Rapid Detection of the *Fusarium oxysporum* Lineage Containing the Canary Island Date Palm Wilt Pathogen

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ABSTRACT

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Fusarium oxysporum f. sp. *canariensis* causes Fusarium wilt disease on the Canary Island date palm (*Phoenix canariensis*). To facilitate disease management, a polymerase chain reaction diagnostic method has been developed to rapidly detect the pathogen. A partial genomic library of *F. oxysporum* f. sp. *canariensis* isolate 95-913 was used to identify a DNA

sequence diagnostic for a lineage containing all tested isolates of *F. oxysporum* f. sp. *canariensis*. Two oligonucleotide primers were designed and used to amplify a 567-bp fragment with *F. oxysporum* f. sp. *canariensis* DNAs. DNA from 61 outgroup isolates did not amplify using these primers. Once the primers were shown to amplify a 0.567-kb fragment from DNA of all the *F. oxysporum* f. sp. *canariensis* isolates tested, a rapid DNA extraction procedure was developed that led to the correct identification of 98% of the tested *F. oxysporum* f. sp. *canariensis* isolates.

Phoenix canariensis (Hort. ex Chabaud.), the Canary Island date palm, is a prized landscape ornamental in major palm-growing regions throughout the world. A single field-grown palm is often worth around \$2,000 if costs of transport and labor are also added to the price (10). Recently, Fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *canariensis*, has become a threat to the Canary Island date palm industry in Florida. Fusarium wilt of *P. canariensis* was first observed in 1970 in France by Mercier and Louvet (11), who named the causal agent *F. oxysporum* f. sp. *canariensis*. Later it was reported in Italy (1974), California (1976), Japan (1977), Australia (1980), Morocco (1987), Florida (1994), and Nevada (1997) (5,9,20; G. W. Simone, unpublished data). Symptoms of the disease are one-sided leaflet dieback of fronds, necrotic and brown streaking on the lower rachis base of older leaves, and necrosis within the vascular bundles. Eventually, the disease kills infected palms, because there is no current treatment for it (20). Other *Phoenix* spp. including *P. dactylifera* (the true date palm) are also susceptible to this pathogen (3).

The fungus spreads through the movement of infested soil and infected trees and through pruning methods. Current disease control, therefore, centers around the prevention of pathogen introduction, destruction of diseased plants, and isolation of susceptible palms from infested sites (20). The fungus also may be seed-transmitted (G. W. Simone, unpublished data).

An early detection method could be used in many ways to prevent the spread of *F. oxysporum* f. sp. *canariensis*. Palm trees, liners, and even seed lots could be screened for the presence of the pathogen prior to transport to nurseries and landscapes. Soil also could be screened prior to planting to prevent the installation of susceptible palm species. Polymerase chain reaction (PCR) has identified the presence of many fungal plant pathogens in a timely and efficient manner including *Colletotrichum gloeosporioides*, *Verticillium albo-atrum*, *F. culmorum*, *F. graminearum*, *F. avenaceum*,

and formae speciales of *F. oxysporum* (4,6,13,16,19). This paper describes the development of a PCR-based method for the detection of *F. oxysporum* f. sp. *canariensis*.

MATERIALS AND METHODS

Fungal isolates used. Seventy-one isolates of *F. oxysporum* f. sp. *canariensis* were used to develop oligonucleotide primers for detection of the fungus (Table 1). These included 13 isolates (70-MercIV, 84-01a, 84-01b, 84-02b, 84-104, 84-207, 84-305, 84-P1, 84-P2, 84-Cala, 87-Guil2, 87-Guil7, and 87-Lago3) that were confirmed to be *F. oxysporum* f. sp. *canariensis* through pathogenicity tests (J. Louvet, personal communication). The remaining isolates used in this study came from symptomatic tissue of *P. canariensis* sent to the Florida Extension Plant Disease Clinic (FEPDC) in Gainesville from locations in Florida and California. Pathogenicity tests were not performed on these isolates, but the genotypes of the remaining isolates were very similar or identical to authentic *F. oxysporum* f. sp. *canariensis* isolates. At least 70% of the isolates belong to a single vegetative compatibility group, and genotypes were confirmed by mitochondrial restriction fragment length polymorphism (RFLP) profiles and multilocus haplotypes determined by single- or low-copy RFLPs (17). Because of their genetic similarity to authentic palm pathogens and their dissimilarity to strains from other hosts, isolates from diseased *P. canariensis* from California and Florida were likewise considered isolates of *F. oxysporum* f. sp. *canariensis*. Isolate 94-868 from Florida additionally has been confirmed through Koch's postulates to be *F. oxysporum* f. sp. *canariensis*.

Diseased tissue containing necrotic streaking, symptomatic of the vascular wilt, was surface-sterilized with a 5.26% sodium hypochlorite solution for 2 to 3 min. This material was rinsed with sterile water, blotted with sterile towels, and then plated on potato dextrose agar (PDA) acidified with lactic acid. In 3 or more days, fungal growth appeared as white, pink, light orange, or purple aerial mycelia. Mycelia were examined by the FEPDC under a light microscope for the presence of two or three septate macroconidia and elliptical microconidia diagnostic of *F. oxysporum* (15).

