AFLP, Pathogenicity, and VCG Analyses of *Fusarium oxysporum* and *Fusarium pseudocircinatum* from *Acacia koa*

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Abstract

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Acacia koa (koa), a native tree in Hawaii, suffers from a dieback caused by *Fusarium oxysporum*. Pathogenicity tests, vegetative compatibility group (VCG) tests, and amplified fragment length polymorphisms (AFLP) analyses were conducted on *Fusarium* isolates recovered from diseased koa. Koa seedling mortality with individual strains ranged from 0 to 85%, with 42% of the strains killing no seedlings. Thus, strains of *F. oxysporum* recovered from dying koa trees may or may not be virulent. In addition to *F. oxysporum*, *F. pseudocircinatum* strains were isolated from diseased koa; however, they were either nonvirulent or had weak virulence. This is the first

report of *F. pseudocircinatum* in Hawaii. The 46 strains of *F. oxysporum* and *F. pseudocircinatum* were grouped into 16 VCGs, but 86% of the highly virulent strains belonged to VCG 2. In AFLP analyses, strains from the same VCG generally clustered with one another. Identification of the same set of strains using VCG, AFLP, and pathogenicity tests showed that the highly virulent strains are genetically close and that high virulence toward koa is not a property of all strains of *F. oxysporum*. Thus, VCG 2 with the corresponding AFLP data is a significant biological entity for which we propose the name *F. oxysporum* f. sp. *acaciae* to reflect its virulence on koa.

Koa (*Acacia koa* A. Gary) is indigenous to Hawaii and is the second most abundant overstory tree in Hawaiian forests in the middle to upper elevations. This species is extremely important in Hawaii in terms of economics, culture, and ecology. The forest industry in Hawaii, which is worth \$30 million per year, relies heavily on timber from koa for the production of wood products (9). Koa also is a key species in the forest ecosystem, as it protects Hawaii's watersheds and provides niches for many native birds and snails, including many of Hawaii's endangered species (9). Koa forests, however, have been experiencing serious wilt and dieback. Decline of Hawaiian forests, which are composed primarily of koa and ohia (*Metrosideros polymorpha* Gaud.), was first reported in the early 1970s (3,16,17). The decline is characterized by a rapid wilt and dieback and/or a slow continuous decline with decreased foliage and dead twigs, followed by complete defoliation and death. Suggested causal agents of the forest decline include the fungi *Fusarium oxysporum* (Schlechtend.) emend. Snyder & Hansen, *Armillaria mellea* (Vahl:Fr.) P. Kumm., *Diatrype princeps* Penz. & Sacc., and *Phytophthora cinnamomi* Rands. The insects implicated in decline include *Xylosandrus compactus* Eichhoff (black twig borer) and *Plagithmysus bilineatus* Sharp, a native wood-boring beetle. The activities of wild pigs and other mammals (presumably rats) also have been suggested as causal agents (16). Abiotic factors such as changes in soil drainage have been mentioned as possible contributing factors (16).

F. oxysporum is one of the most common and important plant pathogens in the world, and causes diseases on many crop plants in Hawaii. Some examples of formae speciales and diseases caused by *F. oxysporum* in Hawaii include: Fusarium yellows on asparagus caused by *F. oxysporum* f. sp. *asparagi*, Panama disease/wilt

on banana caused by *F. oxysporum* f. sp. *cubense*, wilt on carnation caused by *F. oxysporum* f. sp. *dianthi*, wilt on tomato caused by *F. oxysporum* f. sp. *lycopersici* (11,23). *F. oxysporum* also has been suggested as a cause of koa dieback in addition to weather and other environmental factors (1,10). Throughout the world, both pathogenic and nonpathogenic strains of *F. oxysporum* are commonly found in soils. This species is often isolated from asymptomatic crop roots (12).

For *Fusarium*, vegetative compatibility has been used to study population structure, host-pathogen systems, and reproduction within a *Fusarium* species (4,30). Vegetative compatibility is a form of self/non-self recognition in fungi (18,19). Formae speciales of *F. oxysporum* are often correlated with only one or a few vegetative compatibility groups (VCG) (19), whereas soil populations of *F. oxysporum* are much more diverse, with most strains belonging to a unique VCG (8). In addition, the analysis of amplified fragment length polymorphisms (AFLP) is a powerful tool for detecting genetic variation within a *Fusarium* population (17,19,28). Previous studies have reported that subgroups in a *Fusarium* population detected by VCG tests often are the same groups identified following AFLP analyses (2,29).

