sterilized 10% Chelex-100 solution and ground using a 10µl pipette tip. Samples were thermolyzed in a Mastercycler ProS thermocycler (Eppendorf, Hamburg, GE) at 100˚C for 35 minutes. Template DNA was taken from the upper aqueous layer to avoid Chelex-100 resin (Bio-RAD, Hercules, CA) contamination and PCR inhibition. Using this DNA, six conserved regions of the genome were amplified including: 1) translation elongation factor 1- α (*tef1*) (O'Donnell et al., 2004) and 2) RNA polymerase II second largest subunit (*rpb2*) (Liu et al., 1999) using a PCR cycle program of 94˚C for 2 min, 40 cycles of 94˚C for 40s, 58˚C for 40s, and 72˚C for 30s, and 72˚C for 5 min; 3) calmodulin (*CAL*) (Groenewald et al., 2012) and 4) β - tubulin (*β-tub*) (Glass & Donaldson, 1995) using a PCR cycle program of 94˚C for 2 min, 30 cycles of 94˚C for 40s, 54˚C for 40s, and 72˚C for 1 min, and 72˚C for 5 min; 5) internal transcribed spacer (ITS) (Glass & Donaldson, 1995) using a PCR cycle program of 94˚C for 2 min, 35 cycles of 94˚C for 40s, 55˚C for 40s, and 72˚C for 1 min, and 72˚C for 5 min; and 6) mitochondrial small subunit (mtssu) (O'Donnell et al., 2004) using a PCR cycle program of 94˚C for 2 minutes, 30 cycles of 94˚C for 40s, 50˚C for 40s, and 72˚C for 1 min, and 72˚C for 5 minutes. Products were run on a 1.5% agarose gel to visualize amplified PCR product. PCR products were cleaned for sequencing using exonuclease and shrimp phosphate (Affymetrix, Santa Clara, CA) and sent to Eurofins genomics LLC (Louisville, KY) for Sanger sequencing.

These sequences were then used to identify single nucleotide polymorphisms (SNPs) to distinguish known pathogenic *Fo koae* from non-pathogenic *F. oxysporum* collected from koa.

2.3.2 Whole Genome Sequencing and Assembly

In preparation for DNA extraction, one pathogenic isolate to koa (*Fo koae* 44) and one non-pathogenic (*Fo* 170) isolate were first grown on ¼ PDA at 25˚C for three days. Hyphal tips were taken and transferred to 100ml of potato dextrose broth and shaken at 70 rpms for seven days at room temperature. Mycelium was placed in 2ml tubes and frozen at -75˚C for 24 hours prior to extraction. DNA was extracted using a cetyl trimethyl ammonium bromide (CTAB) extraction protocol adapted from Cubero et al. (1999). Tissue preparation was modified from the original protocol such that 0.1g of frozen tissue was pulverized using a FastPrep-24^{Tm} (M.P. Biomedicals LLC, Santa Ana, CA) at 5x speed for 20 seconds for 3 runs. Samples were kept frozen between runs with liquid nitrogen. On the third run, 750µl of CTAB extraction buffer (1% w/v CTAB; 1M NaC1; 100 mM Tris; 20 mM EDTA; 1% w/v polyvinyl polypyrolidone, PVPP) was added to each sample and centrifuged at room temperature. The extracted DNA was gel electrophoresed in a 2% agarose gel and quantified using a Qubit^{TM} fluorometer (Invitrogen, Carlsbad, CA). Due to an observed abundance of remaining polysaccharides after extraction, similarly as described in Huang and others (2018), a Zymo gel cut out kit and a Zymo clean and concentrate column was used to clean the DNA of polysaccharides. Extracted DNA was sent to Macrogen (Seoul, South Korea) for Truseq Illumina shotgun sequencing with reads of 2x151bp.

De novo assembly of both *Fo koae* 44 and *Fo* 170 was constructed using SPAdes 3.11.1 (Bankevich et al., 2012) using default parameters. The SPAdes genome assembly was assessed

for quality using QUAST (Gurevich et al., 2013). Contigs that were identified to have less than 100x coverage were removed, and the remaining contigs were checked for similarity to *Fusarium* spp. using NCBI BLAST to screen for presence of bacterial DNA in samples.

2.3.3 Genome Annotation and Analysis

To determine overall similarity of *Fo koae* 44 and *Fo* 170 isolates, average nucleotide identity was calculated using Pyani (Pritchard et al., 2016). R package 'coRdon' was used to determine codon usage bias (R core team, 2016; Fabijanić & Vlahoviček, 2016). TransposableELMT (Wyka, 2019), a wrapper script for transposable element identification and creation of a comprehensive repeat library using RepeatModeler, RepeatClassifier, LTR_finder, ltr_harvest, and TransposonPSI (http://transposonpsi.sourceforge.net) and subsequently RepeatMasker with this library was used to identify transposable elements and repeat-rich regions for both *Fo koae* 44 and *Fo* 170 (Smit et al., 2015; Ellinghaus et al., 2008).

To identify variants and unique sequences to *Fo koae* 44, putative chromosome contigs were constructed using a closely related reference genome, *F. oxysporum* f. sp. *lycopersici*. Paired reads of the genomes of *Fo koae* 44 and *Fo* 170 were trimmed using BBDuk (decontamination using kmers) (part of BBTools package). The trimmed reads were mapped to the reference genome using Geneious (Geneious Prime 2019.2, http://www.geneious.com/) progressiveMauve genome mapper software at medium-low sensitivity (Darling, Mau, & Perna, 2010). Low and high coverage sites were removed using Geneious' built-in program. Variants and SNPs were identified using FreeBayes (Garrison & Marth, 2012) within Geneious. Variant calling format (VCF) was exported for use in comparison of SNPs and indels between *Fo koae*

44 and *Fo* 170. Synteny maps and MUMmer alignments were made using the synteny mapping and analysis program (SyMAP) (Soderlund, Bomhoff, & Nelson, 2011) to create putative chromosome contigs based on the reference genome.

The *de novo* assembled genomes were annotated for both *Fo koae* 44 and *Fo* 170 using the MAKER 2.31.8 annotation pipeline (Cantarel et al., 2008) with RepeatMasker 4.0.8 (Smit et al., 1996). A *F. oxysporum* specific repeat library was constructed using RepeatModeler 1.0.11 (Smit & Hubley, 2017) to mask interspersed repeats and low complexity DNA sequences. Three gene predictors were used in the pipeline: GeneMark-ES (Ter-Hovhannisyan et al., 2008), SNAP (Zahariaet al., 2011), and AUGUSTUS (Keller et al., 2011). *Fusarium graminearum* was used as a species model for AUGUSTUS. To identify tRNA genes, tRNAscan-SE 1.3.1 was used with default settings.

Predicted transcripts (≥150 bp) and proteins (≥50 amino acids) were analyzed using five databases for analysis of putative genes: OrthoVenn2 for putative non-orthologous proteins (Xu et al., 2019), InterProScan for protein family domains (Quevillon et al., 2005), antiSMASH for putative secondary metabolites (Blin et al., 2019), dbCAN2 for putative carbohydrate-active enzymes (Zhang et al., 2018), and PHI-base for putative virulence-associated proteins (Urban et al., 2017). OrthoVenn2, antiSMASH, and dbCAN2 databases' online servers were used to analyze the predicted genes at default settings. A local BLAST was used for InterProScan and the PHI-base database. Selection of putative proteins from PHI-base was based on a ≥ 85% grade (metric of combining the query coverage, e-value and identity values for each hit with weights 0.5, 0.25 and 0.25 respectively). Putative virulence-associated proteins were tested for exclusivity to *Fo koae* 44 by aligning these sequences to sequences extracted from the NCBI database using MUSCLE in Geneious (Edgar, 2004) (Table 2).

Table 2 - Secreted in xylem (*SIX*) gene sequences of formae speciales of *Fusarium oxysporum* (*Fo*) retrieved from NCBI with Genbank accession numbers. Sequences used to compare identified *SIX* genes in pathogenic isolate *F. oxysporum* f. sp. *koae* 44.

SIX1			***SIX6***	
Species	**Genbank Accession**		**Species**	**Genbank Accession**
F. oxysporum 37	KC296735.1		*Fo* f. sp. *cubense*	KX435008.1
Fo f. sp. *niveum*	KX435036.1		*Fo* f. sp. *vasinfectum*	KR855791.1
Fo f. sp. *fragariae*	KX435028.1		*Fo* f. sp. *radicis-cucumerinum*	KR855755.1
Fo f. sp. *medicaginis*	KR855720.1		*Fo* f. sp. *niveum*	KR855756.1
Fo f. sp. *melonis*	KR811364.1		*Fo* f. sp. *melonis*	GQ268959.1
Fo f. sp. *conglutinans*	KR855720.1		*Fo* f. sp. *pisi*	KR855779.1
Fo f. sp. *lini*	KM893920.1		*Fo* f. sp. *cucumerinum*	KR855770.1
Fo f. sp. *canariensis*	MH616619.1		*Fo* f. sp. *passiflorae*	KR855782.1
Fo f. sp. *cubense*	KM893911.1		*Fo* f. sp. *lycopersici*	KR855786.1
Fo f. sp. *lycopersici*	KR855716.1		*Fo* f. sp. *phaseoli*	KP964967.1

2.3.4 Phylogenetic Analysis

A whole-genome maximum likelihood phylogeny was constructed to elucidate evolutionary history of *Fo koae* 44 and *Fo* 170 to each other and to other *Fusarium* spp. and *F. oxysporum* formae speciales. Realphy 112 (Bertels et al., 2014) and PhyML 3.1 (Guindon et al., 2010) using bowtie2 2.3.4.2 (Langmead & Salzberg, 2012) for alignment to the reference genome of *F. oxysporum* f. sp. *lycopersici* and the general time reversal (GTR) substitution model (Rodriguez et al., 1990), were used to create the whole-genome phylogeny with bootstrap support (n = 200). Whole genomes of *Fusarium* spp. and *F. oxysporum* formae speciales were extracted from the National Center for Biotechnology Information (NCBI) database (Table 3).

Table 3 – Whole genome sequences of *Fusarium* spp. and formae speciales of *F. oxysporum* retrieved from NCBI with Genbank or RefSeq accession numbers. These genomes were used to make the whole genome phylogeny.

Species	**Genbank Accession/ RefSeq**
Fusarium asiaticum	LHTY00000000.1
F. avenaceum	JQGE00000000.1
F. azukicola	MAEG00000000.1
F. Brasiliense	MAEC00000000.1
F. circinatum	CM010400; AYJV00000000
F. commune	BCHB00000000.1
F. culmorum	FJUU00000000.1
F. equiseti	QOHM00000000.1
F. fujikuroi	GCF900079805.1
F. graminearum	AACM00000000.1
F. incarnatum	RBJE00000000.1
F. nygamai	LBNR00000000.1
F. phaseoli	MAEB00000000.1
F. pininemorale	NFZR00000000.1
F. poae	LYXU00000000.1
F. proliferatum	FJOF00000000.1
F. pseudograminearum	GCF_000303195.2
F. sambucinum	LSRD01000000.1
F. solani	NGZQ01000000.1
F. tricinctum	OVTS02000000.1
F. tucumaniae	MAED01000000.1
F. verticillioides	NC_031675.1
Fusarium oxysporum (*Fo*) *47*	AFMM01000000.1
Fo f. sp. *cepae*	CM010800.1
Fo f. sp. *conglutinans*	JH658799.1
Fo f. sp. *cubense* race 1	KB729921.1
Fo f. sp. *cubense* race 4	JH658272.1
Fo f. sp. *cucumerinum*	MABO00000000.1
Fo f. sp. *gladioli*	NJCK00000000.1
Fo f. sp. *lagnariae*	NJCJ00000000.1
Fo f. sp. *lilii*	NJCF00000000.1
Fo f. sp. *luffae*	NJCE00000000.1
Fo f. sp. *lycopersici*	AAXH00000000.1
Fo f. sp. *melongenae*	NJCC00000000.1
Fo f. sp. *melonis*	JH659329.1
Fo f. sp. *momordicae*	NJCB00000000.1
Fo f. sp. *narcissi*	NJCV00000000.1
Fo f. sp. *nicotianae*	NJBZ00000000.1
Fo f. sp. *niveum*	MALG00000000.1
Fo f. sp. *pisi*	JH650968.1
Fo f. sp. *radicis-cucumerinum*	CM008287.1

Fo f. sp. *raphanin*	JH658362.1
Fo f. sp. *tulipae*	NJBS00000000.1
Fo f. sp. *vasinfectum*	JH657918.1
Magnaporthe oryzae	NC_017844.1

2.3.5 Field Collection of *Fusarium* spp.

Fourteen collection sites were selected based on a previous survey conducted by the HARC from 2004-2007 over four of the main Hawaiian Islands including Kauai, Oahu, Maui, and Hawai'i (Dudley et al., 2010). Sites for new isolate collections were selected based on the criteria that moderate to highly virulent isolates, as described previously, of the pathogen were previously collected from a site and were geographically distant from each other to increase the likelihood of collecting more genetically distinct populations of the pathogen. On Kauai, three sites included Kokee, Kapa'a, and Hanalei (Princeville). On Oahu, three sites included Maunawili, Poamoho, and Kahana. On Hawai'i, eight sites included Wood Valley, Pu'u wa'a wa'a, Kona, Wung Ranch, Kaiwiki, Kalopa, Paauilo, and Volcano National Park. Root samples were collected from symptomatic trees at each site. Symptomatic segments of 4-5mm thick roots exhibiting discoloration and black streaks, were targeted for isolation of *Fusarium* spp. Five fine roots and/or pencil-thick roots were plated on selective Komada media (Komada, 1975), for isolation of *Fusarium* spp. Plates were incubated at 25˚C for three days then checked for mycelial growth emanating from the root samples. Hyphal tips were taken from the radiating mycelium and transferred to ¼ PDA and incubated at 25˚C for another three days. Colony morphology was used to determine if the isolate was a *Fusarium* sp. To further characterize these isolates to species, isolates were sequenced at the (*tef1* region.

