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Fusarium spp. diversity associated with symptomatic *Acacia koa* in Hawai'i

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Abstract

Fusarium spp. comprise diverse plant pathogens, which affect both agriculturally and ecologically important plant species, and harmless saprobes. *Fusarium* spp. inhabit widely ranging, geographic regions across the globe and can be found in soil or plant tissue in every climate type, ranging from tropical to polar regions. In their native habitats, *Fusarium* spp. are typically innocuous and ubiquitous soil saprophytes; however, *Fusarium* spp. can become devastating pathogens, especially after introduction into novel environments, where hosts are not adapted to *Fusarium* infection. Hawai'i is among the most isolated regions on Earth. Due to this isolation, many of Hawai'i's native plant species are not well adapted to cope with introductions of novel flora, fauna and pathogens. Pathogens, including *Fusarium* spp., have had a major impact on *Acacia koa* (koa), an iconic tree species in Hawai'i's forests. Multiple *Fusarium* spp. have been identified in association with koa, some of which have been screened for pathogenicity to koa. This study identified new associations of *Fusarium* spp. with koa root samples including *F. verticillioides*, *F. redolens*, *F. anthophilum*, *F. babinda*, *F. commune*, *F. fujikuroi* and *F. lactis* and suggests that a complex of *Fusarium* spp. may contribute to disease severity. Potential spread of putative pathogens across the Hawaiian Islands is suggested on the basis of haplotype network analysis.

1 | INTRODUCTION

Fusarium spp. have been described as devastating pathogens of crops and trees in the Hawaiian archipelago. Multiple *Fusarium* species have been collected and morphologically identified from *Acacia koa* (koa), one of the most iconic forest tree species endemic to Hawai'i (James 2006). Some of these *Fusarium* isolates including *F. oxysporum* f. sp. *koae* (*Fo koae*), the koa wilt pathogen, have been screened for pathogenicity (Dudley et al., 2007). Pathogenic *Fo koae* and non-pathogenic *F. oxysporum* strains have been identified on all islands where koa is grown and planted (Dobbs et al. 2020). Possible inter- and intra-island introductions of pathogenic *Fo koae* may be responsible for the increased genetic variation observed among *Fo koae* strains, and increased genetic variation may exacerbate disease damage, especially within tree populations that lack natural resistance to new *Fo koae* strains.

Koa wilt disease has negatively impacted natural stands and plantations of koa in Hawai'i. The endemic koa tree is of prominent economic, cultural, and ecological importance to Hawai'i. Native Hawaiians used koa trees as the primary wood for the construction of outrigger canoes. Today, koa timber is used in woodcraft, interior design, and fine wood working, which contributes to Hawai'i's timber industry that was last valued at $30 million annually (Dudley et al., 2007; Ishihira et al. 2017). Currently, koa

inhabits only a quarter of its possible habitat examined through climate tolerances across all of the main Hawaiian Islands (except Niihau and Kahoolawe), and its stand density continues to decrease (Gugger et al., 2018). This continuing decrease in koa not only presages future economic hardship to Hawai'i's forest industry, but it could also lead to the extirpation of an endemic Hawaiian species if left unmanaged. For the health and sustainability of koa populations in Hawai'i, it is critical to understand the koa-associated *Fusarium* species and their role in koa wilt/dieback, other diseases and other ecological functions.

Genetic characterization of *Fusarium* isolates provides important information for nursery/timber managers and land stewards for developing management strategies for koa forests and plantations. A broad range of *Fusarium* species have been identified on koa based on morphology (Dudley et al., 2007; James et al., 2006; Nelson et al., 1983). Thirteen morphologically distinct *Fusarium* species have been collected from koa (James et al., 2006), and four have been found to be pathogenic, including *F. oxysporum* Snyder & Hansen, *F. solani* (Martius) Snyder & Hansen, *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas and *F. semitectum* Berkeley & Ravenel (Dudley et al., 2007). These pathogenic *Fusarium* spp. have been found on multiple islands, which raises a question as to whether *Fusarium* spp. could have been transferred to and between islands via anthropogenic means. Identifying routes of *Fusarium* spread is critical for developing informed management strategies to prevent further introductions and limit spread of novel pathogenic strains. One likely avenue of *Fusarium* spread is through the establishment of koa plantations. As an important commercial commodity, establishing koa timber plantations has been increasing, especially in reclaimed sugarcane and pineapple lands (Ishihara et al., 2017). The escalating interest in growing/restoring koa has increased the demand for koa nursery stock for silvicultural operations and other outplantings. This demand may have inadvertently contributed to the spread of undetected pathogens associated with asymptomatic koa seedlings that are grown for inter-island plantings (James et al. 2007a). Nursery stock infected with *F. oxysporum* has been found on all major Hawaiian Islands in tree nurseries, plantations and natural forests (Dudley et al., 2007). For this reason, understanding the composition and diversity of *Fusarium* spp. pathogens and saprobes associated with koa is essential for informed koa breeding and restoration programmes.

