Differentiation by Molecular Analysis of *Elsinoe* spp. Causing Scab Diseases of Citrus and Its Epidemiological Implications

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ABSTRACT

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Genetic differences among three citrus scab pathogens were investigated. The three pathogens were (i) the cosmopolitan *Elsinoe fawcettii* causing citrus scab, (ii) *E. australis* from South America causing sweet orange scab, and (iii) *Sphaceloma fawcettii* var. *scabiosa* from Australia causing Tryon's scab. In a companion study, we were unable to differentiate these taxa morphologically, but were able to differentiate *E. fawcettii*, *E. australis*, and *S. fawcettii* var. *scabiosa* pathologically. Two pathotypes of *E. fawcettii* from Florida and two pathotypes from Australia were distinguished on the basis of host range. Restriction analysis of the amplified internal transcribed spacer (ITS) of ribosomal DNA with several endonucleases and sequence analysis of the ITS readily differentiated *E. australis* from *E. fawcettii* and *S. fawcettii* var. *scabiosa*. Random amplified polymorphic DNA (RAPD) analysis indicated that *E. fawcettii* isolates from Australia and from Florida were more closely related to each other than to *E. australis* isolates from Argentina. However, all Australian isolates were separable from all Florida isolates by their RAPD profiles. There was a good correspondence between the RAPD profiles and the two pathotypes identified in Australia. Molecular analysis will be a rapid, useful tool in identifying exotic types of citrus scab on shipments of fruit and help reduce introductions of these types into new areas.

Scab diseases of citrus produce serious fruit blemishes that cost the citrus industry worldwide millions of dollars in reduced value of the fruit for the fresh market. Three scab diseases have been described on citrus. Citrus scab, caused by *Elsinoe fawcettii* Bitancourt & Jenkins (conidial state is *Sphaceloma fawcettii* Jenkins), is widespread in humid citrus areas of the world (16). In Florida, Whiteside (14) described two biotypes of *E. fawcettii,* one of which was pathogenic to rough lemon (*Citrus jambhiri* Lush.), sour orange (*C. aurantium* L.), grapefruit (*C. paradisi* Macf.), Temple and Murcott tangors (*C. sinensis* (L.) Osbeck × *C. reticulata* Blanco), and sweet orange (*C. sinensis*). The second biotype was pathogenic to all of the above except sour orange, Temple tangor, and sweet orange. Sweet orange scab, caused by *E. australis* Bitancourt & Jenkins, is a problem primarily in southern South America. It affects the fruit of sweet orange and mandarin (*C. reticulata*), but apparently does not attack leaves (16). Whiteside (15) questioned the existence of this species and suggested that sweet orange scab could be caused by the sweet orange-infecting biotype of *E. fawcettii*. Tryon's scab, caused by *S. fawcettii* var. *scabiosa* Jenkins, is primarily a problem on lemon (*C. limon* (L.) Burm. f.) in Australia.

In a companion paper (12), we found that isolates of *E. australis* from Argentina differ pathologically from *E. fawcettii* and *S. fawcettii* var. *scabiosa*. However, the morphological differences among cultures of these fungi were minor (M. Priest, *unpublished data*). We found that *S. fawcettii* var. *scabiosa* could be differentiated from *E. fawcettii* only on the basis of host range. We divided *E. fawcettii* into two pathotypes in Florida based on host reactions: "Florida Narrow Host Range" (FNHR) and "Florida Broad Host Range" (FBHR). Our host range studies also classified Australian isolates into two pathotypes: "Tryon's" pathotype, which included most of the former *S. fawcettii* var. *scabiosa* isolates, and "Lemon" pathotype, which is a newly described pathotype from Australia.

The internal transcribed spacer (ITS) region that occurs between the nuclear small and large subunit rDNA has been useful for distinguishing closely related species in fungi (2,4,6). Some regions of the 26S rDNA gene are relatively less conserved, and frequent length mutations in these have served as useful markers to differentiate fungal strains and populations (8,9,10). Random amplified polymorphic DNAs (RAPDs) with arbitrary primers (13,17) have proven useful at taxonomic levels ranging from cultivars to species (3).

In the present study, we used restriction analysis or sequencing of the ITS, a section of the 26S rDNA, and RAPDs to determine the level of genetic variability of scab isolates from citrus and to develop molecular means of identification of species and pathotypes of *Elsinoe* and *Sphaceloma* spp. on citrus. Some general accounts of these results have been presented elsewhere (1,11).