2.3.6 Primer Development

Putative chromosome contigs from SyMAP were aligned using progressiveMauve (Darling, Mau, & Perna, 2010) in Geneious. Using these alignments, unique sequences to *Fo koae* 44 were identified and extracted. Also, *Fo koae* 44 unique transcripts, comparing the two transcriptomes from the Maker annotation identified from Orthovenn2, were identified as unique by using a local BLAST against *Fo* 170 and the NCBI database. Sequences with no sequence similarity to *Fo* 170 or other *Fusarium* spp. or *F. oxysporum* formae speciales were used to develop primers. Primer3 (Untergasser et al., 2012) was used to develop PCR primers from the unique sequences and transcripts. Ten primer sets (forward and reverse primer) were generated per sequence.

Primers were tested for specificity on two *F. commune* isolates (HARC isolate 85 and Stewart collection FO21), eight characterized moderate virulence of *Fo koae* isolates (HARC isolates 76, 79, 90, 166, 8, 17, 0540K, and 20), three characterized highly virulent *Fo koae* isolates (isolates used in HARC greenhouse screening trials: 44, 77, and 78), two characterized non-pathogenic *Fo* isolates (HARC isolates 27 and 170), and one *F. proliferatum* isolate (HARC isolate 1). Each 25 ul reaction contained 10ng of template DNA or sterile molecular water for the negative control. Primers were run through a Mastercycler ProS thermocycler (Eppendorf, Hamburg, GE) at a program of 94˚C for 2 minutes, 30 cycles of 94˚C for 40s, 59˚C for 40s, and 72˚C for 30s, and 72˚C for 5 minutes. Primers were tested using standard PCR in a 1.5% gel and ran at 60V for 60 minutes.

2.3.7 Genetic Characterization of Field Collected Isolates

To determine the genetic relationships among the field collected isolates and the characterized pathogenic and non-pathogenic isolates, isolates were sequenced at the *tef1* and *rpb2* loci. Sequences were aligned using MUSCLE alignment in Geneious (Edgar, 2004). Two statistical parsimony (TCS) haplotype networks were constructed based on the sequence data of the *tef1* and *rpb2* using PopART with a 95% genetic cut-off (Templeton et al., 1992; Clement et al., 2000). These isolates were tested with the developed primers to identify haplotypes that may contain the putative lineage-specific chromosome identified from *Fo koae* 44.

2.4 Results

2.4.1 Molecular Characterization of Confirmed Pathogenic and Non-pathogenic Isolates

Among the isolates of known virulence (through pathogenicity assays on koa) obtained from HARC, limited variation was observed in the sequenced loci. Two loci, the *tef1* and *rpb2* had the most variation with two and nine conserved SNPs, respectively. Two loci, mtssu and *β-tub* had one SNP each, while the ITS and *CAL* loci had no identified SNPs.

2.4.2 Whole Genome Sequencing and Assembly

Libraries resulted in 397,390,528 reads with 1,245x coverage for *Fo koae* 44 and 385,805,256 reads with 1,150x coverage for *Fo* 170. *De novo* assembly of both *Fo koae* 44 and *Fo* 170 identified the genome size as 48 and 50 Mb respectfully. The QUAST report metrics are shown in Table 4.

Table 4 – QUAST assembly statistics of the SPAdes *de novo* assembly of the pathogenic *Fusarium oxysporum* f. sp. *koae* 44 (*Fo koae* 44) and non-pathogenic *F. oxysporum* 170 (*Fo* 170) isolates.

	***Fo koae* 44**	***Fo* 170**
Genome size (Mb)	48,183,513	50,628,816
Contig Number	492	964
Scaffold Number	330	910
N50	721547	562269
GC%	47.5	47.2

2.4.3 Whole Genome Phylogenetic Analysis

The whole genome phylogeny showed that both *Fo koae* 44 and *Fo* 170 grouped into a well-supported [Bootstrap (BS) = 100] clade with other *F. oxysporum* formae speciales (Figure 1). Interestingly, both were more closely related to other formae speciales of *F. oxysporum* than they were to each other (Figure 1). *Fo koae* 44 clustered in a sub-clade (BS =100) that included *F. oxysporum* f. sp. *cubense* race 1 and *Fo* 170 clustered in a sub-clade (BS =100) with *F. oxysporum* f. sp. *gladioli*.

2.4.4 Genome Annotation and Analysis

The average nucleotide identity (ANIm) between *Fo koae* 44 and *Fo* 170 was 96.58%. Based on the reference genome of *F. oxysporum* f. sp. *lycopersici,* 11 putative core chromosomes shared synteny with *Fo koae* 44 and *Fo* 170, respectively. One putative accessory chromosome was identified in each genome that did not map to *F. oxysporum* f. sp. *lycopersici* or show synteny to each other or other *F. oxysporum* formae speciales (Figure 2). Further analysis comparing the putative chromosomes of *Fo koae* 44 and *Fo* 170 highlighted that variants (SNPs and indels) localized on specific chromosomes (X_44 and X_170) and at chromosome ends (Figure 3). Putative core chromosomes 1, 2, 4, 5, 7, 8, 9, and 10 were the most similar with variants identified localized on chromosome ends. Putative chromosomes 11, 12, 13, and X had

the most variation. Putative core chromosomes 11, 12, and 13 had localized variation at the ends but the putative accessory chromosome X had variation throughout (Figure 3). Analysis of the distribution of repeats and transposons identified *F. oxysporum* transposable elements such as *Foxy*, *Skippy*, *Impala*, *Hop*, *Mariner*, *Hat*, and *Helitron* on the chromosome X of both *Fo koae* 44 and *Fo* 170 (Suga et al., 2013). The average core chromosome was comprised of 3% and 4% of transposable elements and repeats in *Fo koae* 44 and *Fo* 170, respectively. The transposable elements and repeats comprised 19% and 20% of chromosome X of *Fo koae* 44 and *Fo* 170, respectively (Figure 4 A & B).

Using the Maker pipeline, 15,380 and 15,763 transcripts and corresponding proteins were predicted for *Fo koae* 44 and *Fo* 170, respectively. Using Orthovenn2, 1,760 non-orthologous proteins from 78 clusters and 1,576 single genes, were identified to be exclusive to *Fo koae* 44 when compared to *Fo* 170 (Figure 5 & 6). *Fo* 170 had 2,123 non-orthologous proteins from 137 clusters and 1,780 single genes, were found to be exclusive to *Fo* 170. Gene ontology (GO) enrichment was identified individually for *Fo koae* 44, that had enrichment of GO terms for nucleic acid binding, and *Fo* 170, that had enrichment of GO terms for GTP binding, regulation of transcription from RNA polymerase II promoter during meiosis, sporulation resulting in formation of a cellular spore, and regulation of DNA-templated transcription. The 13,500 protein clusters that were shared between *Fo koae* 44 and *Fo* 170 had no identified GO enrichment (Figure 5).

Using InterProScan, 16,429 putative protein family domains were identified in *Fo koae* 44 and 16,487 in *Fo* 170. Thirty of these putative proteins were unique to *Fo koae* 44, whereas thirty-two of these putative proteins were unique to *Fo* 170. A sterigmatocystin biosynthesis

gene was identified in *Fo koae* 44. In *Fo* 170, proteins involved in pathogenesis were identified including oxidoreductases (i.e. monooxygenase) and putrescine biosynthesis.

Using the antiSMASH database, the secondary metabolites identified were similar in both genomes of *Fo koae* 44 and *Fo* 170, however, variation did exist in gene copy number (Figure 7). Non-ribosomal peptide-synthetases (NRPS), NRPS-like, and indoles had more gene copy numbers for *Fo koae* 44. *Fo* 170 had two more copies of genes involved in the production of the *Fusarium* mycotoxin enniatin (Luizzi et al., 2017).

Similar results were observed from genes identified using the dbCAN2 database, except that two copies of a glycoside hydrolase, GH13_22 (characterized glucan synthase), were found in *Fo koae* 44, but not in *Fo* 170. In *Fo* 170, two copies of proteins GH141 (α-L-fucosidase or xylanase), one copy of GH30 (endo-β-1,4-xylanase or β-glucosidase), and one copy of GH43_12 (a glycoside hydrolase involved in hydrolysis and/or rearrangement of glycosidic bonds arabinosidase) were identified (Zhang et al., 2018).

Using PHI-base, shared virulence-associated genes were identified in both *Fo koae* 44 and *Fo* 170 including: *Fusarium* specific transcription factors such as the *Ftf2* (PHI:5484) and the *SIX* gene expression (*SGE1*; PHI:3168); Toxin detoxifiers such as the *GzmetE* (PHI:355) and *Tom1* (PHI:438); A chitin synthase gene, *CHS2* (PHI:336) and a beta-1,3-glucanosyltransferase, *GAS1* (PHI:522). Nine virulence-associated genes were identified as unique to *Fo koae* 44. Multiple *Fusarium* transcription factors, mitogen-activated protein kinases, and ATP-binding cassette (ABC) transporters were identified. The *SIX1* and *SIX6* genes, were only identified on the putative accessory chromosome of *Fo koae* 44. These genes showed sequence similarity to other *F. oxysporum* formae speciales (Table 5 A & B). The *SIX1* gene of *Fo koae* 44 showed highest sequence similarity with *F. oxysporum* f. sp. *fragariae* (85%) and the *SIX6* gene showed

highest sequence similarity to *F. oxysporum* f. sp. *pisi* (97%) and *F. oxysporum* f. sp. *cucumerinum* (97%). There were seven virulence-associated genes identified as unique to *Fo* 170 consisting of signaling proteins, ATP binding cassette transporter, and one of unknown function but related to developing root rot symptoms (PEP2).

2.4.5 Field Isolate Collection

Root samples from 13 of the 14 sites surveyed yielded *Fusarium* isolates (Figure 8). Kapa'a on Kaua'i could not be accessed due to inclement weather conditions. A total of 359 *Fusarium* isolates, including *F. oxysporum*, *F. solani*, *F. proliferatum*, *F. concolor*, *F. lateritium*, *F. commune*, and *F. fujikuroi* (based on *tef1* sequencing data), were collected from 100 trees across the 3 islands (Table 6). Most of the *Fusarium* isolates were from Maunawili, Kalopa, Wood Valley, and Pa'auilo, respectively. The least represented sites were Kaiwiki, Wung Ranch, and Kona, respectively. *Fusarium* spp. isolates were collected from 70.4% of the 142 trees surveyed.

Table 5 A – *SIX1* gene alignment showing percent identity of *Fusarium oxysporum* f. sp. *koae* (*Fo koae* 44) to other *F. oxysporum* (*Fo*) formae speciales.

	Fo niveum	***Fo 37***	***Fo fragariae***	***Fo medicaginis***	***Fo koae 44***	***Fo melonis***	***Fo conglutinans***	***Fo lini***	***Fo canariensis***	***Fo cubense***	***Fo lycopersici***
Fo koae 44	**22%**	**43%**	**85%**	**73%**		**83%**	**77%**	**79%**	**79%**	**78%**	**76%**

Table 5 B – *SIX6* gene alignment showing percent identity of *Fusarium oxysporum* f. sp. *koae* (*Fo koae* 44) to other *F. oxysporum* (*Fo*) formae speciales.

	Fo cubense	***Fo vasinfectum***	***Fo niveum***	***Fo radicis-cucumerinum***	***Fo melonis***	***Fo koae 44***	***Fo pisi***	***Fo cucumerinum***	***Fo passiflorae***	***Fo lycopersici***	***Fo phsaeoli***
Fo koae 44	**71%**	**79%**	**95%**	**95%**	**95%**		**97%**	**97%**	**94%**	**92%**	**92%**

Table 6 –Results of 2018 field collection. Showing number of *Fusarium* isolates collected from symptomatic *Acacia koa*.