Pathogenic *Fusarium* spp. on koa have been identified in previous studies (Dudley et al., 2007; James, 2004; James et al. 2007b), but further analyses of their genetic identification and diversity are needed to determine if pathogenic *Fusarium* spp. are being spread across the Hawaiian Islands, which could undermine the restoration of koa stands. The objectives of this research were to (1) identify *Fusarium* species diversity associated with symptomatic koa roots through genetic characterization; and (2) identify potential spread of haplotypes across the main Hawaiian Islands. Identifying species composition and understanding the spread of *Fusarium* species will encourage the development of measures for proper nursery sanitation and prevention of inter-island spread of pathogenic *Fusarium* spp. associated with koa seedlings.

2 | METHODS

2.1 | Isolate collection

Isolates for this study were provided by the Hawai'i Agriculture Research Center (HARC) from five islands (Kauai, Oahu, Maui, Lanai, and Hawai'i) and also included collections from a survey conducted in 2018 by Colorado State University on symptomatic koa roots from three islands, Kauai, Oahu and Hawai'i (Dobbs et al. 2020). Isolates were grown on ¼-strength Potato Dextrose Agar (PDA) in Petri dishes in the dark for 7 days before they were putatively identified as *Fusarium* species based on colony morphology (Nelson et al., 1983). Isolates morphologically characterized as *Fusarium* were selected for genetic characterization.

2.2 | Genetic characterization of isolates

In preparation for DNA extraction, isolates were sub-cultured on ¼-strength PDA in Petri dishes and maintained at 25°C in darkness for 3 days. DNA was extracted from isolates using a Chelex® extraction protocol, modified from Brewer and Milgroom (2010). A mycelial scrape of each isolate was added to PCR strip tubes with 100 µl of a sterile 5% Chelex-100 resin (Bio-Rad, Hercules) in solution and ground using a 10 µl pipette tip. Samples were thermolysed in a Mastercycler ProS thermocycler (Eppendorf) at 100°C for 35 min. Template DNA was taken from the upper aqueous layer to avoid Chelex-100 resin contamination, which can inhibit PCR. To determine the genetic relationships among isolates, one conserved genomic region, the translation elongation factor 1- α (*tef1*) (O'Donnell et al., 2004), was amplified using a PCR cycle programme of 94°C for 2 min, 40 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 30 s, and 72°C for 5 min. Products were electrophoresed on a 1.5% agarose gel to visualize amplified PCR product using GelRed® (Biotium). Products were sent to Eurofins Genomics for forward and reverse sequencing.

2.3 | Species identification and haplotype network construction

Geneious (Geneious Prime 2019.2, http://www.geneious.com/) was used to generate consensus sequences from 5' and 3' *tef1* contigs. Consensus sequences were BLASTed to the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) and Fusarium-ID (http://isolate.fusariumdb.org/blast.php) to identify *Fusarium* species. Species identities were assigned based on *tef1* sequences with >97% identity and 90% coverage.

Sequences of the *tef1* were aligned using MUSCLE alignment in Geneious (Edgar, 2004). DnaSP 6 was used to estimate number of haplotypes for each *Fusarium* spp. (Rozas et al., 2017). A statistical parsimony (TCS) haplotype network was constructed based on the *tef1* sequence data using PopART with a 95% genetic cut-off, similar to that used in Dobbs et al. (2020).