MATERIALS AND METHODS

Isolates and sources. Isolates used in this study, their source, and classifications are listed in Table 1. They are maintained in the New South Wales Agriculture Plant Pathology Herbarium.

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DNA extraction. Total DNA of each fungal isolate was extracted from five 1-cm-diameter colonies grown on potato-dextrose agar. The colonies were scraped clean of agar and ground in extraction buffer (50 mM Tris-HCl [pH 8.0], 0.7 M NaCl, 10 mM EDTA, 1% [wt/vol] cetyl trimethylammonium bromide [Sigma H-5882; Sigma Chemical Co., St. Louis], and 1% [vol/vol] 2-mercaptoethanol). DNA was then extracted as outlined by Lichtenstein and Draper (7).

Polymerase chain reaction (PCR) amplification. Specific DNA regions were amplified using PCR in a Corbett Research FTS-960 thermal sequencer (Corbett Research, Sydney, Australia) using DNA polymerase from *Thermus aquaticus* (Boehringer GmbH, Mannheim, Germany). The PCR amplification reactions were performed in 40 µl of 50 mM Tris (pH 9.0); 20 mM NaCl; 1% Triton X-100; 0.1% gelatin; 3 mM $MgCl_2$; 200 µM of each of the four deoxynucleotides dATP, dTTP, dCTP, and dTTP (Promega Corp., Madison, WI); 50 ng of each of a primer pair; 10 ng of genomic DNA; and 1.5 units of DNA polymerase. Negative controls included all components except template DNA.

Thermal cycling parameters for amplification of the ITS using primers MK56 (5′GTAGGTGAACCTGCGGAAGGATCATT) and MK57 (5′GTTTCTTTTCCTCCGCT) included an initial cycle of 95°C for 3 min, 57°C for 30 s, and 72°C for 1.5 min; followed by four cycles of 94°C for 1 min, 52°C for 30 s, and 72°C for 1.5 min; followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min; and finally followed by a 4- and 1-min incubation at 72 and 4°C, respectively.

TABLE 1. Classification of citrus scab fungi from Australia, Florida, and Argentina based on host range studies (15) and their random amplified polymorphic DNA (RAPD) profile

DAR[a]	Host	Location	RAPD[b] profile
Australian isolates (*Sphaceloma fawcettii* var. *scabiosa*)			
57541	Bergamot	Narara, NSW	A1
57555	Rough lemon	Nambour, Qld	A2
70027	Eureka lemon	Bundaberg, Qld	A1
70180	Rough lemon	Mulgoa, NSW	A1
70181	Rough lemon	Somersby, NSW	A1
70182	Rough lemon	Somersby, NSW	A1
70183	Rough lemon	Somersby, NSW	A1
70184	Eureka lemon	Somersby, NSW	A1
70186	Eureka lemon	Somersby, NSW	A1
70187	Eureka lemon	Somersby, NSW	A1
70188	Lemon	Somersby, NSW	A3
70189	Eureka lemon	Byron Bay, NSW	A2
70190	Eureka lemon	Kulnura, NSW	A1
70217	Lemon	Thursday Island, Qld	A2
70292	*Citrus* sp.	Lambell's Lagoon, NT	A2
Florida isolates: FNHR pathotype[c] (*Elsinoe fawcettii*)			
70033	Volkamer lemon[d]	Indiantown	F
70254	Marsh grapefruit	Lake Alfred	F
CC-1	Marsh grapefruit	Indiantown	F
70258	Duncan grapefruit	Bowling Green	F
Florida isolates: FBHR pathotype[e] (*E. fawcettii*)			
70034	Sour orange	Indiantown	F
70035	Sweet orange	Tangerine	F
70255	Temple tangor	Lake Alfred	F
70256	Temple tangor	Lake Placid	F
70257	Temple tangor	Lake Placid	F
Argentine isolates (*E. fawcettii*)			
70040	Rough lemon	Entre Rios	A2
70213	Satsuma mandarin	Misiones	F
70214	Satsuma mandarin	Misiones	F
Argentine isolates (*E. australis*)			
70036	Cleopatra mandarin[f]	Entre Rios	R2
70037	Valencia sweet orange	Entre Rios	R2
70038	Navel orange	Entre Rios	R1
70039	Common mandarin	Entre Rios	R1
70041	Sweet orange	Entre Rios	R1
70042	Clementine mandarin[g]	Entre Rios	R3
70210	Satsuma mandarin	Unknown	R1
70211	Valencia sweet orange	Unknown	R1
70212	Satsuma mandarin	Entre Rios	R3
70216	Valencia sweet orange	Corrientes	R1
70259	Satsuma mandarin	Entre Rios	R1