Kauai			
Site	**Number of Trees Sampled**	**Number of Trees Positive for *Fusarium***	**Number of *Fusarium* Isolates per Site**
Hanalei	11	6	8
Kokee	20	13	37
Kapa'a	1	0	0
Oahu			
Site	**Number of Trees Sampled**	**Number of Trees Positive for *Fusarium***	**Number of *Fusarium* Isolates per Site**
Kahana	11	6	9
Maunawili	12	12	64
Poamoho	8	8	27
Hawai'i			
Site	**Number of Trees Sampled**	**Number of Trees Positive for *Fusarium***	**Number of *Fusarium* Isolates per Site**
Wood Valley	20	16	57
Pa'auilo	14	11	48
Kalopa	10	10	60
Kaiwiki	9	4	4
Kona	1	1	1
Pu'uwa'awa'a	5	5	19
Volcano National Park	15	7	21
Wung Ranch	5	1	4

2.4.6 Primer Development

For PCR primer development, a total of 445 sequences were identified as unique to *Fo koae* 44 when compared to *Fo* 170, other *Fusarium* spp. and *F. oxysporum* formae speciales. Thirty-three of these had no BLAST hits to the NCBI database. Further, of the 1,762 *Fo koae* 44 exclusive putative proteins identified in Orthovenn2, 137 transcripts had no BLAST hits to the NCBI database. From these 170 sequences, 3,750 primers were developed *in silico*. Of those, 35 primer pairs consisting of 63 individual primers had no non-target hits *in silico*. Primers were

designed on sequences identified on putative core chromosomes 1, 2, 4, 5, 7, 9 and the putative lineage-specific chromosome X. When PCR tested, six of the seven primers (Table 7) did not amplify *Fo* 170 or other characterized non-pathogenic *F. oxysporum* isolates or *F. commune* or *F. proliferatum* isolates. The primer developed on the core chromosome amplified two of the characterized non-pathogenic isolates.

2.4.7 Genetic Characterization of Field Collected Isolates

One-hundred six isolates (17 characterized for pathogenicity and non-pathogenic and 89 field collected isolates) were sequenced at the *tef1* and 55 isolates (14 characterized and 41 field collected isolates) were sequenced at the *rpb2.* Variation found at these loci were used to construct TCS haplotype networks that resulted in 23 and 10 haplotypes, respectively (Figures 9 and 10). We compared one primer pair that was developed on the core chromosome (primer P4) and one primer pair developed on the X chromosome (primers P22) (Table 8).

Using the haplotype networks of the *tef1* and *rpb2*, we identified that the characterized highly virulent isolates (44, 77, 78, and 79) belonged to the same haplotype (HAP 1). However, in the *tef1* HAP 1, characterized isolates of all virulence ratings including non-pathogens were also found to share this haplotype (Figure 9). We did not analyze the *tef1* haplotype network further due to the lack of a clear pattern in virulence phenotype. There was a clearer pattern of virulence phenotypes in *rpb2* haplotypes (Figure 10). HAP1 had characterized highly virulent and moderately virulent isolates. Non-pathogenic isolates clustered in three haplotypes (HAP 3, 8, and 10). The developed primers amplified two of the ten *rpb2* haplotypes (HAP 1 and 3) (Figure 11). HAP 2 and 4 amplified with only the core chromosome primer pair, P4. P4

amplified highly virulent and moderately virulent isolates but was also found to amplify two non-pathogenic isolates in HAP 3. Uncharacterized isolates in HAP 3 amplified with X chromosome and/or core chromosome primer pairs. X chromosome primer did not amplify non-pathogenic or lowly virulent isolates.

Table 7 – Pathogen-specific PCR primers designed from unique genes and sequences in genome of *Fusarium oxysporum* f. sp. *koae* 44. Isolates were confirmed for pathogen specificity through PCR testing on greenhouse characterized pathogenic and non-pathogenic isolates provided by the Hawai'i Agriculture Research Center.

Primer Description	**Primer Sequence**	**Chromosome**	**Putative Function**
P4 F P4 R	TGTGGCCGCCTTGCAATAAC GGTTTGTCCAACACACCCGT	2	Transcription Factor (25% identity)$^{+}$
P6 F P6 R	GGCGCCAGCGTTTAATGGAA ATTGGGCCTCCTTCGATCCG	X	Saponin detoxification (57% identity)$^{+}$
P17 F P17 R	TAGCGCTGTGTTATCCCGCA GGGAACTGCGGTGTAGTGGA	X	Gylcoside hydrolase*
P22 F P22 R	TGGCGGCTCAAATTATCGCG CCAGGCCGAGGCTATTTCCA	X	Major Facilitator Superfamily (MFS)*
P24 F P24 R	AGGCCTACAAAGCGCACAGA CGTACGTGAGAGGGGCAAGT	X	Fungal transcription factor*
P31 F P31 R	ACTGGAGGCGTTCACTTGCA GCCACTTTTAGCTTCGCCCG	X	Unique Sequence
P32 F P32 R	GTGTTTCAACTTCGCCGGGG TGCGAGGTTGAAGCCGGTAA	X	Unique Sequence

*Pfam identified putative function
$^{+}$PHI-base hit with low identity to genes that were identified as unaffecting pathogenicity

Table 8 – Virulence assayed *Acacia koa* pathogenic *F. oxysporum* f. sp. *koae* (*Fo koae*) and non-pathogenic *F. oxysporum* isolates and 2018 field collection isolates organized into haplotypes by variation found in the translation elongation factor 1-a (*tef1*) and the RNA polymerase II second largest subunit (*rpb2*). Isolates were tested with developed *Fo koae* specific primers for amplification to identify presence or absence of the putative lineage-specific chromosome.

Isolate	Site	Virulence[1]	*tef1* Haplotype	*rpb2* Haplotype	P4	P22	P6	P17	P24	P31	P32
104	HAN 1		1	4	YES	NO			NO		
112	KOK 15		1	9	NO	NO	NO	NO	NO	NO	NO
139	KOK 13		1		YES	YES			YES		
160	KOK 14		1	5	NO	NO			NO		
311	KOK 1		1	3	YES	YES			YES		
333	KOK 19		1	1	YES	YES	NO	YES	YES	YES	YES
366	WV 15		1	2	NO	NO			NO		
515	WV 8		1	2	NO	NO			NO		
693	VNP 9		1		NO	NO			NO		
750	RP 10		1	2	NO	NO			NO		
908	KAH 1		1		YES	YES			YES		
991	MW 8		1		YES	YES			YES		
1003	MW 10		1		YES	YES			YES		
1009	MW 11		1		YES	YES			YES		
1032	MW 5		1	2	YES	NO			NO		
166	Maui	Moderate	1	1	YES	YES			NO		
1802B	Lanai		1	3	YES	NO			NO		
1809A	Lanai		1	1	YES	YES			YES		
34	Hawaii	Low	1	2	NO	NO			NO		
44	Hawaii	High	1	1	YES	YES	YES	YES	YES	YES	YES
72	Kauai	Non-pathogenic	1	3	YES	NO			NO		
76	Kauai	Moderate	1	1	YES	YES			YES		
77	Kauai	High	1	1	YES	YES			YES		
78	Kauai	High	1	1	YES	NO	YES	NO	NO	YES	YES
79	Kauai	High	1	1	YES	YES			YES		

81	Kauai	Non-pathogenic	1	3	YES	NO			NO		
90	Oahu	Moderate	1	1	YES	YES	YES	YES	YES	YES	YES
970	POA 6		1		YES	NO			NO		
185	HAN 7		2		NO	NO			NO		
447	CK 5		2	2	NO	NO	NO	NO	NO	NO	NO
451	CK 8		2		NO	NO			NO		
485	WV 16		2		NO	NO			NO		
491	CK 1		2		NO	NO			NO		
533	TK 1		2	2	NO	NO			NO		
586	RP 8		2	2	NO	NO			NO		
613	VNP 10		2		NO	NO			NO		
676	VNP 11		2		NO	NO			NO		
679	VNP 14		2		NO	NO			NO		
722	HAL 2		2	2	NO	NO			NO		
731	HAL 1		2	2	NO	NO			NO		
736	WV 6		2		NO	NO			NO		
798	WV 18		2	2	NO	NO			NO		
804	WV 14		2	2	NO	NO			NO		
835	WV 20		2	2	NO	NO			NO		
860	CK 10		2	2	NO	NO			NO		
863	CK 9		2		NO	NO			NO		
886	WV 10		2	2	NO	NO			NO		
987	MW 7		2	2	NO	NO			NO		
995	MW 9		2	2	NO	NO			NO		
1015	MW 1		2	2	NO	NO			NO		
1026	MW 4		2	2	NO	NO			NO		
1810	Pepeekeo		2		NO	NO			NO		
1833	VNP 1		2	2	NO	NO			NO		
1850	CK 3		2	2	NO	NO			NO		
1852	RP 2		2		NO	NO			NO		
17	Hawaii	Moderate	2	2	NO	NO			NO		

20	Hawaii	Moderate	2		NO	NO			NO		
53	Maui	Non-pathogenic	2		NO	NO			NO		
456	CK 2		3	2	NO	NO	NO	NO	NO	NO	NO
467	CK 4		3	2	NO	NO			NO		
548	RP 5		3		NO	NO			NO		
571	RP 4		3		NO	NO			NO		
746	RP 12		3		NO	NO			NO		
757	RP 7		3		NO	NO			NO		
1836	CK 7		3		NO	NO			NO		
0540K	Hawaii	Moderate	3	2	YES	NO			NO		
978	POA 8		3		NO	NO			NO		
561	WV 9		4	2	NO	NO	NO	NO	NO	NO	NO
630	WR 1		4		NO	NO			NO		
766	RP 6		4		NO	NO			NO		
796	WV 19		4		NO	NO			NO		
809	CK 6		4	2	NO	NO			NO		
836	WV 3		4	2	NO	NO			NO		
844	HAL 9		4	2	NO	NO			NO		
899	WV 4		4		NO	NO			NO		
134	KOK 13		5		NO	YES	YES	YES	YES	YES	YES
162	KOK 14		5		NO	NO	NO	NO	NO	NO	NO
343	KOK 18		5		NO	NO			NO		
409	KOK 5		5		NO	NO			NO		
1827	HAN 14		5		NO	NO			NO		
1841	KOK 9		5		NO	NO			NO		
411	PW 2		6		NO	NO	NO	NO	NO	NO	NO
418	PW 3		6		NO	NO			NO		
441	WV 12		6		NO	NO			NO		
623	VNP 13		7	10	NO	NO	NO	NO	NO	NO	NO
649	VNP 15		7		NO	NO			NO		
170	Maui	Nonpathogenic	7	10	NO	NO	NO	NO	NO	NO	NO

246	KOK 11		8		NO	NO	NO	NO	NO	NO	NO
8	Hawaii	Moderate	8		YES	NO			NO		
348	PW 4		9		NO	NO	NO	NO	NO	NO	NO
372	WV 15		9		NO	NO			NO		
393	HAN 9		10		YES	YES			YES		
435	RP 11		10		YES	NO	NO	YES	NO	NO	NO
495	WV 7		11		YES	NO	NO	NO	NO	NO	NO
884	WV 13		11	6	NO	NO			NO		
27	Maui	Nonpathogenic	12	8	NO	NO	NO	NO	NO	NO	NO
45	Hawaii	Nonpathogenic	13		NO	NO			NO		
361	PW 5		14		NO	NO			NO		
1011	MW 12		15		NO	NO	NO	NO	NO	NO	NO
1814	MW 3		16	3	NO	YES			YES		
289	HAN 2		17	7	NO	NO			NO		
1830	POA 7		18	1	YES	YES			YES		
188	KOK 8		19		NO	YES	NO	YES	YES	NO	NO
1807	Lanai		20		NO	NO			NO		
951	POA 5		21	9	NO	NO	NO	NO	NO	NO	NO
1816	POA 1		22		NO	NO			NO		
377	PW 1		23		NO	NO	NO	YES	NO	NO	NO
779	RP 1			3	YES	NO			NO		
1803	Lanai			3	YES	YES			YES		

[1] Isolates virulence assayed by the Hawai'i Agriculture Research Center

2.5 Discussion

This study presents the genomic comparison of a pathogenic isolate of *Fo koae* 44 and a non-pathogenic isolate of *Fo* 170 both collected from the roots of symptomatic koa. These isolates were previously characterized as pathogenic or non-pathogenic based on greenhouse virulence assays on koa seedlings (Dudley et al., 2007). Our study performed whole genome sequencing to assess differences that might distinguish the pathogen from the non-pathogen. The observed high coverage of the genomes was consistent with other fungal assemblies made using only short reads (Walkowiak et al., 2016). The *de novo* assembled genomes of the pathogenic *Fo koae* 44 and non-pathogenic *Fo* 170 isolates had similar sized genomes at 48.18 and 50.63 Mb, respectively. Genome size has been shown to be a putative indicator of pathogenicity in filamentous fungi because generally, pathogens have larger genomes than their non-pathogenic counterparts due to the presence of conditionally dispensable, lineage-specific chromosomes (Raffaele & Kamoun, 2012). Despite these reports, this study found that the non-pathogen (*Fo* 170) had a slightly larger genome in comparison to the pathogen (*Fo koae* 44). Though *Fo* 170 had a larger genome, this study identified unique genes and DNA sequences that were exclusive to *Fo koae* 44. These were identified as transport proteins, enzymes, and signaling proteins, etc. These genomic features allowed for the development of potential molecular markers that allowed for the differentiation of pathogenic *Fo koae* isolates from non-pathogenic *Fo* isolates.