It is important to protect koa trees from dieback disease because of their economic, cultural, and ecological value, but little is known about the origin, distribution, or etiology of the disease and its causal agent(s). In this study, we focus on *F. oxysporum*, which has been proposed as a possible cause of the disease (1,10). The objectives of this study were: (i) to confirm that some strains of *F. oxysporum* are pathogenic to koa (strains from the earlier studies [1,10] are no longer available), and (ii) to distinguish pathogenic and nonpathogenic strains of *F. oxysporum* isolated from diseased koa.

Materials and Methods

Sample collection and fungal identification. In October 2008, samples were collected from internal tissues of roots and trunks of five mature koa trees from the College of Tropical Agriculture and Human Resources (CTAHR) Hamakua Research Station on the island of Hawaii. The sample trees showed typical wilt and dieback

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symptoms. Approximately 50% of the branches of each sample tree exhibited wilt and dead tissue. The plant samples were cut into small pieces, approximately 5 mm³, disinfested by soaking in a 0.6% NaOCl solution for 1 min, and then plated on 1.5% water agar in 90-mm petri dishes. After 48 h, fungal hyphae emerged from the specimen pieces and grew on the water agar. Individual hyphal tips were transferred from the water agar plate to a 50-mm petri plate containing 10% (vol/vol) vegetable juice (V8 juice, Campbell Soup Co., Camden, NJ), 2.4 mM CaCO₃, and 1.5% (wt/vol) Bacto agar (Difco, Detroit, MI), and grown at 24°C under continuous fluorescent illumination. After 1 to 3 weeks, the *Fusarium* cultures that emerged were collected and the species of each isolate was determined based on morphology. The DNA sequence of the translation elongation factor-1 α (*tef-1*) gene (19) was used to confirm the identity of isolates morphologically identified as *Fusarium oxysporum*.

Pathogenicity test. Koa seeds were collected from a single open-pollinated mother tree with some dieback symptoms, in the Kona Hema Preserve on the island of Hawaii. To disinfest surface and stimulate germination, the seeds were put in 95°C water, and soaked in the cooling water overnight. Seeds were planted in sterilized tubes (38 × 210 mm) in Sunshine mix (Mix #4, Sun Gro Horticulture Canada Ltd., Vancouver, BC, Canada) and fertilized once every 6 weeks with Osmocote (14-14-14, The Scotts Company LLC, Marysville, OH). One month after germination, seedlings which were approximately 5 cm tall were selected and used for inoculation tests. Forty-three *Fusarium* strains were used for the inoculations. The *Fusarium* cultures were grown in 50-mm petri dishes containing 10% V8 agar at 24°C under continuous fluorescent illumination. After 7 days, sterile distilled water was added to the petri dishes and the spores were gently removed from the agar surface with a rubber spatula. The solution was filtered through a single Kimwipe (Kimberly-Clark Co., Irving, TX) to filter out mycelia and pieces of agar. Spores were counted with a hemacytometer and diluted to 1 × 10⁵ spores/ml with sterile distilled water. Ten milliliters of this spore suspension was used to drench koa seedlings under greenhouse conditions, for a final inoculum level of 10⁶ spores of each strain. Ten seedlings were inoculated per strain. As a control, 10 ml of sterile distilled water was added to each of 10 koa seedlings for each test. Seedlings were evaluated for disease symptoms for 3 months. Symptoms were scored as: 6, dead; 5, wilting and dying; 4, heavy defoliation; 3, dead leaf tip, bleached, partial defoliation, and/or partially wilting; 2, small root system, small stem lesions, stunted, tilted; 1, yellow leaves, and/or small yellow leaf freckling. When a seedling displayed multiple symptoms, the highest symptom score was used as its evaluation. As seedlings died, the fungal pathogen was reisolated from internal tissues of the infected stems and roots. After 3 months, fungi were reisolated from all of the surviving seedlings. Waller-Duncan *k*-ratio *t* tests were used to separate mean differences of symptom scores among isolates. All statistical analyses were conducted at *P* < 0.05, with SAS Version 9.1 (SAS Institute Inc., Cary, NC).

