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Isolation and characterization of an *Isaria fumosorosea* isolate infecting the Asian citrus psyllid in Florida

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ABSTRACT

A fungal pathogen that killed adult *Diaphorina citri* Kuwayama (Asian citrus psyllid) (Hemiptera: Psyllidae) in Florida citrus groves during the fall of 2005 was identified and characterized. Investigation of this pathogen is important because *D. citri* vectors citrus greening disease (Huanglongbing), which was reported in Florida in 2005. The morphological and genetic data generated herein support identification of the fungus as *Isaria fumosorosea* Wize (*Ifr*) (=*Paecilomyces fumosoroseus*) (Hypocreales: Cordycipitaceae) from the Asian citrus psyllid (*Ifr* AsCP). Koch's postulates were fulfilled after the fungus was isolated *in vitro* and transmitted to healthy psyllids, which then exhibited a diseased-phenotype similar to that observed in the field. Both *in vitro* growth characteristics and two *Ifr* AsCP-specific molecular markers discriminated the psyllid pathogen from another local *Ifr* isolate, *Ifr* 97 Apopka. These molecular markers will be useful to track the dynamics of this disease in *D. citri* populations. The potential for utilizing *Ifr* to complement existing psyllid pest management strategies is discussed.

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1. Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, (Hemiptera: Psyllidae) was discovered in Florida in 1998 and apparently arrived without its natural enemies (Halbert, 1998; Knapp et al., 1998; Halbert et al., 2000). Control of *D. citri* is important because this phloem-feeding pest vectors *Candidatus* Liberibacter asiaticus (*Ca.* L. asiaticus), a phloem-residing α–proteobacterium that causes citrus greening disease or Huanglongbing (HLB) (Garnier et al., 2000). HLB was discovered in Florida in 2005 and poses a serious threat to the citrus industry because *Ca.* L. asiaticus infections cause fruit damage and ultimately kill citrus trees (Halbert and Manjunath, 2004; Halbert, 2005; Bouffard, 2006).

To limit the spread of HLB, suppression of *D. citri* populations will require a multi-tactic integrated pest management (IPM) program. Petroleum oil and foliar and systemic insecticides are currently recommended to reduce *D. citri* populations (Rae et al., 1997; Browning et al., 2006; Rogers and Timmer, 2007), but will not likely eliminate HLB transmission. Other control agents include native predators (Michaud, 2004) and the exotic parasitoid *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae) (Hoy et al., 1999; Hoy and Nguyen, 2000). The maintenance of clean nursery stocks and removal of HLB-infected citrus trees are cultural measures used to retard the spread of HLB. Widespread use of chemical insecticides may result in the development of resistant psyllid populations and could negatively affect existing biological control agents in citrus that suppress populations of mites, scales, whiteflies, mealybugs, aphids, and the citrus leafminer. Therefore, new approaches are needed that complement existing management strategies for *D. citri*, such as the potential utilization or augmentation of microbial pathogens that attack the psyllid. Worldwide, diverse species of pathogenic fungi suppress *D. citri* populations, especially during periods of high relative humidity (Samson, 1974; Rivero-Aragon and Grillo-Ravelo, 2000; Subandiyah et al., 2000; Étienne et al., 2001; Xie et al., 1988; Aubert, 1987).

In the fall of 2005, two fungal pathogens were discovered attacking *D. citri* in Florida, and one of these pathogens was identified as *Hirsutella citriformis* (Meyer, 2007; Meyer et al., 2007). The initial goals of this study were to identify the second psyllid pathogen using both morphological and molecular analyses and then determine if the disease could be propagated in a healthy laboratory colony of *D. citri*. For convenience within this manuscript, henceforth the psyllid pathogen will be referred to as *Isaria fumosorosea* Wize (=*Paecilomyces fumosoroseus*) from the Asian citrus psyllid or *Ifr* AsCP (Hypocreales: Cordycipitaceae) (Hodge et al., 2005; Luangsa-ard et al., 2005).