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TABLE 1. *Fusarium oxysporum* f. sp. *canariensis* isolates used in this study and results of detection tests^y

Isolate	Origin, collector ^z	Hybridization to C26 probe	Amplification with HK66 and HK67 (standard DNA extract)	Amplification with HK66 and HK67 (rapid DNA extract)	Amplification with HK66 and HK67 (modified rapid DNA extract)	Amplification with HK66 and HK96	Amplification with HK66 and HK67 and amplicons cut with <i>Afl</i> III
70-MerclV	Provence, France, a	+	+	NT	+	NT	NT
84-01a	Cannes, France, a	+	+	NT	NT	NT	NT
84-01b	Cannes, France, a	+	+	NT	+	NT	NT
84-02b	Cannes, France, a	+	+	NT	+	NT	NT
84-104	Kagoshima, Japan, a	+	+	+	NT	NT	NT
84-207	Kagoshima, Japan, a	+	+	NT	+	–	+
84-305	Kagoshima, Japan, a	+	+	NT	+	NT	NT
84-P1	Cannes, France, a	+	+	NT	+	NT	NT
84-P2	Cannes, France, a	+	+	NT	+	NT	NT
84-Cala	California, a	+	+	NT	+	NT	NT
87-Guil2	Tenerife, Canary Isl., a	+	+	+	NT	NT	NT
87-Guil7	Tenerife, Canary Isl., a	+	+	–	+	NT	NT
87-Lago3	Tenerife, Canary Isl., a	+	+	NT	+	NT	NT
Sicily 1	Sicily, Italy, c	NT	+	+	NT	NT	NT
Sicily 2	Sicily, Italy, c	NT	+	+	NT	+	+
94-275	Manatee Co., FL, b	+	+	NT	+	NT	NT
94-556	Manatee Co., FL, b	+	+	NT	+	NT	NT
94-841	Sarasota Co., FL, b	+	+	NT	+	NT	+
94-865	Manatee Co., FL, b	+	+	NT	+	NT	NT
94-867	Manatee Co., FL, b	+	+	–	+	NT	–
94-868	Manatee Co., FL, b	+	+	–	+	NT	NT
94-869	Manatee Co., FL, b	+	+	NT	+	NT	NT
94-871	Manatee Co., FL, b	+	+	NT	+	NT	NT
94-872	Manatee Co., FL, b	+	+	+	+	NT	NT
94-873	Manatee Co., FL, b	+	+	+	+	NT	NT
94-2140	Sarasota Co., FL, b	NT	+	NT	+	NT	NT
94-2418	Manatee Co., FL, b	+	+	+	NT	NT	NT
95-F050F	California, b	+	+	NT	+	NT	NT
95-F051F	California, b	+	+	+	NT	NT	+
95-F052F	California, b	+	+	+	+	NT	NT
95-F053F	California, b	+	+	+	+	NT	NT
95-456	Lee Co., FL, b	NT	+	NT	+	NT	NT
95-513	Manatee Co., FL, b	+	+	NT	+	NT	NT
95-514B	Manatee Co., FL, b	+	+	+	+	NT	NT
95-514C	Manatee Co., FL, b	+	+	+	+	NT	NT
95-861	Hillsborough Co., FL, b	+	+	–	+	NT	NT
95-913	Manatee Co., FL, b	+	+	NT	+	NT	NT
95-914	Manatee Co., FL, b	+	+	+	NT	NT	–
95-1000	Lake Co., FL, b	+	+	NT	+	NT	NT
95-1001	Lake Co., FL, b	+	+	+	NT	+	+
95-1002	Lake Co., FL, b	+	+	+	NT	NT	NT
95-1003	Lake Co., FL, b	+	+	+	NT	NT	NT
95-1004	Lake Co., FL, b	+	+	+	NT	NT	NT
95-1005	Lake Co., FL, b	+	+	+	NT	NT	NT
95-1006	Lake Co., FL, b	+	+	+	NT	NT	NT
95-1007	Lake Co., FL, b	+	+	+	NT	NT	NT
95-1008	Lake Co., FL, b	+	+	NT	+	NT	NT
95-1009	Lake Co., FL, b	+	+	+	NT	NT	NT
95-1488	Pinellas Co., FL, b	+	+	+	+	+	+
95-1714	Dade Co., FL, b	+	+	NT	+	NT	NT
95-1724	Manatee Co., FL, b	+	+	NT	+	–	NT
95-1725	Manatee Co., FL, b	+	+	NT	+	NT	NT
95-1726	Manatee Co., FL, b	+	+	NT	+	NT	NT
95-1731	Broward Co., FL, b	+	+	NT	+	NT	NT
95-1772	Broward Co., FL, b	+	+	+	NT	NT	NT
95-2121	Palm Beach Co., FL, b	+	+	NT	+	–	–
95-2122	California, b	NT	+	NT	+	NT	NT
95-2123	Santa Anna, CA, b	+	+	NT	+	NT	NT
95-2269	Palm Beach Co., FL, b	+	+	+	NT	NT	NT
96-08	California, b	+	+	+	NT	NT	NT
96-09	California, b	+	+	+	+	NT	NT
96-13	California, b	+	+	+	NT	NT	+
96-14	California, b	+	+	+	+	NT	NT
96-148	Lee Co., FL, b	+	+	NT	NT	NT	NT
96-540	Palm Beach Co., FL, b	NT	+	+	NT	NT	NT
96-847	Sarasota Co., FL, b	NT	+	+	NT	NT	NT
96-1416	Hillsborough Co., FL, b	NT	+	+	NT	NT	NT
96-1672	California, b	NT	+	+	NT	NT	NT
96-1673	California, b	NT	+	+	NT	NT	NT
96-1733	Dade Co., FL, b	NT	+	–	–	NT	NT
96-1889B	Volusia Co., FL, b	NT	+	NT	NT	NT	NT