VCG test. From each *Fusarium* isolate, *nit* mutants were generated on chlorate medium as previously described (6,19). The concentration of KClO₃ used in the medium was 1.5, 2, 2.5, 3, 3.5, or 4%. Normally, 1.6 g of L-asparagine was added per liter of chlorate medium as a nitrogen source. When an isolate produced no *nit* mutants on the medium, either L-proline was added as an alternate nitrogen source (15) or no nitrogen was added at all. The isolates were incubated at 25°C for 14 to 28 days in a 12 h light/12 h dark period incubator. After *nit* mutants were generated, they were phenotyped, and *nit1* and *nitM* mutants from each isolate were paired in a 48-well plate to confirm that the isolate was heterokaryon self-compatible (7,19). After 2 to 3 days of *nit* mutant pairing, heterokaryon formation and stability were scored as robust (+), weak (+/-), or no heterokaryon formation (-).

DNA preparation and sequence analysis. Isolates were grown for 6 to 7 days on potato dextrose agar (PDA) covered with a layer of cellophane (Bio-Rad, Hercules, CA). Mycelia and spores were

harvested from the culture on the cellophane. DNA was extracted using the FastDNA Kit and FastPrep instrument (MP Biomedical, Irvine, CA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed to amplify the *tef-1* gene with a primer pair EF-1 (5'-ATGGGTAAGGAAGACAAGAC-3') and EF-2 (5'-GGAAGTACCAGTGATCATGTT-3') (18). Each PCR reaction contained 0.2 mM deoxynucleotide triphosphates, 2.5 mM MgCl₂, 0.8 μ M of each primer, 1× PCR reaction buffer, and 2 units of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA). The PCR reactions were carried out in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) under the following conditions for 35 cycles: 94°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min. The amplicons were purified by ExoSAP IT (USB Corporation, Cleveland, OH) and sent to the Advanced Studies of Genomics, Proteomics and Bioinformatics sequencing facility at the University of Hawaii for sequencing. The DNA sequences were used for BLASTN searches against the GenBank (<http://blast.ncbi.nlm.nih.gov/>) database for species identification.

DNA extraction and AFLP analysis. DNA was extracted (19) from 46 *Fusarium* isolates recovered from koa trees and used for AFLP analysis. AFLP analysis (28) was conducted as described by Leslie and Summerell (19) with the *EcoRI*-AA/*MseI*-TT primer combination. AFLP bands that were between 300 and 800 bp generated with this primer combination were manually scored. A binary matrix was generated from the banding profile with '1' corresponding to the presence of an allele and '0' indicating the absence of that allele at each potential allele location. A phylogenetic tree of the binary matrix was created by the neighbor-joining method as implemented in PAUP* 4.0 version beta 10 (Sinauer Associates, Inc., Sunderland, MA) with a bootstrap value of 1,000.

Results

Identification based on the morphology and the partial DNA sequence of the translation elongation factor gene. *F. oxysporum* and *F. pseudocircinatum* O'Donnell & Nirenberg are very similar and are difficult to distinguish morphologically. However, *F. pseudocircinatum* produces coiled hyphae, whereas *F. oxysporum* does not (19). Among the 45 isolates, 11 isolates formed coiled hyphal structure on water agar plates and were classified tentatively as *F. pseudocircinatum*. The 11 isolates had a single *tef-1* sequence that was similar but not identical to the *F. pseudocircinatum* sequences in GenBank. In a dendrogram based on the aligned sequences, isolates with the *F. pseudocircinatum* morphological characters all grouped with *F. pseudocircinatum* sequences from GenBank (Fig. 1). Although none of the *F. pseudocircinatum* isolates were highly virulent, isolate 22423 killed 25% of the seedlings inoculated, 22422 and 22418 killed 10 and 5% of the seedlings inoculated, respectively, and isolates 22417, 22413, 22412, 22421, 22416, and 22420 had symptom scores higher than the negative control. Thus, *F. pseudocircinatum* can also be pathogenic to koa. This is the first report of *F. pseudocircinatum* discovery in Hawaii.