[a] Five-digit numbers are those of the collection of the NSW Agriculture Plant Pathology Herbarium, Rydalmere, NSW, Australia. DAR = Department of Agriculture, Rydalmere. Other isolates are not in collection.
[b] RAPD profiles as indicated in Table 2.
[c] FNHR = Florida Narrow Host Range. Attacks rough lemon and grapefruit.
[d] Volkamer lemon, *Citrus volkameriana* Ten. and Pasq.
[e] FBHR = Florida Broad Host Range. Attacks rough lemon, grapefruit, and sour orange.
[f] Cleopatra mandarin, *C. reshni* Hort. ex Tan.
[g] Clementine mandarin, *C. reticulata* × *C. reshni*.

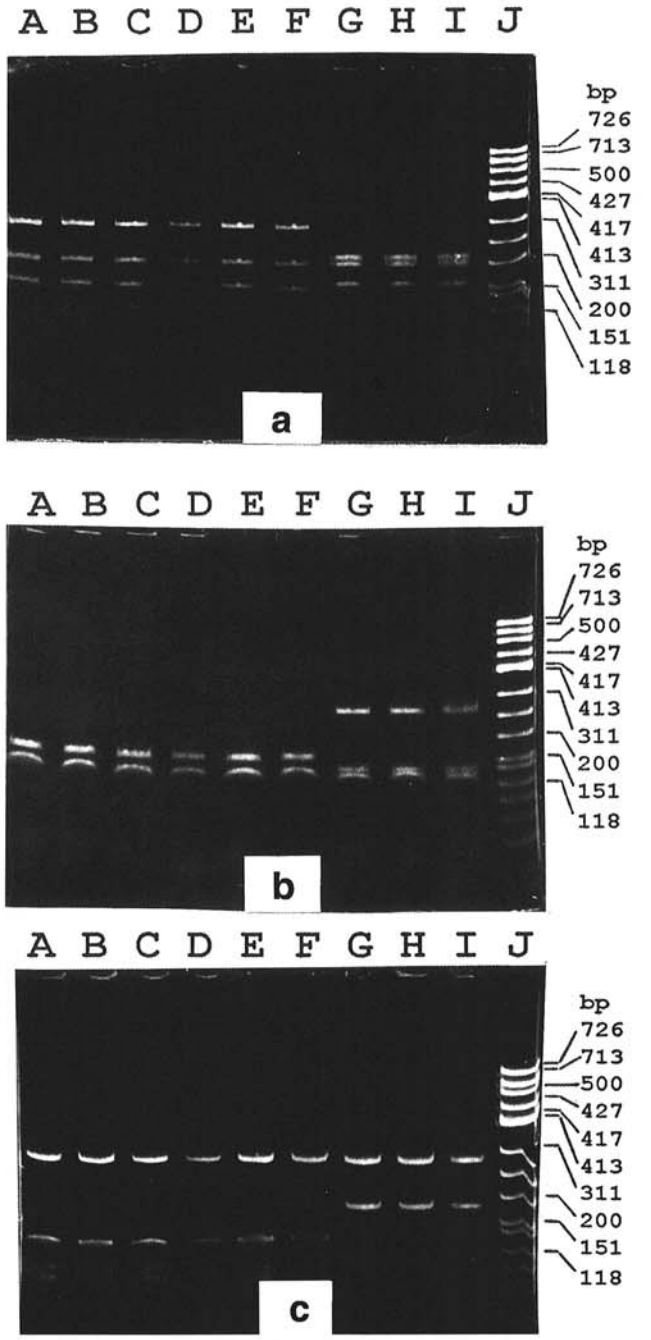

Fig. 1. Electrophoresis of amplified internal transcribed spacer (ITS) fragments restricted by **a,** *Cfo*I; **b,** *Taq*I; and **c,** *Hae*III on a 6% polyacrylamide gel. The Australian isolates of *Sphaceloma fawcettii* var. *scabiosa* (70186, 70184, and 70183) are in lanes A to C; the *Elsinoe fawcettii* isolates from Florida (70035 and 70033) and from Argentina (70040) are in lanes D to F, respectively; and *E. australis* isolates from Argentina (70041, 70036, and 70037) are in lanes G to I. Lane J consists of ϕX174/*Hin*f1 markers.