^y + = Positive hybridization, amplification, or restriction digestion result; – = negative hybridization, amplification, or restriction digestion result; and NT = not tested.^z a = D. Fernandez, Laboratoire de Phytopathologie, ORSTOM, B.P. 5045, 34032 Montpellier Cedex 1, France; b = G. W. Simone, Department of Plant Pathology, PO Box 110680, University of Florida, Gainesville 32611; and c = Q. Migheli, Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Patologia vegetale, Università di Torino, Via Leonardo da Vinci 44, I-10095 Grugliasco (Torino) Italy.

DNA library construction and screening. DNA from *F. oxysporum* f. sp. *canariensis* isolate 95-913 and the vector pUC118 were digested to completion with *Hind*III and heated for 5 min at 65°C to inactivate the restriction enzyme. The 95-913 DNA (0.25 µg/µl) was ligated to the cut vector (0.05 µg/µl) and used to transform competent cells of *Escherichia coli* strain DH5α prepared according to Sambrook et al. (18). Cells were spread on Luria-Bertani (LB) plates containing 75 µg of carbampicillin per ml and 0.8 mg of isopropylthio-β-D-galactoside (IPTG) per 0.8 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and 164 recombinant clones were chosen to represent a partial genomic library of isolate 95-913.

To identify sequences specifically found in *F. oxysporum* f. sp. *canariensis* strains, the 164 colonies were screened to find clones that would not hybridize to a complex probe made of total DNA from eight isolates of seven formae speciales of *F. oxysporum* (725, *F. oxysporum* f. sp. *mathioli*; 3S1 and 144, *F. oxysporum* f. sp. *cubense*; 699, *F. oxysporum* f. sp. *raphani*; DF-1, *F. oxysporum* f. sp. *albedinis*; 73, *F. oxysporum* f. sp. *lycopersici*; 247, *F. oxysporum* f. sp. *pisi*; and 777, *F. oxysporum* f. sp. *conglutinans*). A 1-µg portion of each of the eight above DNA preparations was combined in an Eppendorf tube to create the complex probe. A 2-µg portion of the complex probe was labeled for a chemiluminescence detection method (Renaissance Dupont-NEN, Boston).

To test the complex probe on the partial library of 95-913, plasmid DNA from the 164 clones was isolated using the alkaline lysis method (18). The recombinant DNA was digested with *Hind*III and run on 0.7% agarose gels for 16 to 18 h at 34 V. DNA from each clone was transferred to nylon membranes and screened with the labeled complex probe. In all, 16 clones were found to either weakly hybridize or not hybridize at all with the complex probe. These clones were screened again with the complex probe, and clone C26, which did not hybridize either time to the probe, was selected for further study.

DNA from 59 *F. oxysporum* f. sp. *canariensis* isolates (available at the time of the test) digested with *Hind*III was probed with 0.5 µg of C26 labeled for chemiluminescent detection. C26 hybridized to all tested *F. oxysporum* f. sp. *canariensis* isolates (Table 1). The DNA sequence of C26 was obtained with a Perkin-Elmer/Applied Biosystems apparatus (ABI 373 stretch; Perkin-Elmer/Applied Biosystems, Foster City, CA) at the University of Florida (UF), Interdisciplinary Center for Biotechnology Research, (ICBR), DNA Sequencing Core Facility. Sequence similarities were tested through the National Center for Biotechnology Information (NCBI) using the BLAST network service. Primary DNA sequence comparison was made using the BLASTX algorithm (1).

Design of primers. The insert of clone C26 was 686 bp in length (Fig. 1). To design a PCR primer pair, sequences between 18 to 30 nucleotides were identified at both ends of the insert that would not self anneal or form possible stem-loops or other secondary structures that might inhibit PCR (CloneWorks version 1.92; Anteater Software Corporation, San Diego, CA). Three oligomers that met these conditions and had a melting temperature above 65°C were designed from the insert. One forward primer (HK66) sequence (5'-CATCAGAAGTGCCTTCGTAGG-3') and two reverse sequences, HK67 (5'-CGTTGTAATGGTTTGCTCCTCAC-3') and HK96 (5'-CCAGAAGCGGTGC-3'), were chosen and corresponding oligonucleotides synthesized on an Applied Biosystems ABI 394 synthesizer (Perkin-Elmer/Applied Biosystems) at the UF ICBR DNA Synthesis Core Facility (Fig. 1).

Standard DNA extraction. Cultures were single-spored and placed on full-strength or half-strength PDA (Difco Laboratories, Detroit). After 3 to 7 days of growth on PDA, a 1-cm² block was removed from the plates and transferred to 150- by 20-mm petri plates containing 50 ml of potato dextrose broth (24 g/liter) (Difco Laboratories). Cultures were grown for 5 to 10 days and filtered through sterile cheese cloth for mycelium collection. The filtered product was placed in 13-ml plastic tubes and frozen at -80°C. The tubes were lyophilized for 12 to 48 h, yielding between 0.2 and 0.5 g of mycelium per extraction.