Pathogenicity test. Forty-three of the *Fusarium* isolates were evaluated for pathogenicity to koa seedlings (Tables 1 and 2). Three months after inoculation, the mortality rate of koa seedlings varied from 0 to 85%, and average symptom scores ranged from 0.15 to 5.5 with a maximum symptom score of 6.0 (Table 1). Of the 43 isolates tested, 25 killed at least 5% of the seedlings inoculated, while 18 isolates were of low virulence and caused no mortality and low symptom scores. The most virulent isolate, 22454, killed 85% of the seedlings inoculated and had a symptom score of 5.5. Isolate 22418 had the lowest mortality and killed 5% of the seedlings with a symptom of score 0.3 (negative control had a symptom score of 1). The pathogenicity tests were conducted in the greenhouse where the elevation is lower and therefore the temperature is higher than koa's natural habitat. The vigorous healthy controls used more water and on rare occasions the potting medium was dry. This drying can cause leaf wilting and yellowing a few days later. There were 15 *F. oxysporum* isolates that caused

Fig. 1. Phylogenetic tree constructed from partial elongation factor gene sequence using the neighbor-joining method in ClustalW (25). DNA sequences from data base; *Fusarium oxysporum* f. sp. *vasinfectum* (DQ837695.1), *F. oxysporum* f. sp. *ciceris* (FJ538245.1), *F. oxysporum* (FJ939697.1), *F. oxysporum* f. sp. *lycopersici* (DQ837692.1), *F. oxysporum* isolate BMP1397 (DQ837677.1), *F. pseudocircinatum* strain NRRL (GU737399.1), *F. pseudocircinatum* (GU377298.1), *F. pseudocircinatum* strain NRRL22946 (AF160271.1), *F. pseudocircinatum* (GQ425230.1), *F. oxysporum* isolate FoVal42 (EU313535.1), and *F. oxysporum* strain NRRL (GU170552.1) were used. *Cylindrocladiella peruviana* strain CPC 5614 (AY725736) was used as an outgroup. Bootstrap values only greater than 50% are shown. Origin and vegetative compatibility group of each isolate were indicated in parentheses. Origin refers to the five koa trees from which the *Fusarium* isolates were collected. Isolates with the same number were isolated from the same tree. Isolates were collected from bark, branch, internal root (R), or internal trunk (T) tissues.

significantly higher symptom scores than the negative control (Table 1). Among the 15 isolates, 13 isolates belonged to VCG 2. Three *F. pseudocircinatum* isolates, 22417, 22413, and 22423, caused significantly higher symptom scores than the negative control (Table 2). Many fungi were isolated from the control seedlings, and 70% were *Trichoderma* species. No presumptive pathogens capable of causing dieback in koa were isolated.

VCG test. The 46 *Fusarium* strains were separated into 16 VCGs with two major groups, VCG 1 and 2, to which 8 and 16 isolates belonged, respectively (Table 1). Isolates 22412 (VCG 4), 22413 (VCG 5), 22423 (VCG 7), 22425 (VCG 8), 22426 (VCG 10), 22430 (VCG 12), 22435 (VCG 13), 22437 (VCG 14), 22438 (VCG 15), and 22439 (VCG 16) were the only isolates that were self compatible (Table 1). Isolates 22427, 22440, and 22448 did not produce *nit* mutants, and were not used in the VCG tests. Iso-

late 22434 was grouped into VCG 2; however, this isolate formed only weak heterokaryons when paired with other isolates from this group. Most isolates in VCG 2 were pathogenic (maximum score: 5.5, minimum score: 0.9, mean score: 4.1), but seedlings inoculated with isolate 22434 had a low symptom score and none of the inoculated seedlings died. Thus, isolate 22434 expressed low virulence. The remaining isolates were in VCGs with 1, 2, or 3 isolates per VCG (Tables 1 and 2).