The thermal cycling program for amplification of a section of the 26S rRNA gene using primers 28F (5′GTGATTTCTGCCCAGTGCTCTG) and 28N (5′ACTAACCTGTCTCACGAC) was identical to the parameters above except that the annealing temperature was 57°C throughout the PCR.

The temperature profile for RAPD analysis using two 10-mers, MtR1 (5′GTAAAGGGGG) and MtR2 (5′AAGTTTTTGG), was an initial cycle of 94°C for 3 min, 36°C for 1 min, and 72°C for 3 min; followed by 45 cycles of 94°C for 30 s, 36°C for 1 min, and 72°C for 3 min; and finally followed by a 10- and 1-min incubation at 72 and 4°C, respectively.

Length and restriction analysis of amplified DNA. DNA from amplification reactions was electrophoresed directly in 0.8 or 1% horizontal agarose gels. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet light. For restriction analysis, approximately 0.5 µg of DNA from amplification reactions was digested according to the enzyme manufacturer's recommendations. Digested DNA was electrophoresed in mini (9 × 6.5 cm) 6 or 8% polyacrylamide gels and visualized by ethidium bromide staining.

Sequencing reactions. Amplified ITS fragments of representative isolates of the three types of scab from citrus (Australian isolates 70188, 70189, 70190, and 70292; Florida isolates 70033 [FNHR pathotype] and 70255 [FBHR pathotype]; and Argentine isolates 70037, 70040, 70214, and 70216) were cloned into the plasmid pGEM-T (Promega Corp.). Two ITS clones from each of 70188 and 70037, and one of each of all other isolates were sequenced in both directions using M13 forward and SP6 primers. Sequencing was done automatically using dye-labeled dideoxynucleotides and DNA polymerase in the Applied Biosystems 373A DNA sequencer (Applied Biosystems, Inc., Foster City, CA) at Westmead Hospital, Sydney, Australia. All sequencing traces were checked visually and edited manually.

RESULTS

26S rDNA gene analysis. The distal portion of the 26S rDNA gene, amplified with primers 28F and 28N, was 0.8 kb for all the scab isolates from citrus studied. Restriction analysis using *Hae*III and *Msp*I of this amplified section of the 26S rDNA gene revealed no site differences among the isolates (data not shown).

ITS variation. Primers MK56 and MK57 were used to amplify the ITS from all isolates listed in Table 1. The ITS of *E. australis* was slightly shorter than the other two citrus scab types. The amplified ITS segment was restricted with *Hae*III, *Taq*I, *Rsa*I, and *Cfo*I to analyze for site variation. Restriction analyses divided the scab isolates into two groups. Different profiles were observed for each group with *Hae*III, *Taq*I, and *Cfo*I (Fig. 1). One group consisted of the Australian *S. fawcettii* var. *scabiosa* isolates and the Florida *E. fawcettii* isolates. A subgroup of isolates from Argentina (70040 [Fig. 1 lane F], 70214 [not shown], and 70213 [not shown]) had similar ITS restriction profiles to the Australian and Florida citrus scab. Isolate 70040 was recovered from rough lemon, the usual host of *E. fawcettii* or *S. fawcettii* var. *scabiosa*. Isolates 70214 and 70213 were from Satsuma mandarin (*C. unshiu* (Macf.) Marc.), which is quite often attacked by *E. fawcettii*. The other group of restriction profiles comprised Argentine isolates from sweet orange scab that had been identified as *E. australis* on the basis of host range (12).

*Rsa*I gave a similar pattern for both groups, with the two *Rsa*I fragments (197 and 406 bp) of *E. australis* being slightly lower than the fragments (204 and 427 bp) of *S. fawcettii* var. *scabiosa* and *E. fawcettii*. The sequence alignment (Fig. 2) showed that the two *Rsa*I sites in the ITS of the three scab types from citrus are separated by three bases and are in the same relative positions, thereby giving rise to the similar restriction profiles (data not shown).