DNA from the isolates was extracted according to a method modified from that described by Koenig (7) and Koenig et al. (8). Since the starting amount of mycelium for this experiment was smaller than the amount used by Koenig et al. (8), only 3 to 4 ml of DNA extraction buffer was used. Additionally, LiCl treatments were not used in this experiment, because these did not effect the total yield of DNA. The concentration of DNA obtained from the extraction was estimated using fluorescence in the presence of bis-benzimide measured using a Hoefer TK0/100 DNA fluorometer (Hoefer Scientific Products, San Francisco).

Rapid DNA extraction. A shorter method for obtaining DNA for PCR amplification was investigated (12). A 1-cm² block was removed from the colony edge of single-spored cultures and placed in a 1.5-ml Eppendorf tube containing 150 µl of Tris-EDTA (TE) buffer. Mycelium was ground in the buffer with sterile wooden sticks. The tubes were then placed in liquid nitrogen for 3 to 4 min. Following the freeze, the tubes were thawed in 22°C water for 5 min and then returned to liquid nitrogen. Freezing and thawing to 22°C was repeated three to four times and then the tubes were placed in a 65°C water bath for 15 min. After the tubes were spun for 10 min at 11,500 × g, the supernatant was removed and placed in a new Eppendorf tube. An equal volume of 24:1 chloroform/octanol was added to the supernatant, and the two were mixed with vigorous shaking. The tubes were again centrifuged for 10 min at 12,000 × g, after which the supernatant was not quantified, but diluted 10-fold for use in PCR reactions.

After DNA preparations of some of the *F. oxysporum* f. sp. *canariensis* isolates failed to amplify by PCR using this rapid extraction method, additional steps were added to the procedure. To this end, 24 µl of the final supernatant and 16 µl of isopropanol were combined in a separate tube to precipitate the DNA, and the tubes were spun for 5 min. After draining isopropanol from the tubes, 16 µl of 70% ethanol was added to wash the pellet and the tubes were spun for 5 min. The pellet was allowed to dry under a laminar flow hood and resuspended in 25 µl of TE buffer.

PCR protocol. A 26-µl reaction mixture contained 13 ng of each primer, 0.2 U of *Taq* polymerase, 0.115 mM deoxynucleotide triphosphates (Boehringer Mannheim Biochemicals, Indianapolis, IN), 1.8 mM MgCl₂ (Promega Corp., Madison, WI), and reaction buffer (50 mM KCl; 10 mM Tris-HCl, pH 9; and 0.1% Triton X-100). The template DNA concentrations used ranged from 5 to 40 ng per reaction. Template DNA concentration was not calculated when the template was obtained by the rapid DNA extraction method; instead, a 10-fold dilution of the DNA in water was made. The

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1   AAGCTTACCA AGAAATGGAA AATCGATAAG CTCTGGCAAC ATATTGTTTC
      HK66 primer →
51  AGCAAAGCGA TGTCATCAGA AGTGCCTTCG TAGGAAATTGC AGGCCACAAC
101 ACCGGAAGAA CTATCAGCGA TCTTACCGGG CCTACAACAG AATTCGTCCC
151 ATATGGATGG AAGCCTACCA TCTTTGTGCA CGGCACCTTC GATCTGSTAT
201 CTGGCTGTGC CAAAGCCCCA GATAAAGTCC TTGGGAAGCG ACATGCTGAA
251 AAACAGATGA ATAATAATA ATAATAATAG TAATAGTAAT GATAGTAATA
301 ATAATAATAA AACTGACTGG ACTAAGAGCC TGATGAAAGT CGTTTATGAA
351 GGTATCCCGT CACAGACGGA ACTTCCTTTT TTGAATCTCC GAAAACGTTC
401 TTGACTGTAG GAACTTCTCC TTTGGGAAGT ACCGCATATT TAAGCCTCCA
451 CTACACGTTA GAGCATTCTG AGTATGTTTC ACCACCGGAA CCAATATCCC
501 TTTTGGGAAC ACGGGGACTC AGCCAAATCA CGCCTTGCTT GGATCTCGTC
      ← HK96 primer
551 AGATGTAGCG GCCGTATTGC ACGCCTTGCA CCGCCTTCTGGCTCGTACA
      ← HK67 primer
601 TGGAAGGGGA GTGAGGCAAA CCATTACAAC GCCAGATTGA AAACGCTTAG
651 TAATTCTGAG AGAAGAGAGT GACTAATAAA AAGCTT

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Fig. 1. Sequence and primer selection for clone C26. C26 is a clone from the partial library of *Fusarium oxysporum* f. sp. *canariensis* isolate 95-913. The GenBank accession number for C26 is AF118442.