3 | RESULTS

3.1 | Genetic characterization of isolates

A total of 213 isolates including 131 from the 2018 survey and 82 provided by HARC were collected from symptomatic koa (Table 1). The majority of isolates, 112, were collected from the island of Hawai'i; 44 isolates were collected from Oahu; 36 from Kauai; 15 from Maui; and six isolates from Lanai. Based on BLAST results of *tef1* sequences from NCBI and Fusarium-ID database, we identified 13 *Fusarium* species from the five islands surveyed: *F. anthophilum* (A. Braun) Wollenweber (Hawai'i; one haplotype), *F. babinda* Summerell, Rugg & Burgess (Hawai'i and Kauai; three haplotypes), *F. commune* (Maui and Hawai'i; two haplotypes), *F. fujikuroi* Nirenberg (Hawai'i; two haplotypes), *F. lactis* Pirotta & Riboni (Hawai'i; one haplotype), *F. oxysporum* (Kauai, Oahu, Maui, Lanai, and Hawai'i; 14 haplotypes), *F. proliferatum* (Matsushima) Nirenberg (Kauai, Maui, and Hawai'i; five haplotypes), *F. solani* (Kauai, Oahu, and Hawai'i; 16 haplotypes), *F. sterilihyphosum* Britz, Marasas & Wingfield (Hawai'i; one haplotype), *F. subglutinans* (Hawai'i; one haplotype), *F. verticillioides* (Saccardo) Nirenberg (Lanai; one haplotype), *F. redolens* Wollenweber (Hawai'i; two haplotypes) and *F. lateritium* Nees (Kauai; one haplotype) (Table S1). Most of these species have been described as plant pathogens (Leslie & Summerell, 2006). *Fusarium* species composition differed among the islands (Table S2). Hawai'i island had the most diversity in *Fusarium* spp.; however, more diverse sites were sampled on this island. Likewise, Lanai had the least diversity in *Fusarium* spp., but isolates were only collected from a single location.

3.2 | Haplotype network

To identify potential spread of *Fusarium* isolates between or among Hawaiian Islands, haplotype networks were constructed, based on *tef1* sequence data, for eight (*F. oxysporum*, *F. babinda*, *F. commune*, *F. fujikuroi*, *F. proliferatum*, *F. solani*, *F. subglutinans* and *F. redolens*) of the 13 *Fusarium* species that were represented by multiple isolates collected from symptomatic koa roots from five of the main Hawaiian Islands (Figure 1). The *F. oxysporum* network analysis resulted in 14 haplotypes that comprised 168 isolates overlaid with island distribution. The haplotype composition of each island was analysed to identify possible spread. The distribution of haplotypes 1, 4, 6, 8, 9, 10, 13 and 14 provided indications of potential spread among the Hawaiian Islands, because these haplotypes were observed across multiple islands. *Fusarium oxysporum* was collected from each island surveyed and had the highest genetic diversity; however, this species is considered the koa wilt pathogen, which was a focus in the surveys of symptomatic koa (Table S1). The *F. solani* network analysis resulted in 16 haplotypes that comprised 18 isolates from three islands (Hawai'i, Kauai, and Oahu). Although genetic diversity was high within sites on different islands, only one *F. solani* haplotype (Hap_12) showed an indication of potential spread across the Hawaiian Islands. The haplotype network of *F. solani* shows more genetic variation compared to *F. oxysporum*. The *F. proliferatum* network analysis resulted in five haplotypes that comprised nine isolates from three islands (Hawai'i, Kauai and Maui) with no indication

TABLE 1 Number of *Fusarium* isolates collected from symptomatic koa (*Acacia koa*) roots from five of the main Hawaiian Islands

Fusarium/Fungal species	Kauai (#)	Oahu(#)	Maui (#)	Lanai (#)	Hawai'i (#)	All (#)	All (%)	Haplotypes (#)
F. anthophilum					1	1	0.5	1
F. babinda	1				2	3	1.4	3
F. commune			1		1	2	0.9	2
F. fujikuroi					3	3	1.4	2
F. lactis					1	1	0.5	1
F. oxysporum	25	36	12	5	90	168	78.5	14
F. proliferatum	4		2		3	9	4.2	5
F. solani	5	8			5	18	8.4	16
F. sterilihyphosum					1	1	0.5	1
F. subglutinans					2	2	0.9	1
F. verticillioides				1		1	0.5	1
F. lateritium	1					1	0.5	1
F. redolens					4	4	1.9	2

Note: Species identification based on translation elongation factor 1- α (*tef1*) sequences using BLAST against the NCBI and confirmed with Fusarium-ID database. Estimated number of haplotypes was calculated for each *Fusarium* spp.