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Sfs  gtaggtgaacctgcggaaggatcattaACGAGGTAGGGCCTCCTTCGGGAGCCCGAACTCCCCACCCTTTGCTGTTGCGA 80
Ef   ..............................................................................  80
Ea   ..............................A....TT...GAAA.....................................  80

Sfs  ATCACGTTGCTTCGGCGGGACCTCCCCCCCTCGAGAGGGGGGCGGGGGGCCGGGCCACCGCGCCCGGACAGCGCCCGCCG 160
Ef   ..............................................................................  160
Ea   ...T-............C..G.......ATCCCT......GC..CC.GGCCT-.....--.................  156

Sfs  GGGGACCGAACCAACTCTTGTCTTGTGAAACCTTTGCAGTCGGAGTACAACCGTACAATCAAATCAAAACTTTCAACAAC 240
Ef   ..............................................................................  240
Ea   .A........T.......-..T..-.-..-..A.AAT-..........TTTT............T..............  231

Sfs  GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC 320
Ef   ..............................................................................  320
Ea   ..............................................................................  311

Sfs  GAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAGCGTCATTTCACCAATCAAGCCCCG 400
Ef   ..............................................................................  400
Ea   ..............................................................................  391

Sfs  CTTGGTATTGGGTGCGACAGCCCGCCCCCCGTGGCCGGCCCGCCCGAAATGCATCGGCGAGGCACCGACCCCCGGCGTGT 480
Ef   ..............................................................................  480
Ea   .........A..C..CTGGC.G.C.....................................................  471

Sfs  TAGAATTTCGAAACGTCAGGAGCACCGGTG--ACCCTCCGCCGTCAACCCGGACCCCTCCCCCCCGCGGGCGGGGCCCAC 558
Ef   ..............................--..............................................  558
Ea   ..............................TT.A....T.....G..A..TT-----.------------------..-  526

Sfs  CCCCC--------TTC-AAGGttgacctcggatcaggtaggaatacccgctgaacttaagcatatcaataagcggaggaa 629
Ef   .....--------...-..............................................................  629
Ea   -----AACTATTT...T..............................................................  601

Sfs  aagaaac 636
Ef   ....... 636
Ea   ....... 608
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Fig. 2. Alignments of internal transcribed spacer (ITS) I and ITS II including the 5.8S gene of *Sphaceloma fawcettii* var. *scabiosa* (Sfs; representing isolates 70190, 70292, 70188, 70189, and 70040); *Elsinoe fawcettii* (Ef; representing isolates 70255, 70033, and 70214); and *E. australis* (Ea; representing isolates 70216 and 70037). A dot (.) indicates identity with the *S. fawcettii* var. *scabiosa* sequence. A dash (–) indicates alignment gaps. Sequences in bold are those of the 5.8S gene, sequences in lower case at the 5′ and 3′ ends represent parts of gene sequences of the 18S and 26S rDNA, respectively. Sequences in uppercase upstream and downstream of the 5.8S gene are the ITS I and ITS II, respectively.

TABLE 2. Amplified fragments scored in the random amplified polymorphic DNA (RAPD) profiles of the scab fungi from citrus in Table 1

	RAPD profiles[a]						
	Australia			Florida	Argentina		
Band (bp)	A1	A2	A3	F	R1	R2	R3
1,950	+	+	+[b]	+	–	–	–
1,160	+	+	+	+	–	–	–
1,000	–	–	–	–	–	–	+
800	–	–	–	–	+	+	+
770	–	–	+	–	–	–	–
740	–	–	–	–	+	–	–
720	+	–	+	–	–	–	–
600	–	+	+	–	–	–	–
580	–	–	–	–	–	–	+
510	+	–	+	2+[c]	6+[c]	–	–
480	–	+	–	–	–	–	–
430	+	–	+	+	–	–	–
<430	–	–	–	–	–	–	+

[a] + or – indicates the presence or absence of the amplified fragment.
[b] Extremely faint.
[c] 2+ and 6+ indicates the amplified fragment is two and six times, respectively, as intense as that scored for the A1 and A3 profile.