samples were amplified via PCR using the following program: 95°C for 30 s and 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min, followed by an additional extension time for 10 min at 72°C. The annealing temperature used to test a subset of isolates with HK66 and HK67 primers initially was 60°C (84-01a, 84-01b, 84-02b, 84-104, 84-305, 87-Guil7, 94-841, 95-913, 95-914, 95-1000, 95-1001, 95-1002, 95-1003, 95-1004, 95-1005, 95-1006, 95-1007, 95-1008, 95-1009, 95-1772, 95-2122, 95-2269, 96-08, 96-13, *F. oxysporum* f. sp. *raphani* isolate 699, and *F. oxysporum* f. sp. *cubense* isolate 3S1). A portion of these isolates at this temperature with these primers is shown in Figure 2A. *F. oxysporum* f. sp. *raphani* isolate 699 gave amplification of two bands with 60°C; therefore, the annealing temperature was raised to 62°C. At 62°C, *F. oxysporum* f. sp. *raphani* isolate 699 did not amplify, but isolates of *F. oxysporum* f. sp. *canariensis* did. DNA from Figure 2A was probed with 0.5 µg of C26 labeled for chemiluminiscent detection (Fig. 2B). *F. oxysporum* f. sp. *raphani* isolate 699 did not hybridize, but the 12 *F. oxysporum* f. sp. *canariensis* isolates did. Reactions were first run on a Gene Machine II thermal cycler (USA/Scientific Plastics, Ocala, FL) and a subset was repeated on a Coy thermal cycler (Coy Laboratory Products, Grass Lake, MI).

PCR reaction samples (10 µl per well) were electrophoresed on a 0.7% LE NuSieve (FMC Corp., Rockland, ME) agarose gel at 80 V for 3 to 4 h. Tris-borate-EDTA (5.4 g of Tris per liter, 2.75 g of boric acid per liter, and 2 ml of 0.5-M EDTA per liter, pH 8) was used as the running buffer and 100 ng of ethidium bromide per ml was added to the gel prior to the run.

RESULTS

To identify a potential diagnostic clone, a partial genomic library of *F. oxysporum* f. sp. *canariensis* isolate 95-913 consisting of 164 re-

combinant clones was screened for the inability to hybridize to labeled total DNA from eight strains representing seven different formae speciales of *F. oxysporum*. Based on Southern hybridization, recombinant clone C26 consistently did not hybridize to the complex probe. Moreover, when C26 was used as a probe for Southern hybridization, it hybridized to a corresponding band in all 59 *F. oxysporum* f. sp. *canariensis* isolates tested, but did not hybridize to 7 other *F. oxysporum* isolates from other hosts (*Rhododendron* sp., *Elaeagnus* sp., *Hibiscus* sp., *Phoenix* sp., *Lycopersicon* sp., *Cyrtomium* sp., and *Pisum sativum*). Based on these results, C26 was considered a potential diagnostic clone and its DNA sequence was obtained. The sequence of the insert of clone C26 was 686 bp in length (Fig. 1), and a BLASTX search of DNA sequence databases indicated it had significant sequence similarity ($P = 1.6 \times 10^{-26}$) to a portion of a microbial beta-glucosidase gene (accession no. X60268). Three oligonucleotide primers were synthesized corresponding to either the 5' or 3' end of the clone (Fig. 1). The predicted amplification product size using primers HK66 and HK67 was 567 bp, while the predicted product size using primers HK66 and HK96 was 528 bp.

PCR using DNA extracted by the standard procedure. Primers HK66 and HK67 were initially selected from the sequence of clone C26 and were tested using template DNA from isolates of *F. oxysporum* f. sp. *canariensis*. DNA from 64 isolates of *F. oxysporum* f. sp. *canariensis* was tested at least twice. Each test resulted in an amplification of a 567-bp band two or more times. For four isolates (87-Guil7, 87-Guil2, 84-Lago3, and 70-MercIV), amplification of the 567-bp fragment occurred only once out of three or more reactions. Problems with amplification of these DNAs were determined to be due to the first template DNA preparation, because DNA extracted by the rapid method (described below) resulted in repeatable amplification of the 567-bp band from these isolates. DNA from the remaining seven isolates of *F. oxysporum* f. sp. *canariensis* was tested once with HK66 and HK67, and each produced a 0.567-kb band.

An illustration of DNA from 12 *F. oxysporum* f. sp. *canariensis* isolates amplified with primers HK66 and HK67 is shown in Figure

Fig. 2. A, Polymerase chain reaction (PCR) amplification of DNA from isolates of *Fusarium oxysporum* f. sp. *canariensis* at a 60°C annealing temperature using primers HK66 and HK67. The isolates (beginning in lane a) are 84-01b, 94-841, 95-1007, 84-02b, 84-305, 84-01a, 95-1001, 84-207, 95-1006, 95-2122, 95-914, and 84-104. DNA from strains of *F. oxysporum* f. sp. *raphani* (699) and *F. oxysporum* f. sp. *cubense* (3S1) are in lanes m and n, respectively. B, Southern blot of PCR products in A probed with clone C26.

Fig. 3. Polymerase chain reaction (PCR) amplification of DNA from isolates of *Fusarium oxysporum* f. sp. *cubense* at a 62°C annealing temperature using primers HK66 and HK67. Lane a, PCR-negative control consisting of reaction mix without DNA template. Lane b, DNA from a strain of *F. oxysporum* f. sp. *raphani* (699). Lanes c to o, Amplification products from isolates of *F. oxysporum* f. sp. *cubense* (SABA, 3S1, A2, PW7, GM, F9127, STPA, F0001, B2-1, DAVAO, 22615, STNP1, and STA2). Lanes p to r, *F. oxysporum* f. sp. *canariensis* isolates (94-2140, 95-1004, and 95-1004).