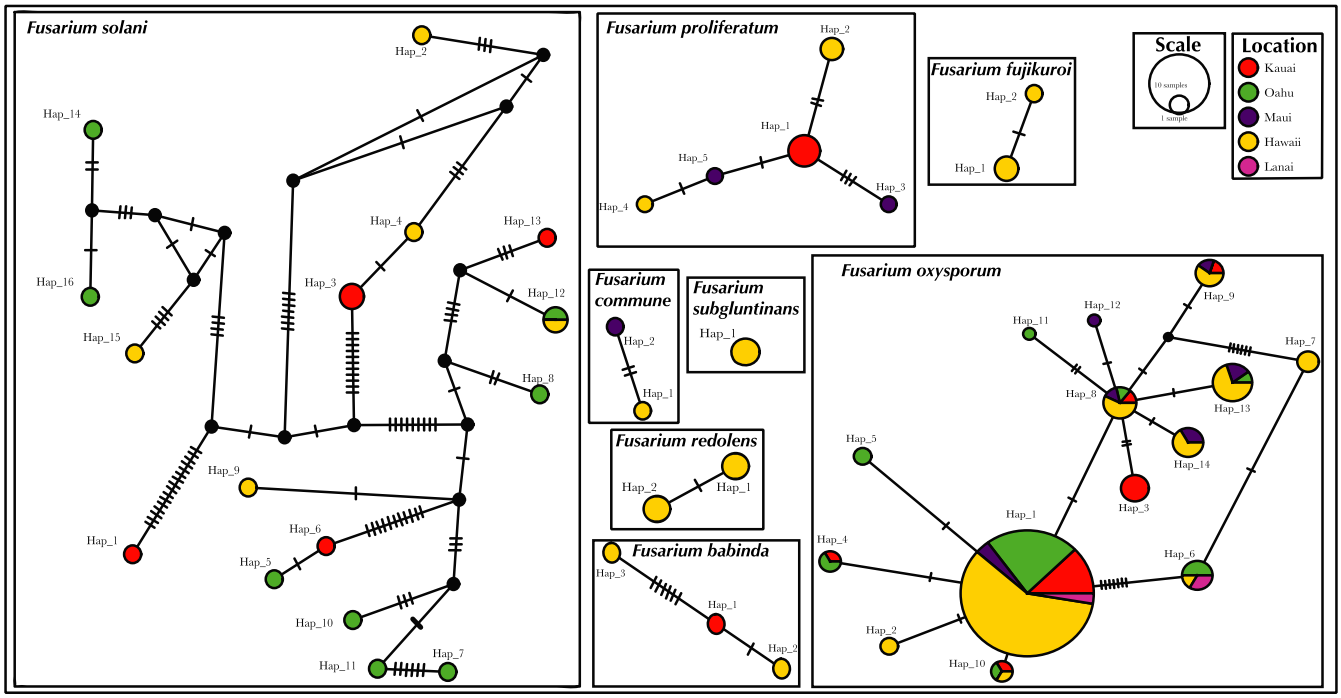

FIGURE 1 Translation elongation factor 1-α (*tef1*) haplotype networks were constructed for eight [*F. oxysporum* (168 isolates), *F. babinda* (3 isolates), *F. commune* (2 isolates), *F. fujikuroi* (3 isolates), *F. proliferatum* (9 isolates), *F. solani* (18 isolates), *F. subglutinans* (2 isolates) and *F. redolens* (4 isolates)] of the 13 *Fusarium* species collected from symptomatic koa (*Acacia koa*) roots from five Hawaiian Islands (Kauai, Oahu, Maui, Hawai'i and Lanai). *Fusarium verticillioides*, *F. lateritium*, *F. sterilihyphosum*, *F. anthophilum* and *F. lactis* only had a single isolate. All haplotypes were interconnected with identified single nucleotide polymorphisms denoted by hash marks. Each haplotype (denoted Hap) was analysed by inter-island composition to identify possible spread. *Fusarium oxysporum* had the most haplotypes that indicate possible spread, likely because koa wilt was a target for the survey with multiple symptomatic koa trees surveyed per island. Haplotypes 1, 4, 6, 8, 9, 10, 13 and 14 show indications of potential spread across the Hawaiian Islands. One haplotype, Hap_1, in the *F. solani* network showed an indication of spread. No indications of spread were found in other *Fusarium* spp., but these species were underrepresented in the surveys