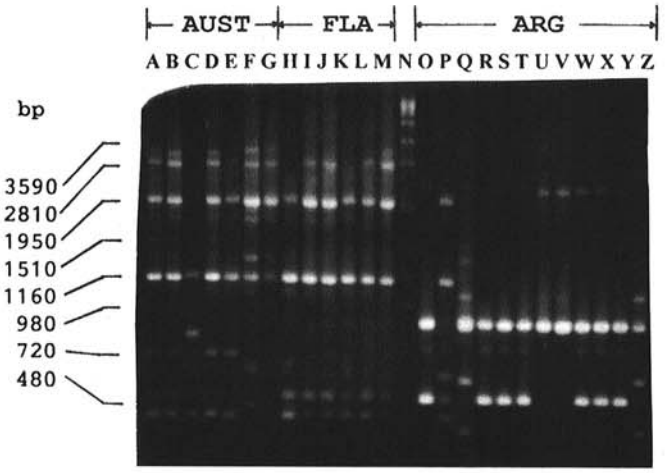

Fig. 3. Electrophoresis of random amplified polymorphic DNA fragments on a 2% agarose gel. The Australian (AUST) isolates 70184, 70186, 70188, 70027, 57541, 70217, and 70292 are in lanes A to G, respectively. The Florida (FLA) isolates 70034, 70254, CC-1, 70256, 70257, and 70258 are in lanes H to M, respectively. The Argentine (ARG) isolates 70216, 70213, 70212, 70211, 70210, 70259, 70036, 70037, 70038, 70039, 70041, and 70042 are in lanes O to Z, respectively. Lane N consists of SPP1/*Eco*RI marker fragments.

Amplified ITS regions generated by primers MK56 and MK57 were sequenced for eight representative isolates of *S. fawcettii* var. *scabiosa* (from Australia) and *E. fawcettii* (from Florida and Argentina) and two *E. australis* isolates. Sequence analysis confirmed the results from restriction analysis. Identical sequences were found in the 636-bp PCR fragment of the representative isolates of *S. fawcettii* var. *scabiosa* (70190, 70292, 70188, and 70189) and *E. fawcettii* (70255, 70033, 70040, and 70214). The ITS including the 5.8S gene of *S. fawcettii* var. *scabiosa* and *E. fawcettii* was 543 bases (Fig. 2). Similarly, no substitutions were found in the 608-bp PCR segment sequenced for the representative isolates of *E. australis* (70037 and 70216). The ITS including the 5.8S gene of *E. australis* was 515 bp, which was 28 bp shorter than that of *S. fawcettii* var. *scabiosa* and *E. fawcettii* (Fig. 2).

The 5.8S gene was conserved in all the scab isolates from citrus sequenced. The ITS I of *E. fawcettii* and *S. fawcettii* var. *scabiosa* was 200 bp and that of *E. australis* was 191 bp. There were 37 base differences between the ITS I of *E. fawcettii* (and *S. fawcettii* var. *scabiosa*) and that of *E. australis* (Fig. 2). The ITS II of *E. australis* (166 bp) was also shorter than that of the other two citrus scab types (185 bp). Substitutions included 11 transversions and four transitions (Fig. 2). There was a 32-bp stretch of G-C bases at the 3′ end of the ITS II in *E. fawcettii* and *S. fawcettii* var. *scabiosa* compared with a short AT stretch of 11 bp at the corresponding positions in *E. australis* (Fig. 2). Overall, the ITS of *E. fawcettii* and *S. fawcettii* var. *scabiosa* was found to be slightly more G-C rich (59% compared with 54% G-C content of *E. australis*).

RAPD analysis. Amplifications were performed using two 10-mer primers, MtR1 and MtR2. No amplification product was observed with the MtR2 primer under the reaction conditions used. RAPD product patterns with MtR1 primer were useful in differentiating the three scab types on citrus. The RAPD fragment profiles obtained were reproducible in time for five replicates across isolates. Altogether, 13 amplified fragments were scored in the analysis of the RAPD profiles (Table 2). Fragments above 2 kb were not consistent or well-defined and were not considered. Each band was scored for its presence or absence, and the intensity variation for each amplified fragment across isolates was noted by eye. Densitometric analysis would be necessary if a quantitative analysis was made. There is, at present, insufficient characters for a comprehensive quantitative analysis.

The RAPD profiles broadly divided the isolates into two major groups that corresponded with the restriction and sequence analysis of the ITS. One group, characterized by two fragments of 1,950 and 1,160 bp, comprised isolates of *S. fawcettii* var. *scabiosa* and *E. fawcettii* (Fig. 3; Tables 1 and 2) except isolate 70188, which had an extremely faint fragment of 1,950 bp. A subgroup of Argentine isolates comprising 70040, 70214, and 70213 (Fig. 3 lane P and Fig. 4 lanes E and H) was again classified with this group, agreeing with the ITS analysis. The other group, characterized by a fragment of approximately 800 bp (Fig. 3), comprised sweet orange scab isolates from Argentina (presumably *E. australis*).