AFLP analysis. A phylogenetic tree based on the AFLP results (Fig. 2) showed that isolates in the same VCG were more closely related to one another than they were to isolates in other VCGs. Members of VCG 1 formed an exclusive cluster that contains only isolates in VCG 1. Of the 16 isolates in VCG 2, only 22434 was not in the same clade with the other members of this VCG. Isolate 22448 did not produce *nit* mutants but clustered with the other

Table 1. Host mortality, symptom score, vegetative compatibility group (VCG), and Blastn results of the *Fusarium oxysporum* (F.o.) isolates

Isolate number	Mortality (%) ^a	Symptom score ^r	N ^s	VCG ^t	Blastn result ^u	Accession number	Deposit number
22454	85	5.5 a	20	2	F.o.	JF979445	ATCC ^v : MYA-4833, FGSC ^w : 16090, NRRL ^x : 54716
22445	70	5.1 a	20	2	F.o.	JF979437	
22444	70	4.55 abc	20	2	F.o.	JF979436	
22451	70	4.5 abc	20	2			
22453	65	4.7 ab	20	2	F.o.	JF979444	
22443	65	4.65 ab	20	2	F.o.	JF979435	
22441	65	4.45 abc	20	2	F.o.	JF979433	
22450	60	3.9 abcde	20	2	F.o.	JF979442	
22455	55	4.55 abc	20	2	F.o.	JF979446	
22447	55	4.3 abcd	20	2	F.o.	JF979439	
22433	55	3.85 abcde	20	2	F.o.	JF979425	
22452	50	4.3 abcd	20	2	F.o.	JF979443	
22442	30	2.8 cdefg	10	2	F.o.	JF979434	
22431	10	2.4 efghi	20	2	F.o.	JF979423	
22449	10	1.4 fghij	10	2	F.o.	JF979441	
22434	0	0.9 hij	10	2	F.o.	JF979426	
22414	10	1.6 fghij	10	3	F.o.	JF979406	
22415	0	1.4 fghij	10	3	F.o.	JF979407	
22432	40	2.6 defgh	20	6	F.o.	JF979424	
22424	0	0.7 ij	10	6	F.o.	JF979416	
22425	0	4 abcd	20	8	F.o.	JF979417	
22456	0	0.2 j	20	9	F.o.	JF979447	
22428	-	-		9	F.o.	JF979420	
22429	-	-		9	F.o.	JF979421	
22426	70	4.45 abc	20	10	F.o.	JF979418	ATCC: MYA-4830, FGSC: 16086, NRRL: 54712 ATCC: MYA-4832, FGSC: 16089, NRRL: 54715
22446	50	3.15 bedef	20	11	F.o.	JF979438	
22436	0	0.6 ij	20	11	F.o.	JF979428	
22430	0	0.15 j	20	12	F.o.	JF979422	
22435	0	1.2 ghij	10	13	F.o.	JF979427	ATCC: MYA-4834, FGSC: 16088, NRRL: 54714
22437	20	1 ghij	10	14	F.o.	JF979429	
22438	10	1 ghij	10	15	F.o.	JF979430	
22439	0	0.3 j	20	16	F.o.	JF979431	
22440	0	1.4 fghij	10	NM ^y	F.o.	JF979432	
22427	0	0.8 hij	10	NM	F.o.	JF979419	
22448	20	2.3 efghi	10	NM	F.o.	JF979440	
Control ^z	0	1 ghij					

^a Mortality percentage was based on koa seedlings killed in pathogenicity tests.

^r Symptom score was calculated for each isolate based on a 1 to 6 disease scale 3 months after inoculation: 6 = dead; 5 = wilting and dying; 4 = heavy defoliation; 3 = dead leaf tip, bleached, partial defoliation, and/or partially wilting; 2 = small root system, small stem lesions, stunted, tilted; and 1 = yellow leaves, and/or small yellow leaf freckling. Symptom scores are averages of 10 or 20 plants. Values followed by different letters are significantly different at a minimum $P < 0.05$ based on Waller-Duncan's k -ratio t test.

^s N represents the number of seedlings used in pathogenicity tests.

^t VCG was determined by pairing of complementary *nit* mutants.

^u Blastn result was from a partial DNA sequence of the translation elongation factor-1 α (*tef-1*) gene from each of the *Fusarium* isolates.

^v American Type Culture Collection (ATCC), Manassas, VA.

^w Fungal Genetics Stock Center (FGSC), at the University of Missouri, Kansas City, MO.

^x ARS Culture Collection (NRRL), USDA, Peoria, IL.

^y NM: no *nit* mutant generated.