Since both isolates were collected from the same host and are of the same species, based on sequencing data, we assumed that they would share a high level of sequence similarity of conserved genes. However, van Dam et al. (2016 & 2017) found that conserved genes like the *tef1* were good at distinguishing *Fusarium* species but not *F. oxysporum* formae speciales. The polyphyletic nature and the clonal habit of *F. oxysporum* formae speciales allows conserved

(housekeeping) genes to share more sequence similarity with other formae speciales than they do with their own, suggesting that other genes, such as virulence-associated genes, are needed to distinguish them (van Dam et al., 2017). The whole genome phylogeny identified that *Fo koae* 44 and *Fo* 170 were more closely related to other *F. oxysporum* formae speciales than they were to each other. In this study, we found *Fo koae* 44 and *Fo* 170 had a shared average nucleotide identity (ANIm) of 96% and little variation was observed in housekeeping genes. Further, when examining codon usage bias, little variation was observed. The observed 4% variation was not found within the housekeeping genes housed on core chromosomes but rather the conditionally dispensable, lineage-specific chromosomes.

Synteny analyses showed that the 11 putative core chromosomes of *Fo koae* 44 and *Fo* 170 shared synteny with the core chromosomes of the reference strain *F. oxysporum* f. sp. *lycopersici* and to each other. This analysis identified a non-syntenic putative lineage-specific chromosome, labeled chromosome X for *Fo koae* 44 and *Fo* 170. Host-specific virulence-associated genes necessary for developing koa wilt disease were likely localized to chromosome X. The whole genome differences and the presence of putative lineage-specific chromosomes indicated that there was enough variation between *Fo koae* 44 and *Fo* 170 to evaluate virulence factors that may be important for causing disease on koa.

Lineage-specific chromosomes in *F. oxysporum* are found to be usually ~2Mb in length (Ma et al., 2013). The chromosomes X identified here are likely to be a combination of multiple lineage-specific chromosomes, but due to the methodologies used in this study to map to the reference genome, the chromosomes X likely also contain contigs from the assembly that did not map to the reference that may be found on core chromosomes. However, a portion of DNA

found on the X chromosome likely represents one or more lineage specific chromosome for both Fo-koae 44 and Fo 170.

The localization of transposable elements and the lack of housekeeping genes have been used to identify lineage-specific chromosomes in *F. oxysporum* formae speciales (Daboussi & Langin, 1994; van Dam et al., 2017). The putative lineage-specific chromosomes identified in *Fo koae* 44 and *Fo* 170 were found to be repeat- and transposon-rich. Most of the transposons identified localized to chromosome X; Chromosome X of *Fo koae* 44 and Fo 170 had 33% and 63% of the identified transposable elements, respectively, while the core chromosomes had on average 6% and 3% each, respectively. This data, taken together suggests that chromosome X of both isolates are lineage-specific chromosomes. Presti et al. (2015) suggested that these repeat- and transposon-rich chromosomes rely heavily on point mutations for diversity in virulence genes.

Even though 1,353 and 2,369 predicted proteins were identified on the putative chromosomes X of *Fo koae* 44 and *Fo* 170, respectively, the function of most of these genes, unfortunately, could not be determined from the databases we used in this study (Figure 12). It may be that these genes are highly specific to *Fo koae* 44 and *Fo* 170 and are possibly important for pathogenicity and have yet to be described.

Shared virulence-associated genes were identified in *Fo koae* 44 and *Fo* 170. Overall, secondary metabolites and carbohydrate active enzymes were found to be very similar for *Fo koae* 44 and *Fo* 170, suggesting that some mechanisms, such as toxin-production, may not determine pathogenicity to koa. Some well-characterized virulence-associated genes involved in transport, toxin detoxifiers and producers, *GzmetE* (PHI:355) and *Tom1* (PHI:2364), effectors, and transcription factors, such as the *Ftf2* (PHI:5484) and *SGE1* (PHI:3168), were identified in

both *Fo koae* 44 and *Fo* 170. These genes indicate that *Fo koae* 44 and *Fo* 170 both have the capacity for disease development. Virulence-associated genes on the chromosome X of *Fo koae* 44 may be the signifiers of disease development on koa.

Fifty-seven unique predicted genes from *Fo koae* 44 were identified on chromosome X. Various virulence-associated genes involved in transmembrane transport, cell structure, signaling, and transcription regulation were identified (Deng et al., 2017; Thatcher et al., 2016; Ramos et al., 2007). A well-characterized mitogen-activated protein kinase pathway (with genes *Fmk1*, *Ste11*, & *Ste7*) was identified that has been reported as important for host penetration and pathogenicity (Di Pietro et al., 2004). The most interesting *Fo koae* 44 exclusive genes were the *SIX* genes. *SIX* genes have been well-characterized as important host-specific pathogenicity factors in various *F. oxysporum* formae speciales (Ma et al., 2010; Schmidt et al., 2013). Sequence variation and the combination of the 14 known *SIX* effectors have shown to highly contribute to host specificity of pathogenic *F. oxysporum* (Carvalhais et al., 2019; Meldrum et al., 2012). These *SIX* genes in conjunction with their associated transcription factor (*SGE1*) and the mitogen-activated protein kinase pathway may be key characteristics that distinguish pathogenicity on koa.

Fifty-nine unique predicted genes from *Fo* 170 were identified on the putative chromosome X and, similar to *Fo koae* 44, were identified as involved in transmembrane transport, transcription, and signaling. There was a stronger putative emphasis in *Fo* 170 for secondary metabolite production and transport than in *Fo koae* 44. Genes involved toxin production such as trehalose, putrescine, cyclodipeptides, and monooxygenases were identified in *Fo* 170. The presence of more unique toxin producing genes signifies that toxin production may be more important for disease development on the host that *Fo* 170 is pathogenic to.

Interestingly, *Fo* 170 lacked the *SIX* genes identified in *Fo koae* 44 suggesting *Fo* 170 may have lost these important effectors putatively necessary for disease development in koa. However, another secreted peptide, PEP2 (PHI:224), was identified as exclusive to *Fo* 170 and has been found to be important for development of root rot on pea (Han et al., 2001) and was identified on chromosome X of *Fo* 170. These results suggest that *Fo* 170 may not cause wilt symptoms but may be a pathogen on another host.

Since *F. oxysporum* does not have an observed sexual stage, it is thought that horizontal chromosome transfer and repeat and transposon-rich lineage-specific chromosomes are necessary for genetic diversity (Mehrabi et al., 2011; Raffaele & Kamoun, 2012; Rep & Kistler, 2010). Rep and Kistler (2010) described that effector genes localize on a transposon-rich lineage-specific chromosome and that these transposons may be responsible for chromosomal rearrangements and influence effector gene expression through chromatin restructuring. Raffaele and Kamoun (2012) describe that *SIX* gene effectors are found exclusively on lineage-specific chromosomes of other *F. oxysporum* formae speciales. These genes are surrounded by repeat- and transposon-rich regions which allows for higher chances of mutation and duplication of these important effectors (Raffaele & Kamoun, 2012). Our results concur with these observations as we observed the localization of the two *SIX* genes on the repeat- and transposon-rich chromosome X of *Fo koae* 44 (Suga et al., 2013).

To detect pathogenic isolates of *Fo koae*, pathogen-specific primers were developed based on *Fo koae* 44 unique non-coding sequences and exclusive predicted genes. *Fo koae* 44 exclusive predicted pathogenicity-related proteins were not used for the development of pathogen-specific primers due to sequence similarity to other *F. oxysporum* formae speciales. The *SIX1* and *SIX6* genes were analyzed to develop primers but were found to share sequence

similarity of 85% and 97%, respectively to other *F. oxysporum* formae speciales. We chose to develop primers that would amplify genes unique to *Fo koae* 44 that did not have an identified gene ontology terms to reduce the risk of false positive amplification of other *F. oxysporum* formae speciales isolated from koa.

Genes on chromosome X were target for primer development to detect the presence of the lineage-specific chromosome that house putative genes necessary for disease development on koa. We observed that primers developed for the chromosome X were more reliable to distinguish pathogenic isolates than unique genes identified on the core chromosomes. Primer pair P4, which was developed to amplify a unique gene on *Fo koae* 44 putative chromosomes 2, was found to be less stringent than the X chromosome primer pair, P22, that only amplified highly virulent and moderately virulent isolates. P4 amplified two characterized non-pathogenic isolates (HARC: 72 and 81) in addition to the highly and moderately virulent isolates. The highly virulent isolates that amplified were used in HARC's disease resistance breeding program (Dudley et al., 2015). In the greenhouse pathogenicity trials, these highly virulent isolates (HARC:44, 77, 78, and 79) with three moderate isolates (HARC: 76, 90, and 166) are combined evenly by weight and used in combination as a soil inoculum (Dudley et al., 2015). These seven isolates were collected on Hawai'i (44 and 166), Kauai (76, 77, 78, and 79), and Oahu (90) (Dudley et al., 2007). Driven by this methodology (mixing of inoculum from multiple isolates), horizontal chromosome transfer could have shared this putative lineage-specific chromosome among the isolates during inoculation trials conducted by HARC (Han et al., 2001; Mehrabi et al., 2011). This method of genetic transfer may be the cause for the observed relationship of haplotypes with the developed primers.

Of the tested 10 *rpb2* haplotypes, only two haplotypes amplified, respectively. These results indicate that these haplotypes may possess the putative lineage-specific chromosome. The haplotypes that only amplified with primer pairs P4, may contain a homologous gene in their core chromosomes that may not be necessary for virulence. Perhaps, since these primers were developed on unique genes on chromosome X of *Fo koae* 44, rearrangement of this gene across the chromosomes may have allowed these isolates to gain these genes on one of their core chromosomes when they putatively lost the highly virulent lineage-specific chromosome (Mehrabi et al., 2011).

Future studies should include greenhouse validation, population genomic analyses, transcriptomics and karyotyping. Greenhouse virulence assays should be conducted to characterize field collected isolates that amplified with the X chromosome primers to confirm they are highly virulent isolates. Further population genomic analyses would identify how genetically distinct *Fo koae* pathogens and *F. oxysporum* non-pathogens are across the Hawaiian archipelago and would determine if a direct comparison of one pathogen and one non-pathogen is sufficient or if whole genome sequences of representatives of each haplotype are needed to develop a more robust primer. Transcriptomics would identify variation in expression of virulence-associated genes that may be necessary for development of disease on koa to identify better gene targets for primer development and to better inform breeding programs interested in gene for gene interactions. Gene knockouts would contribute to transcriptomics data to allow for the identification of currently unknown genes that were unique to the pathogen to determine their importance for pathogenicity. Since the chromosome X was targeted in this study, karyotyping assays would validate the presence and quantity of the putative lineage-specific chromosome(s) of *Fo koae* 44.

2.6 Conclusion

This study presents, through whole genome analyses, predicted virulence-associated genes that may be necessary for the development of koa wilt disease and developed PCR primers that putatively distinguish highly virulent *Fo koae* isolates from non-pathogenic *F. oxysporum* isolates. The *SIX1* and *SIX6* genes have been well characterized in their role in causing wilt diseases in other hosts and may be necessary for *Fo koae* to cause wilt in koa. In the comparison of *Fo koae* 44 and *Fo* 170, genes identified as exclusive to *Fo* 170 were found to be evidence that *Fo* 170 may be a pathogen to a different host but may not be a wilt-inducing pathogen. Putative lineage-specific chromosomes were identified but further research is needed to further characterize and quantify these chromosomes. Developed pathogen-specific primers were found to amplify haplotypes of characterized pathogenic isolates of *Fo koae*.

Since virulence assays were not conducted on haplotypes from the field isolate collection, further research is needed to determine the robustness of the developed primers and to determine if the predicted virulence-associated genes are necessary for disease development. These results provide the framework for understanding the genes necessary for development of koa wilt disease and the genetic variation of *F. oxysporum* f. sp. *koae* populations across the Hawaiian Islands.