can	1	CACAACACCGGAAGAACTATCAGCGATCTTACCGGGCTACACAGAATT
cub		CACAACACCGGAAGAACTATCAGCGATCTTACCGGGCTACACAGAATT
can	51	CGTCCCATATGGATGGAAGCCTACCATCTTTGTGCGAGCGCACCTTCGATC
cub		CGTCCCATATGGATGGAAGCCTACCATCTTTGTGCGAGCGCACCTTCGATC
can	101	TGGTAGCTGGCTGTGCCAAGCCCCAGATAAAGTCCTTGGGAAGCGACAT
cub		TGGTAGCTGGCTGTGCCAAGCCCCAGATAAAGTCCTTGGGAAGCGACAT
can	151	GCTGAAAAACAGATGAATAAATAATAATAATAATAGTAATAGTAATGATA
cub		GCTGAAAAACAGATGAATAAATAATAATAATAATAATAGTAATAGTA-----
can	201	GTAATAATAATAATAAACTGACTGGACTAAGAGCCTGATGAAAGTCGTT
cub		---ATAATAATAATAAACTGACTGGACTAAGAGCCTGATGAAAGTCGTT
can	251	TATGAAGGTATCCCGTCACAGACGGAACCTTCCTTTTGAATCTCCGAAA
cub		TATGAAGGTATCCCGTCACAGACGGAACCTTCCTTTTGAATCTCCGAAA
can	301	ACGTTCTTGACTGTAGGAACCTTCCTTTGGGAAGTACCGCATATTTAAG
cub		ACGTTCTTGACTGTAGGAACCTTCCTTTGGGCAGGACCGCATATTTAAG
can	351	CCTCCACTACACGTTAGAGCATTCTGAGTATGTTTCACCAACCGGAACCAA
cub		CCTCCACTA-ACGTTAGAGCATTCTGAGTATGTTTCACCAACAGGAACCAA
can	401	TATCCCTTTTGGGAACACGGGGACTCAGCCAAATCAGCCCTTGCTTGGAT
cub		TATCCCTTTTGGGAACACGGGGACTCAGCCAAATCAGCCCTTGCTTGGAT
can	451	CTCGTCAGATGTAGCGGCCGTATTCGACGCCCTTCACCGCCTTCTGGGTC
cub		CTCGTCAGATGTAGCGGCCGTATTCGACGCCCTTCACCGCCTTCTGAGTC
can	501	GCTACATGGAAGGGAGTGAGGCAAAACCATTAACAA
cub		GCTACATGGAAGGGAGTGAGGCAAAACCATTAACAA

Fig. 4. Alignment of sequences amplified by primers HK66 and HK67 for *Fusarium oxysporum* f. sp. *canariensis* (can) isolate 95-913 and *F. oxysporum* f. sp. *cubense* (cub) isolate GM. The variable nucleotide positions are in bold type. The *Afl*III restriction site and HK96 primer site within the *F. oxysporum* f. sp. *canariensis* sequence are indicated.

2A. All 12 isolates amplified; variation in the intensity of the 0.5-kb amplicon was due to differences in starting DNA template concentrations. All 12 hybridized with clone C26 (Fig. 2B). At 60°C, DNA from a strain of *F. oxysporum* f. sp. *raphani* (699) gave amplification of two bands other than the 0.5-kb amplicon, but neither of these hybridized to C26; amplification products from 699 were not seen at 62°C, and this became the standard annealing temperature. While no amplification product was detected in the ethidium bromide-stained gel, DNA from *F. oxysporum* f. sp. *cubense* strain 3S1, which was amplified at 60°C, hybridized faintly to labeled C26.

Sixty-one isolates of *F. oxysporum* from other hosts were included to test the specificity of the primers developed for *F. oxysporum* f. sp. *canariensis*. These included 13 genetically diverse *F. oxysporum* f. sp. *cubense* isolates; 3 isolates of *F. oxysporum* f. sp. *lycopersici*; 9 isolates of *F. oxysporum* f. sp. *vasinfectum* (all representatives of distinct *F. oxysporum* f. sp. *vasinfectum* lineages); 5 isolates of *F. oxysporum* f. sp. *elaeidis*, 1 isolate each of *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *albedinis* (representative of the unique lineage of this special form), *F. oxysporum* f. sp. *raphani*, *F. oxysporum* f. sp. *conglutinans*, and *F. oxysporum* f. sp. *matthioli*; and 26 *F. oxysporum* isolates from various ornamental hosts. DNA from three additional fungal species (*Serenomyces phearrii*, *F. solani*, and *F. verticillioides*) also was included as a control. DNA from these strains failed to produce a 567-bp fragment when amplified with HK66 and HK67, with the exception of 7 of the 13 *F. oxysporum* f. sp. *cubense* isolates (Fig. 3). Amplicons from isolates of *F. oxysporum* f. sp. *cubense* that amplified were from a clonal lineage that previously had been determined to be most closely related to *F. oxysporum* f. sp. *canariensis* based on comparison of DNA sequences from the nuclear and mitochondrial genome (14).

To determine the reproducibility of the PCR results, the same procedure was performed using a different thermocycler in a different laboratory for DNAs from a subset of 13 *F. oxysporum* f. sp. *canariensis* isolates (95-50, 95-1000, 96-09, 95-914, 96-540, 84-02b, 95-1006, 95-1731, 87-Guil2, 87-Guil7, 96-148, 95-2123, and 84-P2). The 567-bp fragment was amplified from DNA of all 13 isolates examined. Because of the previously mentioned failure of DNA templates for 87-Guil2 and 87-Guil7, the standard DNA extraction was repeated for these isolates to give a different starting template preparation than had been used on the previous tests.