of potential inter-island spread. The network analyses for *F. fujikuroi* and *F. redolens* each resulted in two haplotypes that comprised three and four isolates, respectively, from Hawai'i island. The *F. commune* network analysis also resulted in two haplotypes that comprised two isolates, one from Hawai'i and one from Maui, with no indication of potential spread. The *F. babinda* network analysis resulted in three haplotypes, each isolate from two islands (Kauai and Hawai'i) represented its own haplotype. The *F. subglutinans* network analysis resulted in one haplotype from Hawai'i island that comprised two isolates. Only single isolates of *F. verticillioides*, *F. lactis*, *F. lateritium*, *F. anthophilum* and *F. sterilihyphosum* were collected, which did not warrant a network analysis.

4 | DISCUSSION

This study presents findings of 13 *Fusarium* species collected from symptomatic koa roots and identifies possible spread of some *Fusarium* spp. across the Hawaiian Islands. The genus *Fusarium* comprises species complexes that alone and/or in association can cause diseases on many important plant species, which are both agriculturally and ecologically important worldwide (Leslie & Summerell, 2006). Some of the identified *Fusarium* species (i.e. *F. proliferatum*, *F. oxysporum*, *F. subglutinans* and *F. solani*) from this study have been shown to have low-to-moderate virulence on koa and may work in concert to cause disease and mortality along with the highly virulent strains of *Fo koae* (James et al., 2006; Dudley et al., 2007; unpublished data). Some of the *Fusarium* species, including *F. oxysporum*, were found on multiple islands and showed genetic diversity that may also contribute to disease severity as some haplotypes are likely more virulent than others (Figure 1). *Fusarium solani* and *F. oxysporum* had the highest genetic diversity based on our haplotype analyses. Haplotypes of *F. solani* showed less spread, potentially indicating limited movement between islands or that extraneous haplotypes were maladapted to survive transport or growth in other sites. Greater genetic variation in the haplotype network of *F. solani* compared to *F. oxysporum* could be the result of higher levels of single nucleotide polymorphisms (SNPs) among haplotypes or more introductions of distinct genotypes of *F. solani*. In contrast, haplotypes of *F. oxysporum* were identified on all the islands studied, which may indicate that concurrent spread to other islands occurred with *Fo koae* isolates belonging to one haplotype, Hap_1. Also, noteworthy is that known non-pathogenic isolates were found in limited distribution, belonging to distinct haplotypes (Hap_12, 13 and 14), suggesting a different host range or recent introductions. Movement of

Fusarium isolates, which are not pathogenic to koa, into koa forests may have been limited because of a variation in host preference, such as when agricultural plants are hosts of the isolate. Characterizing *Fusarium* composition allows for the development of informed management programmes by providing a better understanding of the distribution and genetic relationships of pathogenic *Fusarium* spp.

Morphology has been widely used for species identification of *Fusarium*, but morphology alone is considered unreliable because some diagnostic characteristics are inconsistent (e.g. polyphialides in *F. commune*; Stewart et al., 2006). Genetic characterization through DNA barcoding has become more widely used for *Fusarium* species identification due to its increasing accuracy, reliability and affordability. The *tef1* locus used in this study to genetically characterize isolates has been described as a reliable genetic barcode for fungal diversity, especially *Fusarium* spp. (Boutigny et al., 2019). James et al. (2004, 2006, 2007a, 2007b) primarily used colony morphology to identify 16 *Fusarium* spp. [*F. oxysporum*, *F. graminearum* Schwabe, *F. semitectum*, *F. equiseti* (Corda) Saccardo, *F. solani*, *F. proliferatum*, *F. subglutinans*, *F. avenaceum* (Fries) Saccardo, *F. acuminatum* Ellis & Everhart, *F. sambucinum* Fückel, *F. sporotrichioides* Sherbakoff, *F. sterilihyphosum*, *F. chlamydosporum* Wollenweber & Reinking, *F. decemcellulare* Brick and *F. lateritium*] collected from various components of koa trees, from the rhizosphere to the phyllosphere. Many of these koa-associated *Fusarium* species are described as plant pathogens (James, 2004). Similarly, we also collected *F. oxysporum*, *F. lateritium*, *F. proliferatum*, *F. solani*, *F. sterilihyphosum* and *F. subglutinans* in this study. In contrast, however, we collected a greater species diversity that included *F. verticillioides*, *F. redolens*, *F. anthophilum*, *F. babinda*, *F. commune*, *F. fujikuroi* and *F. lactis*.