In addition, the RAPD fragment profiles were able to distinguish the Australian citrus scab from the Florida citrus scab (Figs. 3 and 4). Three RAPD patterns, A1, A2, and A3, were observed for the Australian isolates (Figs. 3 and 4; Table 2). The 2,810- and 3,000-bp fragments were amplified from all *S. fawcettii* var. *scabiosa* isolates from Australia (Figs. 3 and 4). The RAPD profile A1 (Table 2) included a major subgroup of *S. fawcettii* var. *scabiosa* isolates (70180, 70181, 70182, 70183, 70184, 70186, 70187, 70190, 70027, and 57541). The A2 profile included the Australian isolates 57555, 70189, 70217, and 70292, and an Argentine isolate, 70040. The A2 profile differed from the A1 pattern by five fragments (Table 2). The host range of 70040 was similar to the Australian isolates (1,11,12). Profile A3 includes only 70188 (Table 1) and had a unique fragment of 770 bp in addition to all fragments scored for A1 and A2, except the 480-bp band (Table 2).

In distinguishing some Florida isolates, it was observed that the consistency of two fragments, 2,810 and 3,000 bp, needs to be confirmed with a bigger sample size and, hence, were not included in the RAPD analysis. All the Florida isolates displayed four common fragments of 1,950, 1,160, 510, and 430 bp (Table 2 profile F), all of which were present in the Australian A1 profile, with the 510 bp being less intense in the Australian isolates (Fig. 3). There were only two differences noted between the Australian A1 and the Florida F profiles. One was attributed to the absence of the 720-bp fragment and the other was due to the greater intensity of the 510-bp band in the F profiles. Two Argentine isolates, 70213 and 70214, had the F profile (Fig. 3 lane P and Fig. 4 lane H), and their host range corresponded with the FBHR pathotype.

The *E. australis* isolates from Argentina were subdivided into three groups. The largest subgroup, R1 (70216, 70211, 70210, 70259, 70038, 70039, and 70041), had two characteristic fragments of 740 and 510 bp (Fig. 3; Tables 1 and 2). The 510-bp

band was visually about six and three times more intense than the fragment of corresponding size present in the Australian profiles (A1 and A3) and the Florida profile (F), respectively. This 510-bp fragment was absent in the subgroup R2 isolates of 70036 and 70037 (Fig. 3 lanes U and V) and the subgroup R3 isolates of 70212 and 70042. The R3 profile had three distinct fragments of approximately 1,000, 580, and less than 480 bp (Fig. 3 lanes Q and Z).

DISCUSSION

Molecular methods used in this study uncovered three levels of genetic variation for differentiation of the pathogens that cause the three described types of scab on citrus. In contrast, studies (1,11,12) on colony morphology and conidial shape and size could not conclusively distinguish the Argentine sweet orange scab from the Australian and Florida citrus scab isolates.

All the scab isolates from citrus showed conservation of *Hae*III and *Msp*I sites in the amplified 0.8-kb segment of the distal end of the 26S rDNA gene. More work is needed to clarify whether conservation of these sites characterizes the *Elsinoe* spp.

A higher level of variation revealed by ITS restriction, ITS sequence, and RAPD analyses separates *E. australis* (causing sweet orange scab) from the scab fungi from Australia and Florida. The Australian and Florida isolates were grouped together into one group by the molecular similarity observed in ITS I and II. The RAPD analysis also suggests that the Florida isolates and the Australian isolates are much more closely related than either is to the Argentine sweet orange scab fungus. Jenkins (5) mentioned the need for further taxonomic study to determine whether *S. fawcettii* var. *scabiosa* should be accorded specific rank distinct from *S. fawcettii*, the conidial state of *E. fawcettii*. The results presented here strongly suggest that *S. fawcettii* var. *scabiosa* may be conspecific with *E. fawcettii*. However, a taxonomic study including other *Elsinoe* spp. will be necessary to clarify the amount of molecular difference that exists between the species in this genus.

E. fawcettii (and *S. fawcettii* var. *scabiosa*) can be differentiated from *E. australis* by restriction analysis of amplified ITS with *Hae*III, *Cfo*I, and *Taq*I (Fig. 1). Sequence analysis determined that the ITS of *E. australis* is 28 bp shorter than the ITS of *E. fawcettii* (and *S. fawcettii* var. *scabiosa*), which is 543 bp long. The 5.8S gene is conserved in both *Elsinoe* spp. The base differences in ITS I and II indicate that the ITS of *E. fawcettii* is slightly more G-C rich. The MtR1 primer used in the RAPD analysis is G rich, and it amplified more RAPD fragments from *E. fawcettii* (and *S. fawcettii* var. *scabiosa*) than *E. australis*. This suggests that not only is the ITS of *E. fawcettii* (and *S. fawcettii* var. *scabiosa*) more G-C rich, but the whole genome may be generally so.