FIGURES

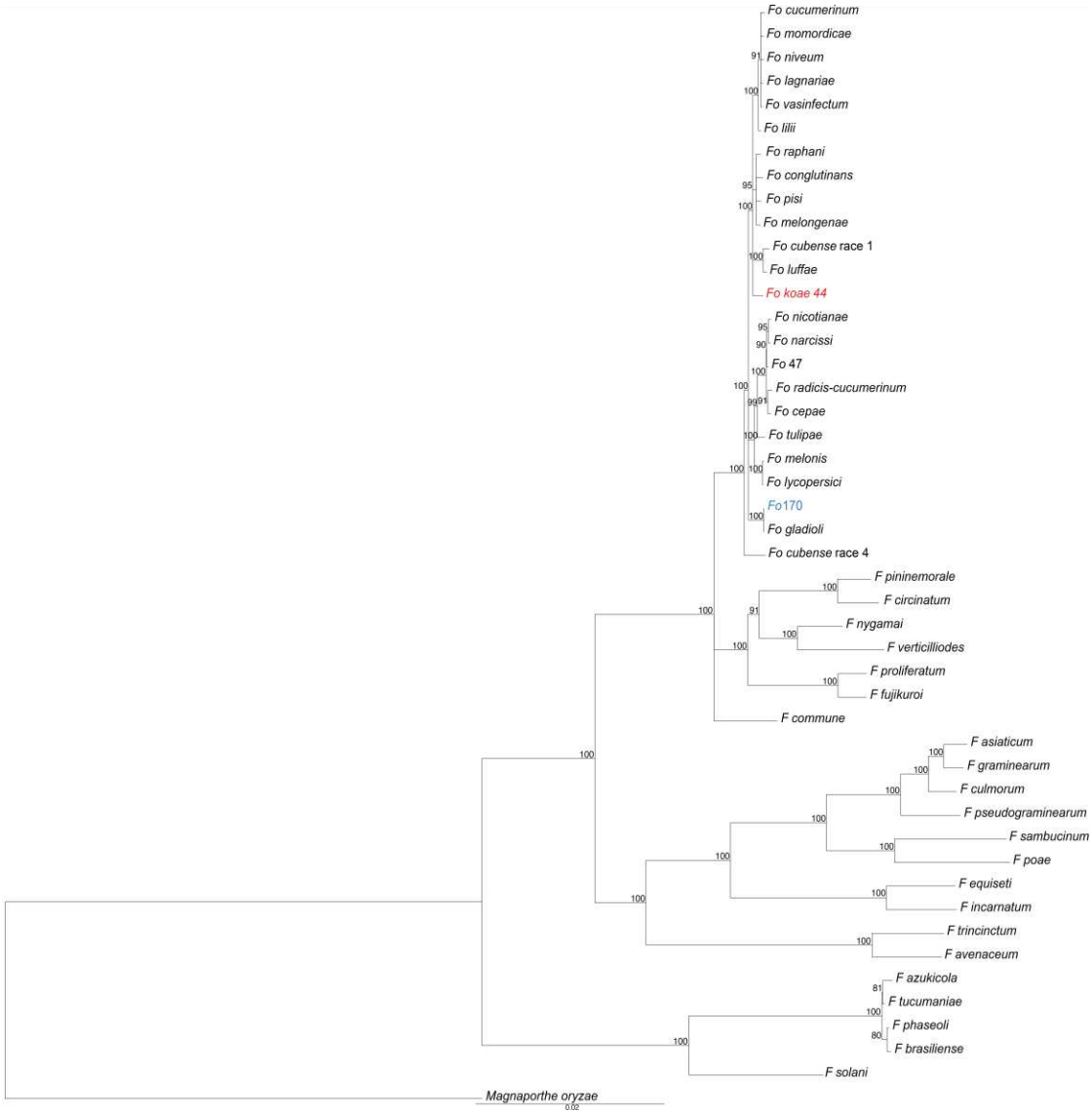

Figure 1 – Maximum likelihood of whole genome phylogeny based on *Fusarium* spp. and *F. oxysporum* (*Fo*) formae speciales. Pathogenic isolate *F. oxysporum f. sp. koae* (*Fo koae* 44) indicated in red and non-pathogenic isolate *F. oxysporum* (*Fo* 170) indicated in blue. Numbers indicate bootstrap values from 200 replicates.

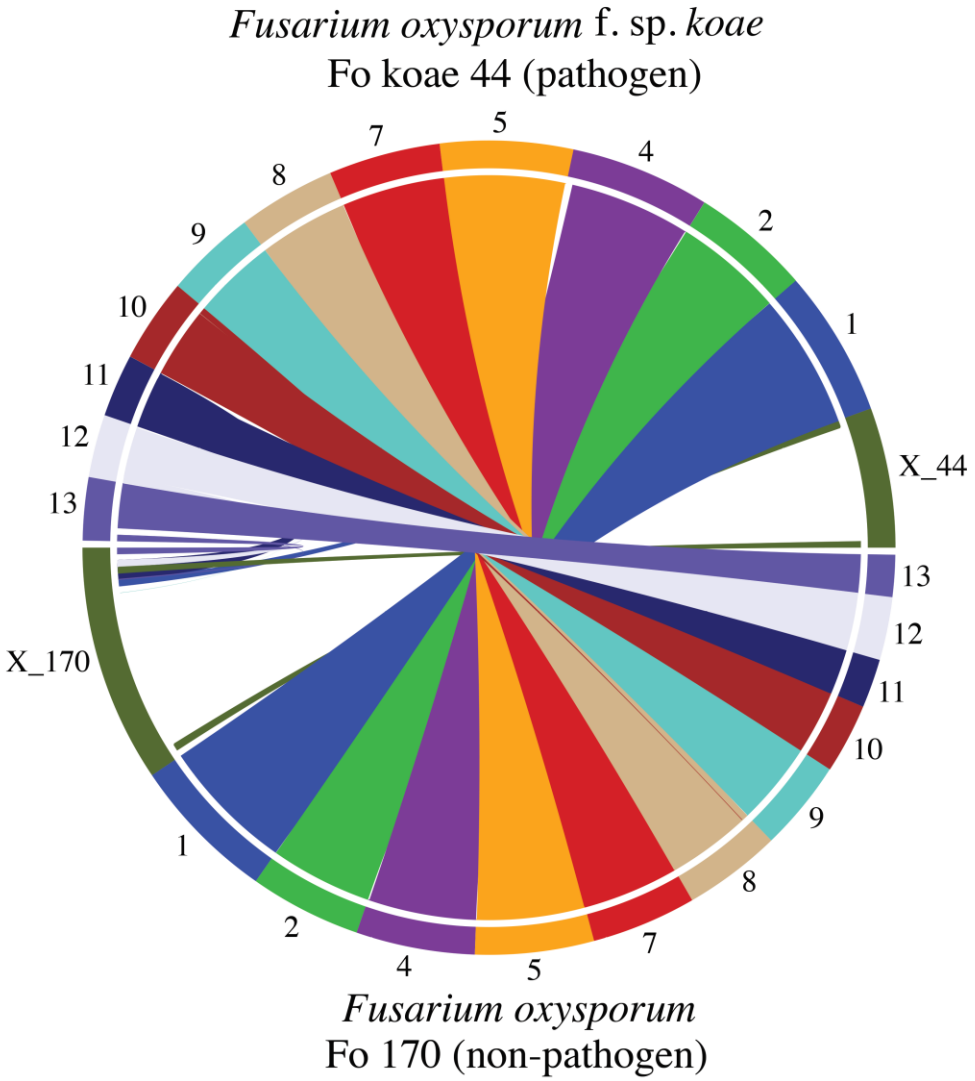

Figure 2 – Synteny map showing synteny between eleven of the putative core chromosomes that mapped to the reference strain of *Fusarium oxysporum* f. sp. *lycopersici*. Most of the putative twelfth chromosome (denoted as X_44 for *F. oxysporum* f. sp. *koae* 44 and X_170 for *F. oxysporum* 170) does not show synteny between the pathogen and non-pathogen and did not map to the reference strain. This lack of synteny on the putative twelfth chromosome is indicative of a conditionally dispensable, lineage-specific chromosome.

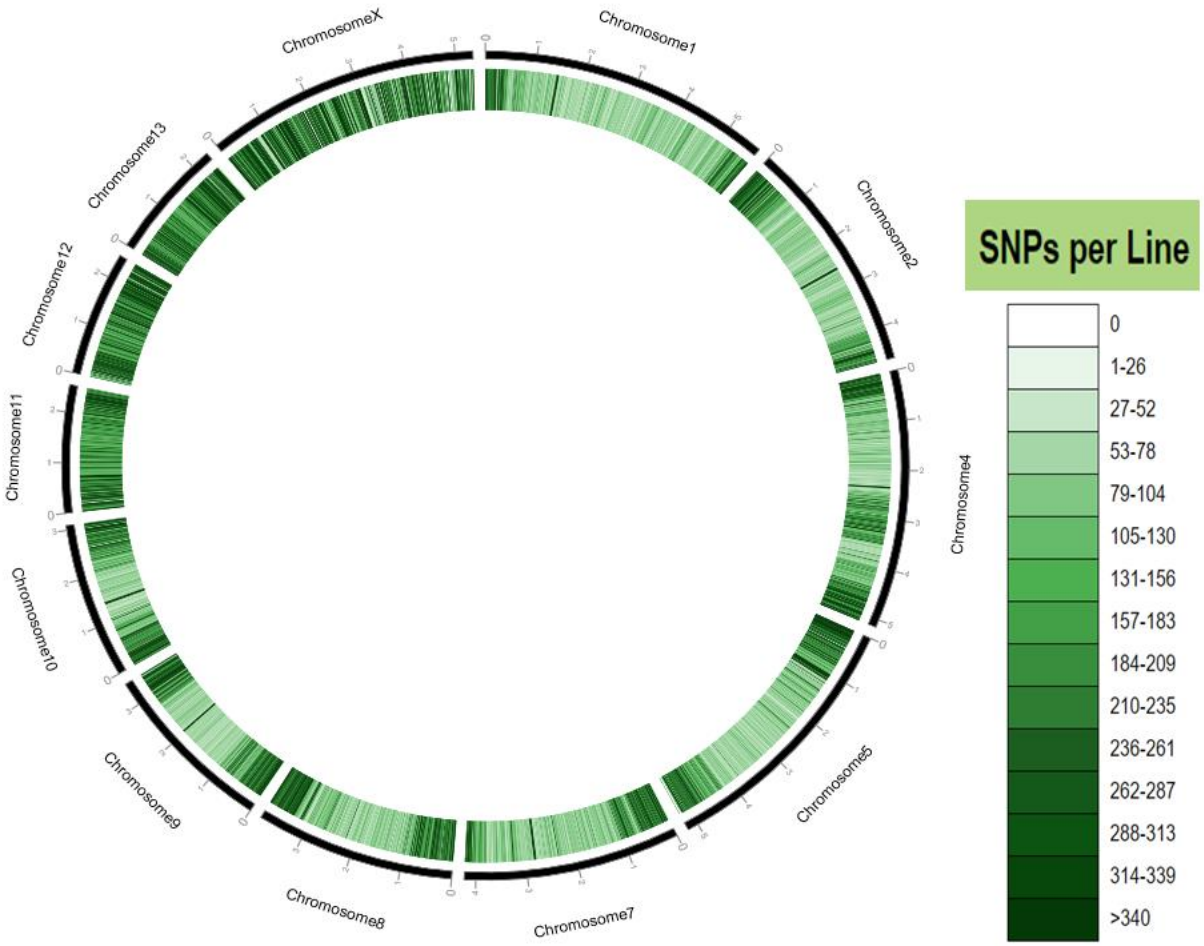

Figure 3 – Distribution of variants (single nucleotide polymorphisms, SNPs) comparing the putative chromosomes of the pathogenic *Fusarium oxysporum* f. sp. *koae* isolate (*Fo koae* 44) and non-pathogenic *F. oxysporum* isolate (*Fo* 170). Darker bars indicate more SNPs identified in that region. Chromosomes 11, 12, 13, and X show the highest SNP densities.

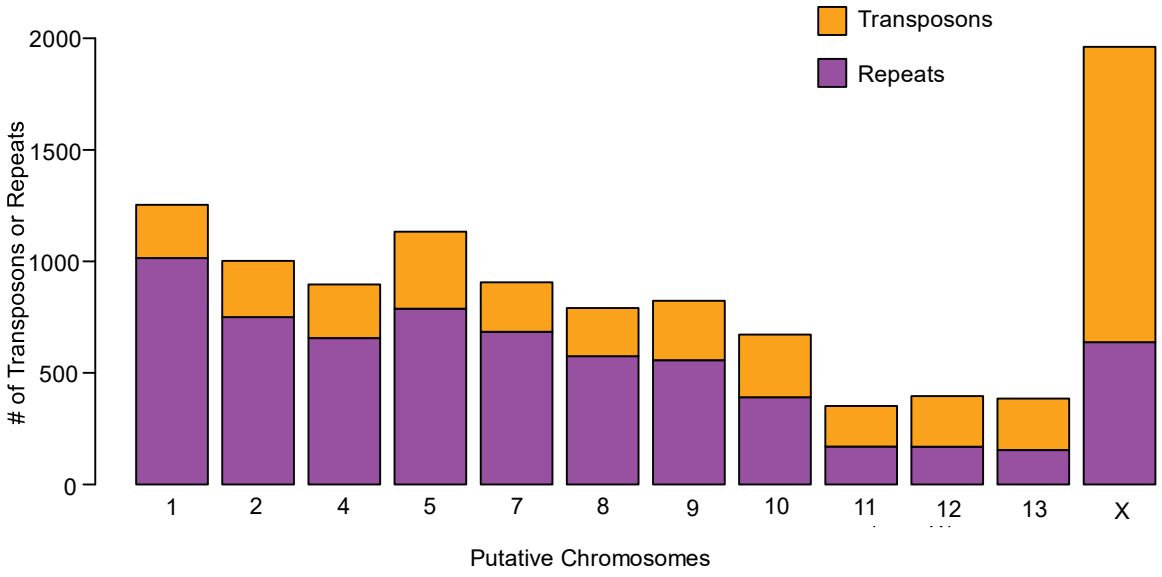

Figure 4 A – Distribution of transposons (orange bars) and repeats (purple bars) across the putative chromosomes in *Fusarium oxysporum* f. sp. *koae* (*Fo koae* 44). Majority of the transposons localize on the putative lineage-specific chromosome X.