Amplification using an alternative primer. Using the forward (HK66) and reverse primer (HK67) pairs listed above, DNA from some isolates of *F. oxysporum* f. sp. *cubense* were also amplified (Fig. 3). To understand this, the amplification product of *F. oxysporum* f. sp. *cubense* isolate GM was sequenced and compared with the corresponding sequence from *F. oxysporum* f. sp. *canariensis* (Fig. 4). Only a few differences in the aligned sequence were found. There were six 1-bp sequence changes, one 1-bp insertion-deletion, and an insertion-deletion containing a 9-bp palindrome (ATGATAGTA). A region that contained two 1-bp changes

14 nucleotides apart was selected for creating a new primer. This region was 19 bp 5' to the sequence complementary of reverse primer HK67. The new primer (HK96) was 14 bp in length with the sequence 5'-CCAGAAGGCGGTGC-3'.

Primer HK96 was tested in combination with the forward primer HK66 under the same PCR reaction protocol. The expected amplification product was 528 bp in length. Annealing temperatures of 62, 60, and 58°C were tried with isolates of *F. oxysporum* f. sp. *albedinis*, *F. oxysporum* f. sp. *cubense* (GM, 3S1, SABA, PW7, DAVAO, STA2, STNP1, A2, 22615, F0001, STPA2, and B2-1), and *F. oxysporum* f. sp. *canariensis* (84-207, 95-1001, 95-1488, 95-1724, 95-2121, and Sicily 2). At all three temperatures, the *F. oxysporum* f. sp. *cubense* isolates that were positive with HK66 and HK67 failed to amplify using HK96 and HK66 (Table 2). DNA from *F. oxysporum* f. sp. *albedinis* also failed to amplify with these primers. Some of the *F. oxysporum* f. sp. *canariensis* isolates tested, however, also did not amplify at these annealing temperatures. Only three of the six isolates tested (95-1001, 95-1488, and Sicily 2) produced the 528-bp fragment at all three temperatures.

HK96 and HK66 failed to amplify all *F. oxysporum* f. sp. *canariensis* isolates; therefore, a restriction site distinctive for the *F. oxysporum* f. sp. *canariensis* sequence was found using the MAP program of GCG (2). An *Afl*III restriction enzyme site found in the sequence of the *F. oxysporum* f. sp. *canariensis* amplicon produced by HK66 and HK67 was absent in the *F. oxysporum* f. sp. *cubense* sequence. DNA digested with *Afl*III was predicted to produce 359- and 178-bp fragments for *F. oxysporum* f. sp. *canariensis*, but not for *F. oxysporum* f. sp. *cubense*. Amplicons from *F. oxysporum* f. sp. *cubense* isolates (SABA, A2, PW7, GM, F0001, and 22615) using HK66 and HK67 did not cut with *Afl*III. Amplicons of *F. oxysporum* f. sp. *canariensis* isolates (95-1001, 95-1488, 96-13, 94-841, 84-207, Sicily 2, and 95-51) using HK66 and HK67 were cut by *Afl*III into the predicted products but, unfortunately, other isolates (95-2121, 94-867, and 95-914) were not cut by the restriction enzyme.

PCR of DNA from the rapid extraction procedure. A procedure for rapidly obtaining fungal DNA for PCR detection is available (12). DNA suitable for PCR amplification can be obtained in 1 day using this method. Samples obtained by this method were used directly or diluted 1:1, 1:10, 1:25, 1:50, or 1:500 (DNA sample/water). No major differences among the amplification intensity from these dilutions was observed. Of the 38 isolates of *F. oxysporum* f. sp. *canariensis* prepared in this manner, DNA from 5 isolates (95-861, 96-1733, 94-868, 87-Guil7, and 94-867) did not amplify using HK66 and HK67 as primers using this procedure. All 10 strains of *F. oxysporum* isolated from sources other than *P. canariensis* also were negative.

Given the low efficiency (87%) of amplification of *F. oxysporum* f. sp. *canariensis* isolates using this method, additional steps were

TABLE 2. *Fusarium oxysporum* f. sp. *cubense* strains tested and results of detection tests^z

Isolate	Amplification with HK66 and HK67 (standard DNA extract)	Amplification with HK66 and HK67 (modified rapid DNA extract)	Amplification with HK66 and HK96	Amplification with HK66 and HK67 and amplicons cut with <i>Afl</i> III
DAVAO	—	—	—	NA
PW7	+	+	—	—
A2	+	+	—	—
SABA	+	+	—	—
B2-1	—	—	—	NA
STNP1	—	—	—	NA
STPA2	—	—	—	NA
STA2	—	—	—	NA
GM	+	+	—	—
22615	+	+	—	—
F0001	+	+	—	—
F9127	+	+	—	NT
3S1	+	—	—	NT

^z + = Positive amplification; — = no amplification or amplification product not cut with *Afl*III; NA = not applicable; and NT = not tested.

added to the rapid procedure to clean the DNA before the remaining *F. oxysporum* f. sp. *canariensis* isolates were tested. Isopropanol and ethanol were used to precipitate the DNA to remove any residual chloroform that might inhibit the reaction. With this modification, a 10-fold dilution of the DNA in water was used as a template for 44 *F. oxysporum* f. sp. *canariensis* isolates. Amplification using the modified rapid extraction procedure is shown in Figure 5. With this method, amplicons were obtained in 43 of these 44 isolates of *F. oxysporum* f. sp. *canariensis*. All DNAs that gave no amplification with the unmodified rapid extraction procedure were repeated with the modification, and all but isolate 96-1733 amplified using HK66 and HK67 primers.