In RAPD analysis using the 10-mer, MtR1 provided the appropriate level of genetic variation to enable clear demarcation between the Australian and Florida scab fungi (Figs. 3 and 4). The RAPD patterns also enabled subdivisions to be made within the Australian isolates. There appeared to be at least two distinct RAPD subpatterns that characterized the Australian isolates. Profile A1 (Table 1) was more common, whereas profile A2 (Table 1) included isolates from northern Australia (northern NSW, Qld, and NT), all of which represent a second pathotype identified in Australia (12). Three Argentine isolates were identified as *E. fawcettii* (or *S. fawcettii* var. *scabiosa*) on the basis of ITS and RAPD analyses. Two of these (70213 and 70214) had RAPD profiles typical of the Florida isolates, whereas the third (70040) had the A2 profile of Australian isolates (Table 1). Their molecular differentiation corresponded with the results of host range studies (11,12). Hence, the entire range of scab fungi from citrus is present in Argentina.

Most citrus-producing countries regulate importation of fresh fruit from other areas to avoid introduction of exotic pathogens. Sweet orange scab caused by *E. australis* is of considerable concern, because of its ability to attack oranges and mandarins. The ability to distinguish sweet orange scab from both Tryon's scab and citrus scab by molecular analysis of the ITS will facilitate regulation of movement of infected fruit into citrus areas free of this pathogen, which currently includes most citrus production areas of the world except South America. The molecular technique is sensitive, reliable, and much more rapid than the host range differentiation studies, thereby enabling a quicker decision regarding perishable produce.

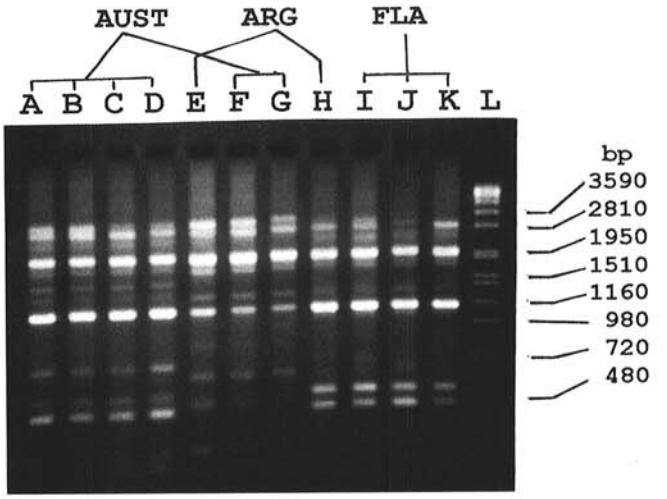

Fig. 4. Electrophoresis of random amplified polymorphic DNA fragments on a 2% agarose gel. The Australian (AUST) isolates 70182, 70190, 70187, 70180, 57555, and 70189 are in lanes A to D, F, and G, respectively. The Florida (FLA) isolates 70033, 70035, and 70255 are in lanes I to K, respectively. The Argentine (ARG) *Elsinoe fawcettii* isolates 70040 and 70214 are in lanes E and H, respectively. Lane L consists of SPP1/*Eco*RI marker fragments.

The genetic differences revealed by RAPD analysis between the Australian and the Florida citrus scab isolates correlate with their geographical origin and the results of host range studies (12). The molecular evidence presented suggests that the Australian *S. fawcettii* var. *scabiosa* and the Florida *E. fawcettii* are very likely different pathotypes of the same species. The Australian citrus scab pathotypes have a narrower host range than the Florida pathotypes. The citrus species affected by Tryon's scab that are not affected by citrus scab in Florida are bergamot (*C. bergamia* Risso & Poit.) and *Microcitrus* spp. (19). These species are of little economic significance and, hence, Tryon's scab poses little if any threat to Florida citrus. Moreover, there is no doubt from the studies presented here that strict quarantine precautions should be taken to avoid moving the Florida citrus scab fungi into Australia, either from the United States, from South America, or from other countries where citrus scab is known to occur. Since many as yet unidentified pathotypes may exist in localized areas, precautions should also be taken not to move scab-infected nursery stock and budwood even within countries and states.

Molecular analysis of scab fungi has so far only included isolates from Australia, Florida, and South America. Because the citrus scab has a widespread distribution (18), pathological and molecular studies should be extended in future work to isolates from other geographical regions that might be of quarantine and trade significance.

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