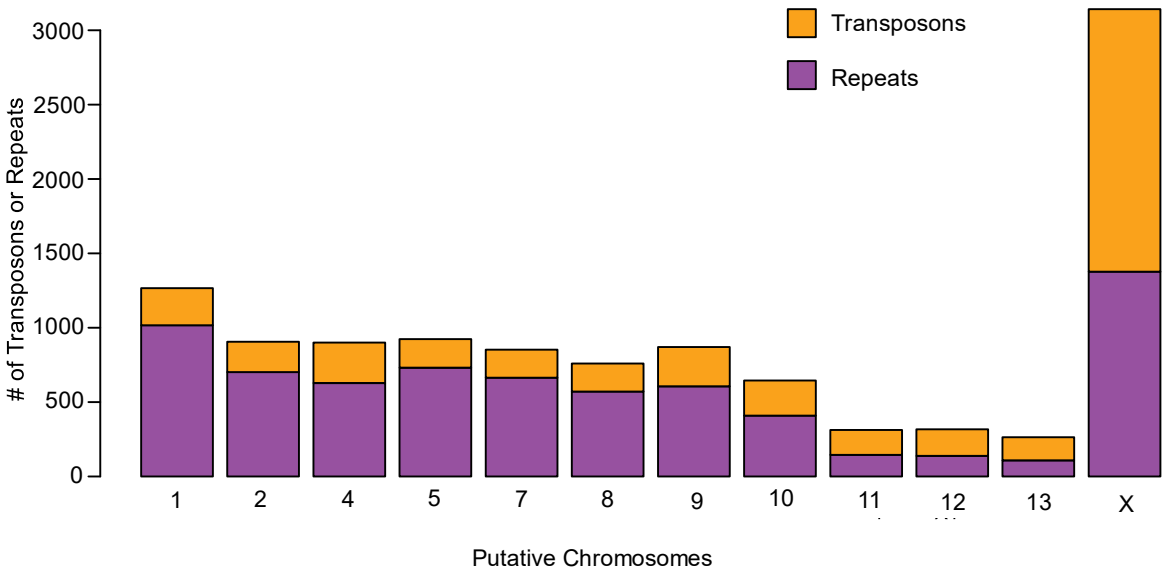

Figure 4 B – Distribution of transposons (orange bars) and repeats (purple bars) across the putative chromosomes in *Fusarium oxysporum* (*Fo* 170). Majority of the transposons localize on the putative lineage-specific chromosome X.

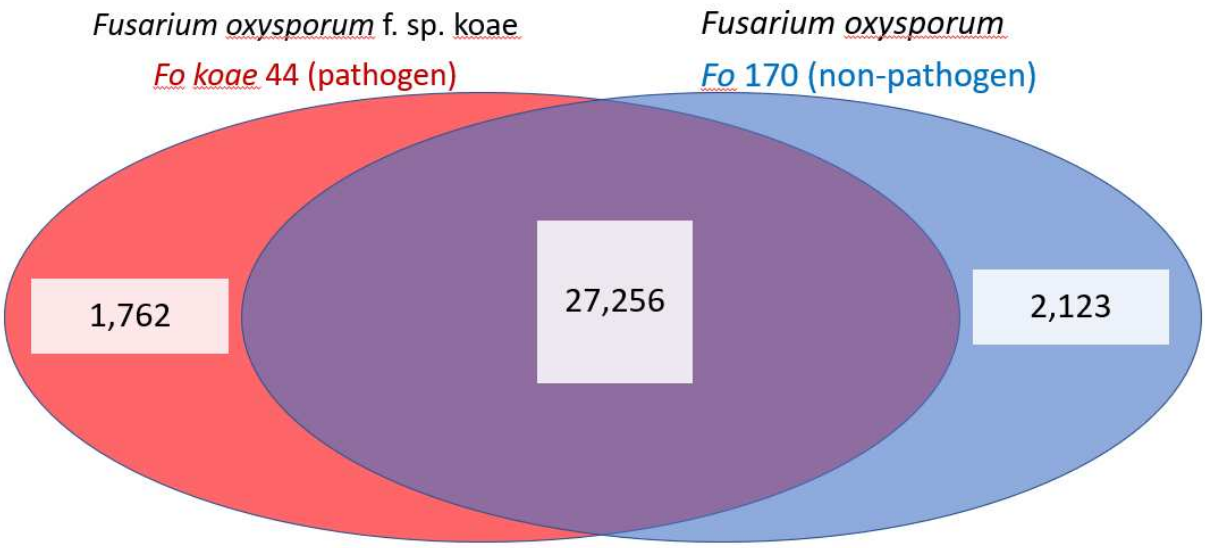

Figure 5 – Distribution of orthologous and non-orthologous proteins comparing the koa wilt pathogen, *Fusarium oxysporum* f. sp. *koae* (*Fo koae* 44) and the non-pathogenic isolate of *Fusarium oxysporum* (*Fo* 170). Numbers are indicative of proteins unique (to either side) or shared (center column).

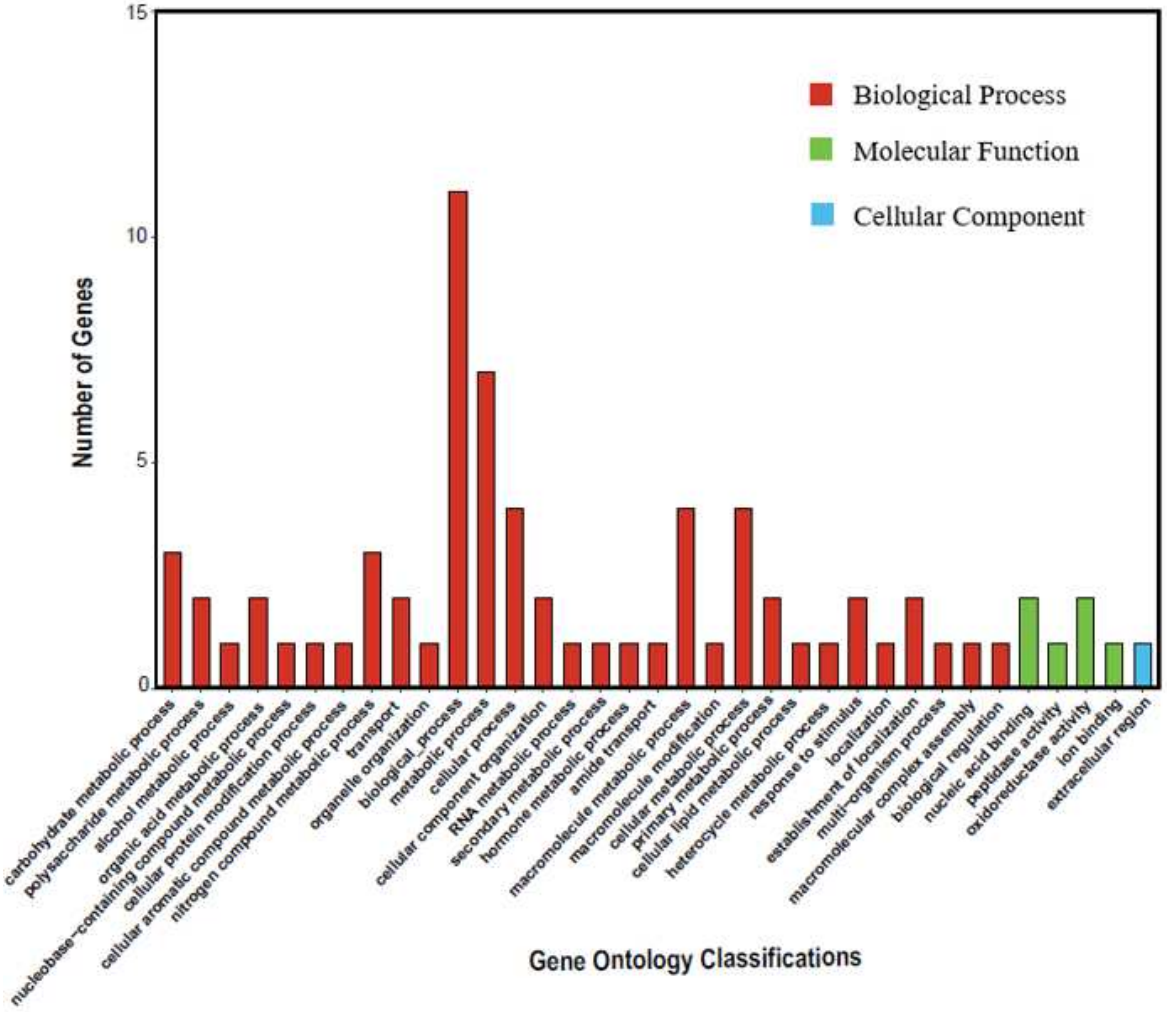

Figure 6A – Putative biological, molecular, and cellular function of the predicted non-orthologous proteins identified as unique to the pathogenic isolate of *Fusarium oxysporum* f. sp. *koae* (*Fo koae* 44) when compared to non-pathogenic isolate of *F. oxysporum (Fo* 170). Identified function based on gene ontology (GO) terms.

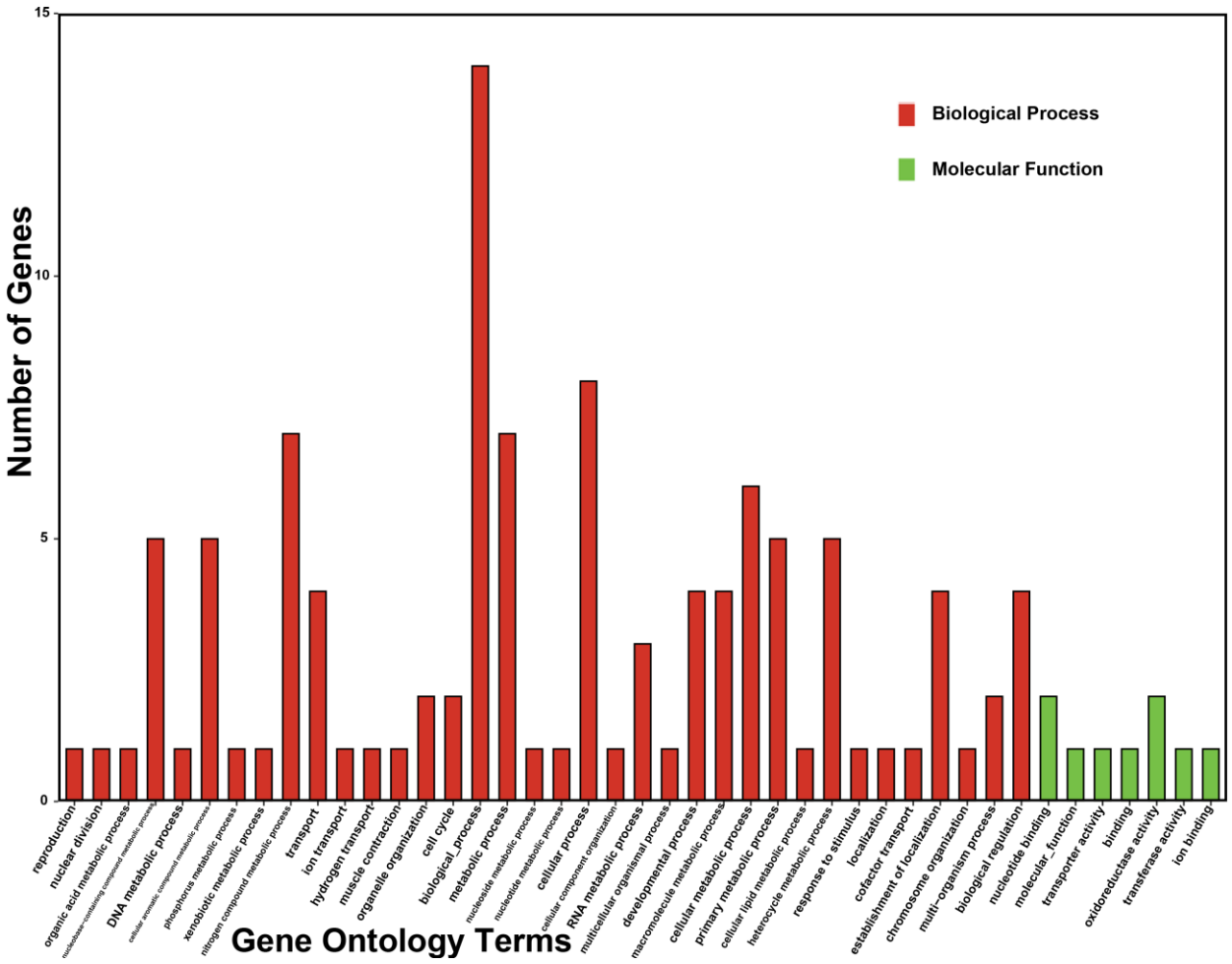

Figure 6B – Putative biological process and molecular function of the predicted non-orthologous proteins identified as unique to the non-pathogenic *Fusarium oxysporum* isolate (*Fo* 170) when compared to the pathogenic isolate of *F. oxysporum* f. sp. *koae* (*Fo koae* 44). Function based on gene ontology (GO) terms.