DISCUSSION

The standard DNA extracts of all *F. oxysporum* f. sp. *canariensis* isolates produced a 567-bp fragment with HK66 and HK67 primers. All of these isolates also gave the 567-bp fragment with these primers when template prepared by the rapid extraction method was used, except for DNA from isolate 96-1733. This isolate amplified with the standard DNA extraction method but failed to amplify three times when the DNA was extracted using the rapid method. This particular isolate apparently contains inhibitors to PCR that are not removed by the shortened procedure.

The primer pair HK66 and HK67 also consistently amplified a 567-bp product from DNA from 7 of the 13 isolates of *F. oxysporum* f. sp. *cubense*. Sequence information from translation elongation factor (EF-1 α) and mitochondrial small subunit ribosomal DNA (mtSSU rDNA) indicates that *F. oxysporum* f. sp. *canariensis* and some strains of *F. oxysporum* f. sp. *cubense* are very closely related (14). Sequences from *F. oxysporum* f. sp. *canariensis* isolate 87-Guil7 fall within the same clonal lineage (Clade 1, lineage C4) as a subset of *F. oxysporum* f. sp. *cubense* isolates. All of the *F. oxysporum* f. sp. *cubense* isolates that amplified were within this group. Isolates of *F. oxysporum* f. sp. *cubense* that did not amplify were assigned to several other clades based on the DNA sequence of EF-1 α and mtSSU rDNA. Of the 332 genetically diverse *F. oxysporum* isolates analyzed by DNA sequence analysis (14; K. O'Donnell, unpublished data), only four fall within this lineage and might be expected to be amplified using HK66 and HK67.

In an attempt to find a method even more specific to *F. oxysporum* f. sp. *canariensis*, amplification products were digested with the restriction enzyme *Afl*III. A unique *Afl*III site was discovered in the *F. oxysporum* f. sp. *canariensis* amplicon that was not present in the amplicon of *F. oxysporum* f. sp. *cubense*. This enzyme did not cut any of the DNAs amplified from *F. oxysporum* f. sp. *cubense* isolates but, unfortunately, also failed to cut a subset of the *F. oxysporum* f. sp. *canariensis* isolates tested. Another approach to develop a greater detection specificity was to design a new oligonucleotide incorporating polymorphisms in the sequences derived from *F. oxysporum* f. sp. *canariensis* and *F. oxysporum* f. sp. *cubense*. When a 14-nucleotide primer (HK96) was designed that contained a nucleotide difference at each end and was used for PCR, the DNA from isolates of *F. oxysporum* f. sp. *cubense*

was not amplified. However, this primer combination also failed to amplify a subset of the *F. oxysporum* f. sp. *canariensis* isolates. Taken together, the similarities between the *F. oxysporum* f. sp. *cubense* and *F. oxysporum* f. sp. *canariensis* isolates suggest that these lineages evolved relatively recently from a common ancestor.

The PCR technique using primers HK66 and HK67 detected all *F. oxysporum* f. sp. *canariensis* isolates recovered from symptomatic palm tissue. Fifty-six other isolates of *Fusarium* spp. included in this study failed to amplify by this method, including a nonpathogenic isolate of *F. oxysporum* from the roots of *P. canariensis*. On the other hand, all isolates of *F. oxysporum* causing symptoms of Fusarium wilt on *P. canariensis* and isolated from symptomatic vascular tissue amplified with this primer set. *F. oxysporum* f. sp. *albedinis* and *F. oxysporum* f. sp. *elaedis* also produce vascular wilt on palms; DNA from isolates of these formae speciales also were tested with HK66 and HK67. *F. oxysporum* f. sp. *albedinis* potentially may infect *P. canariensis*, and it is difficult to distinguish the pathogens by symptoms alone (9, 17). The PCR method described here clearly distinguished *F. oxysporum* f. sp. *canariensis* from isolates of other formae speciales that cause disease on palms or from nonpathogenic isolates of *F. oxysporum* that may be found on palms. This method currently is being extended to detect the presence of *F. oxysporum* f. sp. *canariensis* by PCR amplification directly from infected palm tissue (Q. Migheli and H. C. Kistler, unpublished data).

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Fig. 5. Polymerase chain reaction (PCR) amplification of DNA from *Fusarium oxysporum* f. sp. *canariensis* strains isolated using the rapid extraction procedure. PCR primers HK66 and HK67 were used at an annealing temperature of 62°C. Lane a, Negative control without template DNA. Lanes b and c, DNA extracted by the standard method (95-1009 and 95-1001). Lanes d to k, DNAs extracted by the rapid extraction procedure (95-50, 84-Lago3, 95-1000, 94-871, 94-2140, 95-913, 84-P1, and 95-1008). M = bacteriophage λ DNA cut with *Pst*I.

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