The newly found koa-associated *Fusarium* spp. have been found as plant pathogens on various hosts and innocuous soil saprophytes. *Fusarium verticillioides* is found worldwide as a pathogen on maize (Leslie & Summerell, 2006). *Fusarium redolens* is a common soil-borne fungus causing root rot on many hosts including beans (*Phaseolus*), peas (*Pisum*) and carnations (*Dianthus*) (Leslie & Summerell, 2006). *Fusarium commune* was found to be the causal agent of root rot and damping-off in conifer species (Stewart et al., 2006). *Fusarium fujikuroi* has been found to be a rice (*Oryza*) pathogen that causes bakanae disease (Leslie & Summerell, 2006) and *F. lactis* causes fig (*Ficus*) endosepsis (Leslie & Summerell, 2006). *Fusarium babinda* is common in rainforest soils and plant debris (Leslie & Summerell, 2006), while *F. anthophilum* is also a common saprophyte and not considered as a plant pathogen (Leslie & Summerell, 2006). Strains of these plant pathogenic *Fusarium* species have displayed varying virulence that influences their impact as a cause or contributor to disease development.

Haplotype networks are useful tools to identify potential spread of pathogens (Ramdial et al., 2017). Using conserved genomic regions, such as *tef1*, is useful because these regions are not subjected to high selective pressure and typically do not develop SNPs rapidly, like other regions of the genome (e.g. virulence genes; Ramdial et al., 2017). The generated haplotype networks based on the *tef1* provide evidence that *Fusarium* isolates of some pathogenic species, including *F. proliferatum*, *F. oxysporum*, *F. subglutinans* and *F. solani* have spread among islands. Haplotypes of *F. oxysporum*, *F. proliferatum* and *F. solani* were identified on multiple islands surveyed, and these species have been found to be pathogenic to koa with varying levels of virulence (Dudley et al., 2007; James, 2004). The spread of *F. oxysporum* and *F. solani* haplotypes could have easily occurred through anthropogenic means (e.g. inter-island tourism, trade and movement of plants and/or soil) and the spread of these pathogen haplotypes has likely contributed to the reduction in health of koa stands within their native range.

Koa-dominated forests have been diminishing across the Hawaiian archipelago due to increasing disease impacts and overharvesting of natural stands. To prevent the loss of an iconic Hawai'i tree species, millions of koa seedlings were grown in a few state and federal nurseries, including the Bishop Estates Nursery, and dispersed across the Hawaiian Islands beginning in the early 1900s (Walters, 1978). The harvesting of koa seed and distribution of regenerated seedlings across the islands represents a potential pathway for introducing pathogenic *Fusarium* to diverse areas across the Hawaiian Islands, where native koa stands may possess little resistance to the introduced pathogenic *Fusarium* species and strains.

This study provides a framework for identifying *Fusarium* and assessing the putative spread of potentially pathogenic *Fusarium* among the Hawaiian Islands. Resistance breeding and restoration programmes for koa should incorporate a diversity of *Fusarium* pathogens with representative genetic diversity and genetically diverse koa to potentially limit koa susceptibility to novel *Fusarium* strains that could be introduced. High-resolution genetic markers could monitor/assess pathogen spread more precisely at the species/sub-species level, and pathogenicity tests on koa are needed to establish pathogenicity of the untested *Fusarium* spp. Genetic tools are valuable for identifying pathogenic strains of *Fusarium* and reducing the need for lengthy greenhouse virulence trials (Dobbs et al. 2020). Development of tools to identify diverse pathogenic genotypes of *Fusarium* would greatly aid breeding programmes in the development of robust, disease-resistant koa and improve forest health by reintroducing koa to regions under potentially high disease conditions.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data is submitted to NCBI Genbank. Genbank numbers for sequences are listed in Supplemental Table 1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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