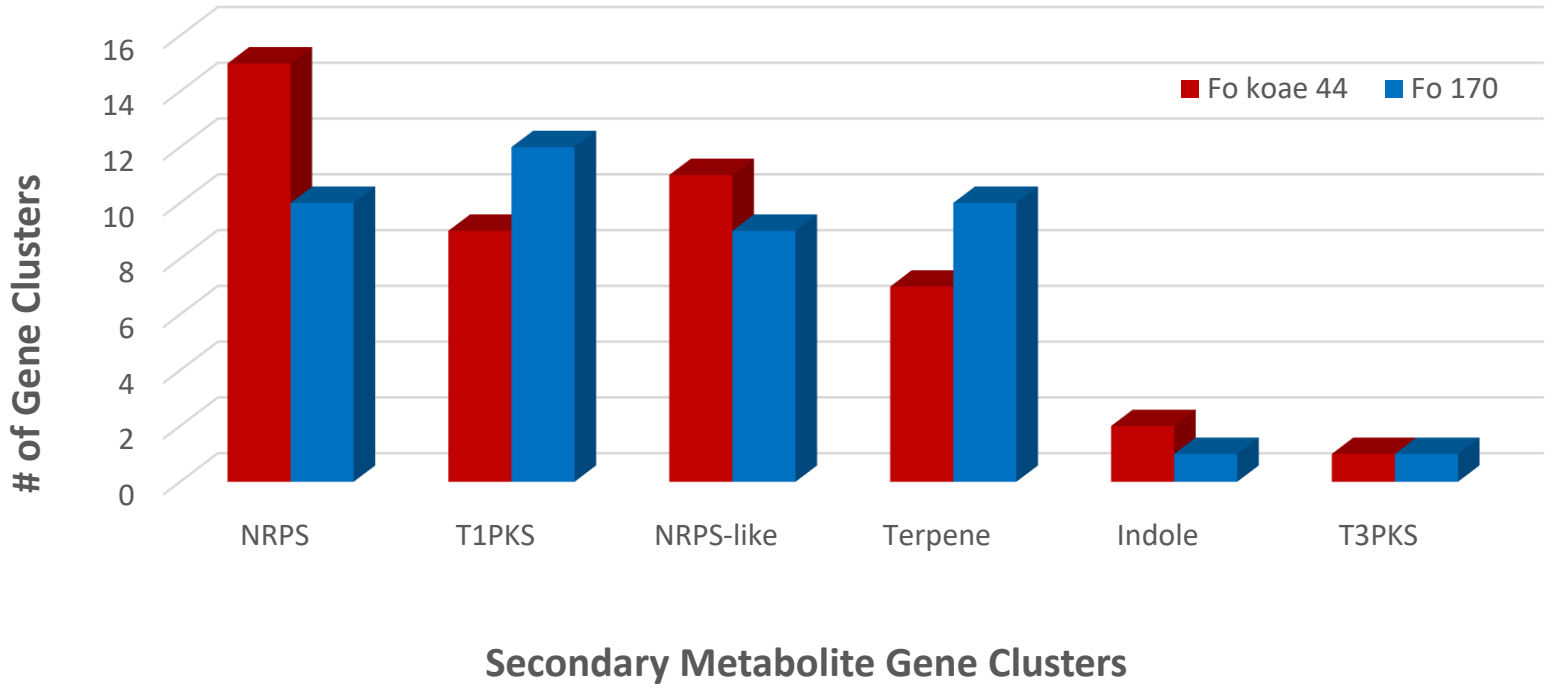

Figure 7 – Copy number of secondary metabolite genes including nonribosomal peptide synthetases (NRPS), type 1 and type 3 polyketide synthases (T1PKS and T3PKS), terpenes, and indoles for the pathogenic *Fusarium oxysporum* f. sp. *koae* isolate (*Fo koae* 44, red bars) and non-pathogenic *F. oxysporum* isolate (*Fo* 170, blue bars).

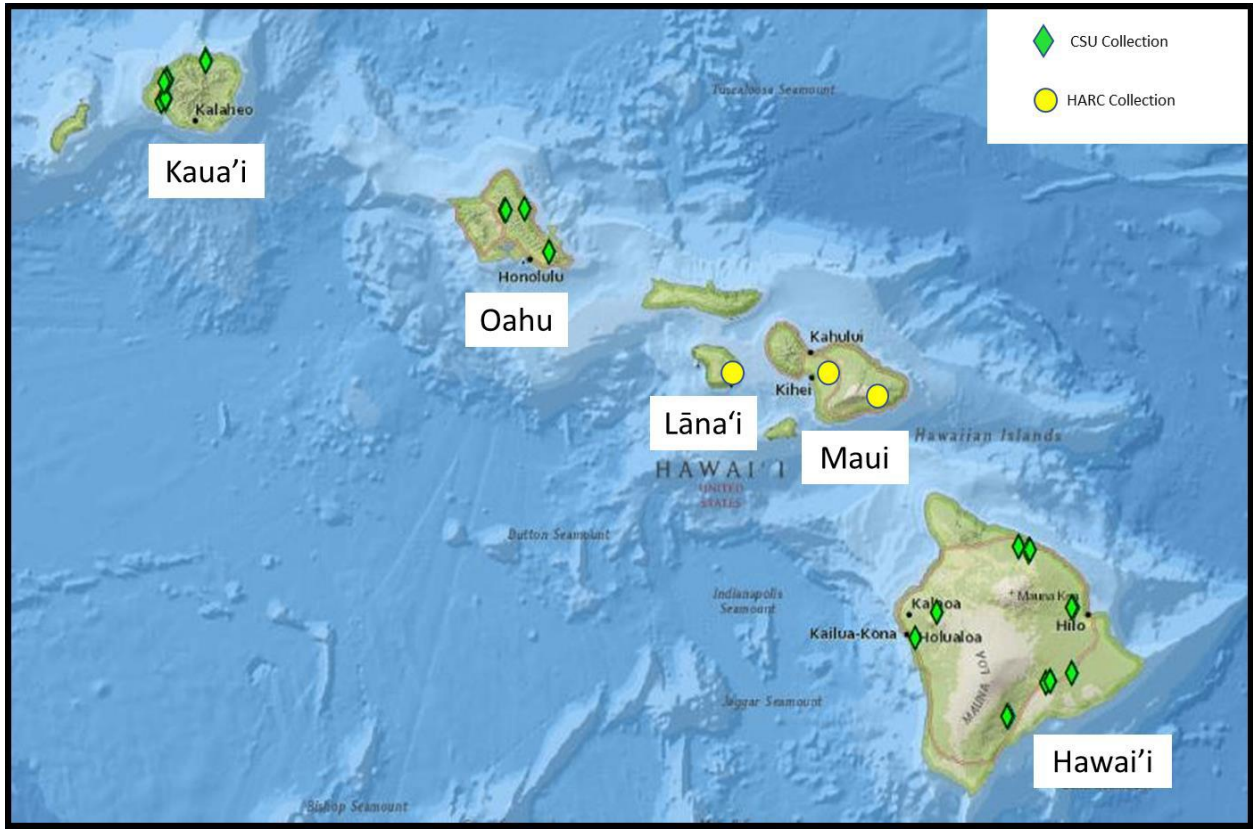

Figure 8 – Field isolate collection map identifies individual trees that were sampled for the pathogenic *Fusarium oxysporum* f. sp. *koae* isolates in this study (green diamonds). Two field sites on Kauai, three field sites on Oahu, and eight field sites on Hawai'i island. Yellow circles indicate Hawaii Agriculture Research Center's (HARC) 2017 & 2018 collection from 2 two field sites on Maui and one on Lāna'i.

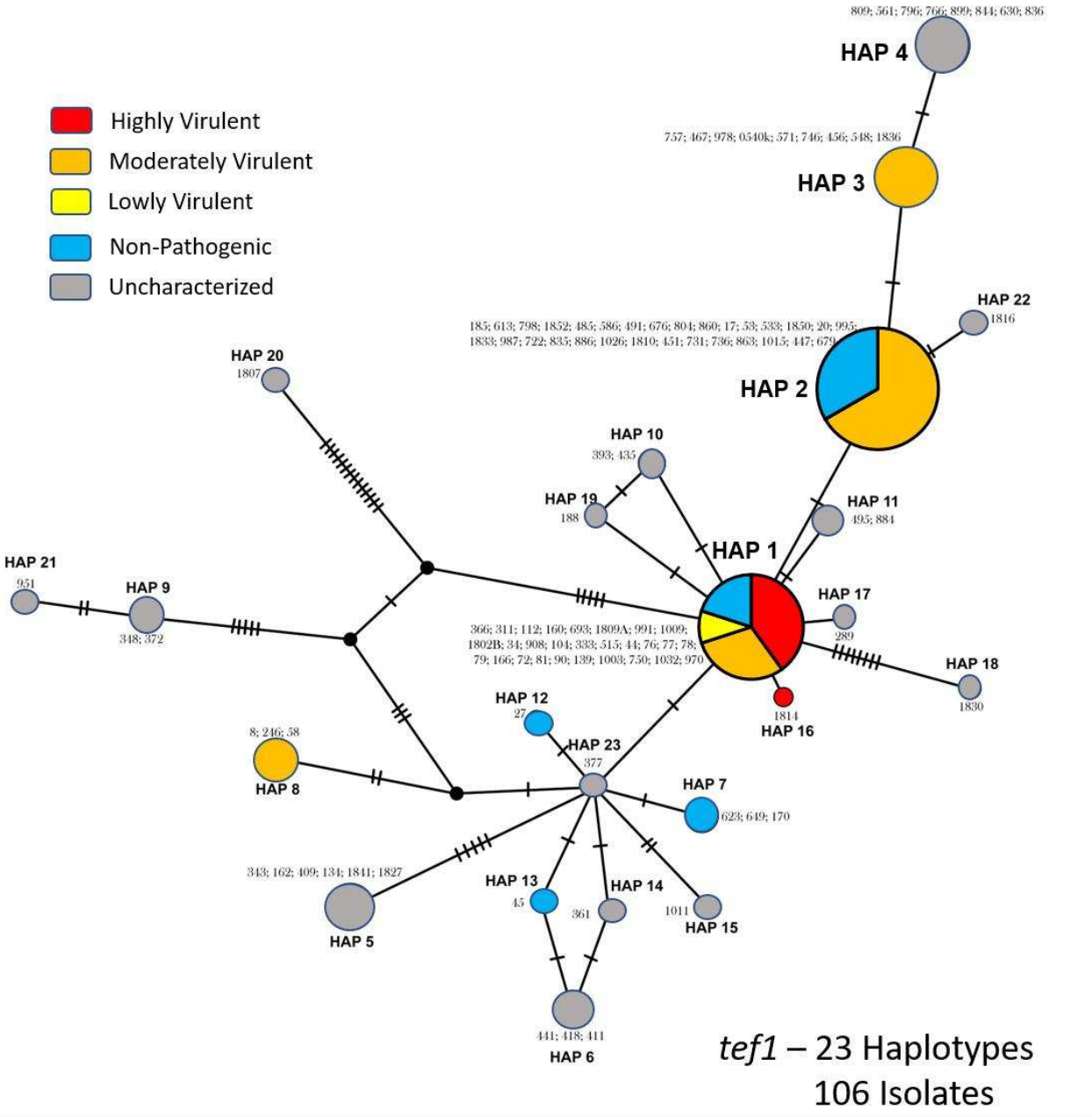

Figure 9 - Translation elongation factor 1 - α (*tef1*) haplotype network consisting of 23 haplotypes identified from 106 *Fusarium oxysporum* and *F. oxysporum* f. sp. *koae* isolates including 89 field collected isolates and 17 characterized isolates (high, moderate, and low virulence and non-pathogenic). All highly virulent isolates localized to HAP 1 but this haplotype also consisted of characterized moderately and lowly virulent and non-pathogenic isolates. *Tef1* haplotypes were inconsistent with virulence phenotype.