^z Controls: healthy and uninoculated plants.

sexual reproduction within a *F. subglutinans* f. sp. *pini* population in South Africa (27). No sexual stage is known for *F. oxysporum* or *F. pseudocircinatum*. Based on the VCG analysis, sexual reproduction could theoretically have generated the observed patterns. Asexual genetic transfer could occur between isolates in the same VCG, but would be more meaningful if it occurred in highly transient heterokaryons that are formed prior to death when isolates in different VCGs fuse asexually. It seems more likely, however, that isolates with pathogenic capability have evolved locally, perhaps in parallel, and then have been selected for growth by the additional resources (koa trees) that virulence makes available to them. Such evolution to higher levels of host resistance could continue in a native setting at a slow pace for a relatively long time.

In the AFLP analyses, some VCGs clustered with isolates in different VCGs. For example, isolates 22435, 22437, and 22439 formed a cluster, even though they belong to a different VCG. Isolates 22438, 22424, 22425, and 22436, which are all in different VCGs, were in the same clade with two isolates from VCG 9. Isolates 22426, 22430, and 22446 formed an individual cluster, although all of them are in different VCGs. In asexual populations, isolates in the same VCGs usually are genetically close and thus should be clustered into the same group in the AFLP analyses (20,26). Isolates that cluster with others in different VCGs, therefore, may be genetically similar to each other but have different alleles controlling vegetative compatibility (5). On the other hand, isolates in VCG 6 and VCG 11 showed long genetic distances from other isolates within the same VCGs. Similar mismatching between VCGs and AFLP dendrograms has been observed in previous studies in *Fusarium* species (2,24). Vegetatively compatible isolates must share certain alleles in the *vic* loci (19); however, they are not necessarily clones of each other. Therefore, these results indicate that some isolates in this population are not genetically close but have compatible alleles for heterokaryon formation.

Isolates from VCG 1, 2, and 3 clustered with one another in the AFLP analyses except for 22434, which was not in the cluster. This isolate exhibited low virulence and formed only weak heterokaryons when paired with other VCG 2 isolates. In the present study, there were several virulent isolates that were not in VCG 2. It is known that nonpathogenic *F. oxysporum* isolates can be transformed into pathogenic isolates (13). Active exchange of genetic materials could occur asexually in plants harboring coexisting populations of pathogenic and nonpathogenic isolates. Of the 25 pathogenic isolates, 14 killed 50% or more of the inoculated koa seedlings, and of these 14, 12 belonged to VCG 2. Isolate 22448 did not produce *nit* mutants; however, we could place it in VCG 2 because of the AFLP results (Fig. 2). AFLP analysis is an alternative method for the identification of genetic similarity if *nit* mutants are not available. AFLP analyses of a larger set of isolates also may lead to the identification of one or more conserved AFLP fragments that can serve as the basis for a diagnostic molecular test for this pathogen. Although a previous study reported recovery of pathogenic *F. oxysporum* isolates from *A. koa* and identified those as *F. oxysporum* f. sp. *koae* (1), the isolates used in that study are not available. This is the first study on *F. oxysporum* isolated from *A. koa*, examining the pathogenicity, *tef-1* gene DNA sequence, VCG, and AFLP. Based on the results of these tests, and the identification of pathogenic *F. oxysporum* isolates, we propose a new forma specialis, *F. oxysporum* f. sp. *acaciae*, f. sp. *nov.*

In conclusion, we found a range of isolates of *F. oxysporum* colonizing koa and exhibiting high to low virulence. These isolates can be separated by using VCG tests. Most of the highly virulent *F. oxysporum* isolates belong to VCG 2. We also found that *F. pseudocircinatum* appears to play a role in koa wilt. The VCG information obtained in this study could help koa growers and researchers because VCG tests could be used to screen *F. oxysporum* isolates from field soil prior to planting, thereby increasing the likelihood of establishing healthy koa plantations. In future work, identification of AFLP fragments associated with isolates of *F. oxysporum* virulent to koa may lead to the development of a potent tool for detection and identification of

pathogenic isolates in the field. Also, conducting VCG tests with the 16 VCG representative isolates obtained from this study and *F. oxysporum* isolates of known VCGs from different origins would help to address the etiology of the *F. oxysporum* f. sp. *acaciae*, f. sp. *nov.* isolates infecting koa.

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