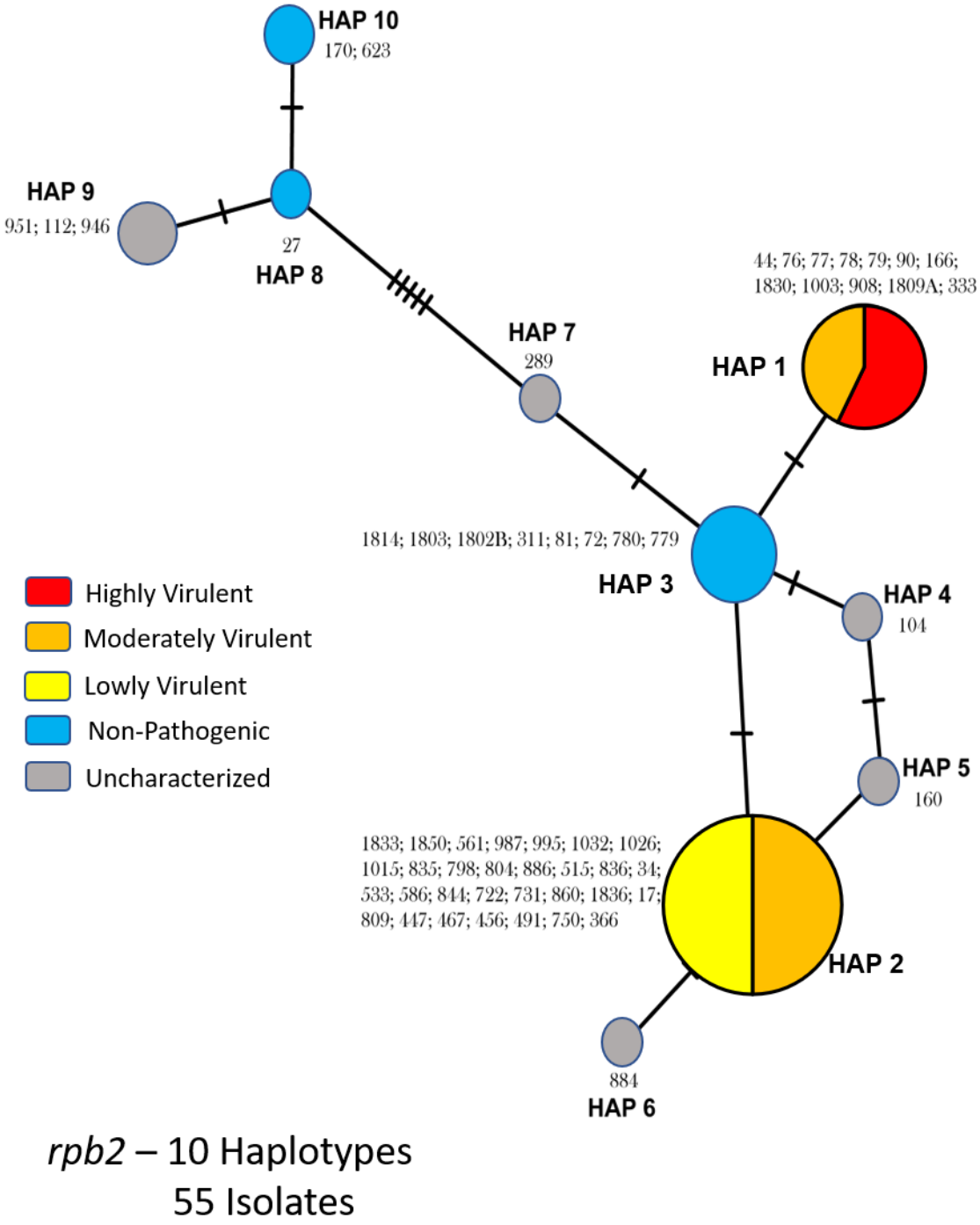

Figure 10 - RNA polymerase II second largest subunit (*rpb2*) haplotype network. Ten haplotypes were identified from 55 *Fusarium oxysporum* and *F. oxysporum* f. sp. *koae* isolates including 41 field collected isolates and 14 characterized isolates (high, moderate, and low virulence and non-pathogenic). All highly virulent isolates clustered in the HAP 1 haplotype along with characterized moderately virulent isolates. Characterized non-pathogenic isolates clustered in three separate haplotypes (HAP 3, 8, & 10). One characterized lowly virulent and one

moderately virulent isolate clustered in HAP 2. *Rpb2* haplotype network showed a clear pattern of pathogenic and non-pathogenic haplotypes.

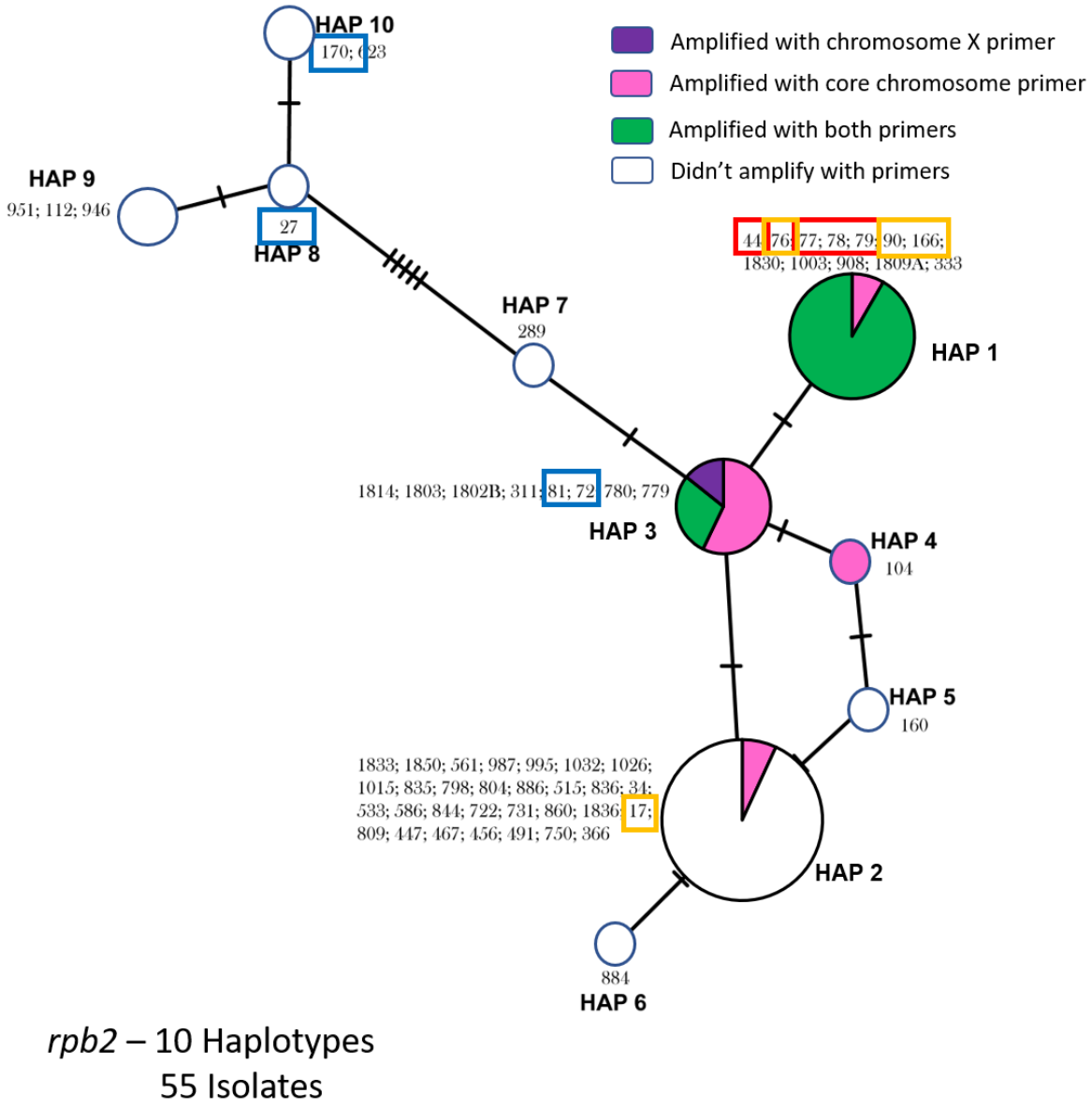

Figure 11 - RNA polymerase II second largest subunit (*rpb2*) haplotype network overlaid with core chromosome primer pair (amplification in pink) and X chromosome primer pair (amplification in purple). Of the 10 haplotypes identified only two amplify with the X chromosome primer pair. All but one of the highly virulent *F. oxysporum* f. sp. *koae* (*Fo koae*) amplified with the X chromosome primer. Uncharacterized isolates in HAP 3 also amplified with the X chromosome primer. Core chromosome primers amplified four haplotypes including the all of the highly virulent isolates but also two non-pathogenic isolates in HAP 3.

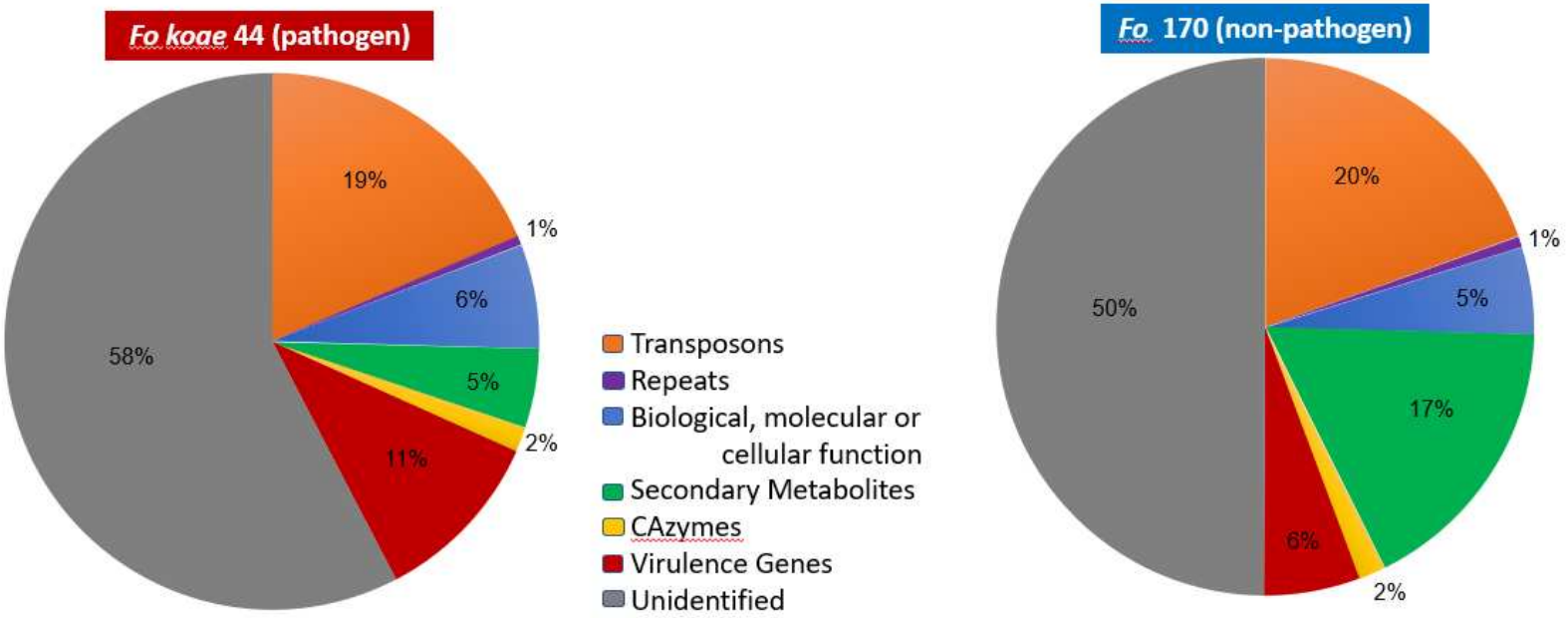

Figure 12 – Proportion of predicted genes with putative function on the putative lineage-specific chromosome X of the *Acacia koa* wilt pathogen *Fusarium oxysporum* f. sp. *koae* (*Fo koae* 44) and the *F. oxysporum* (*Fo* 170) isolate characterized as non-pathogenic to *A. koa*. Both isolates had similar proportions of transposons, repeats, carbohydrate active enzymes (CAZymes), and genes identitifed through gene ontology terms and protein family as having a biological, molecular, or cellular function. *Fo koae* 44 had a higher proportion of virulence associated genes and *Fo* 170 had a higher proportion of secondary metabolite genes on the X chromosome.

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