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Once determined, the morphological and molecular characteristics of *Ifr* AsCP were compared to a reference strain, *Ifr* 97 (=*Pfr* 97), which was isolated from *Phenacoccus* sp. (Hemiptera: Pseudococcidae) in Apopka, FL. *Ifr* 97 was commercially developed as the microbial insecticide Pre-FeRal W.R. Grace and Co., Columbia MD; Thermo Trilogy/Biobest N.V., Belgium) (Vidal et al., 1998; Faria and Wraight, 2001). These comparisons were conducted to determine if *Ifr* AsCP was the same local strain as the well-characterized *Ifr* 97. If the two fungi were the same strain, then we could pursue field trials with a commercially-available microbial formulation. If they were different strains, then *Ifr* AsCP, the naturally occurring *D. citri* pathogen, could be formulated and compared with *Ifr* 97 in laboratory and field trials for psyllid control. Collectively, this research contributes the first steps towards evaluating *Ifr* for its potential use in an IPM program to suppress *D. citri* populations in Florida or elsewhere.

2. Materials and methods

2.1. Collection of psyllid cadavers and description of the fungus

Cadavers of adult *D. citri* were collected from a single orange grove in Polk county, FL (28°06′295″ N, 81°42.895′ W) during September and October 2005. The cadavers were placed into sterile 50-mL centrifuge tubes (USA Scientific, Ocala, FL) and held at 4 °C during transit to the Entomology and Nematology Department at the University of Florida, Gainesville, FL. Mycosed *D. citri* were photographed using a dissecting microscope linked with the Auto-Montage Pro system using software ver. 5.02 (Synoptics, Frederick, MD) and with scanning electron microscopy (SEM) on a Hitachi 4000 FE-SEM operating at 4–6 kV (Quattlebaum and Carner, 1980). Differential interference contrast (DIC) microscopy (360–1000×) was used to analyze the hemolymph of infected adult *D. citri* and mature conidia isolated from *in vitro* cultures of *Ifr* AsCP. SPOT software 3.4.3 (Diagnostic Instruments, Sterling Heights, MI) was used to measure structures of the psyllid pathogen captured digitally.

2.2. Laboratory propagation of the psyllid disease

A laboratory colony of *D. citri* was maintained according to Skelley and Hoy (2004) in a greenhouse at 20–32 °C with a 16L:8D photoperiod. Adult *D. citri* were allowed to oviposit on the tender new growth (flush) produced by small potted orange trees held in mesh cages (0.8 m × 0.9 m × 1.1 m). Upon emergence, adult *D. citri* were collected to produce another generation.

Initially, healthy adult *D. citri* from the greenhouse colony were either exposed to the field-collected cadavers (20 individuals) or not exposed (20 controls) to test if the infection could be propagated using the following procedure. Adult *D. citri* were collected from the colony in a sterile 50-mL centrifuge tube and placed on ice for 10–15 min. The immobilized psyllids, held with fine-tipped forceps, were touched to the conidia present on cadavers of mycosed *D. citri*. Inoculated insects were held in a sterile 50-mL centrifuge tube containing a single mature orange leaf and a water-soaked cotton ball to maintain approximately 100% relative humidity (RH). The inoculated psyllids were held in a growth chamber at 24–25 °C with a 16L:8D photoperiod. Once infections were established, fresh cadavers produced in the laboratory were used to inoculate 10–20 psyllids each week to maintain the culture.

2.3. *In vitro* culturing

To culture the fungus *in vitro*, conidia were isolated from a single field-collected cadaver to inoculate 6-cm plates containing quarter-strength Sabouraud dextrose agar +1% yeast extract (SDY) or malt extract agar (MEA) media. Another local isolate of *Ifr*, *Ifr* 97 Apopka, was kindly supplied by Dr. L. Osborne and maintained on psyllids and *in vitro* according to the methods described above. Transparent tape-mounts of sporulating cultures were stained with acid fuchsin and examined under a light microscope at 400× to examine the structural characteristics of the fungi grown *in vitro* (Koneman and Roberts, 1985).

The growth rate and conidia yield of *Ifr* AsCP and *Ifr* 97 were compared 1-week post-inoculation on SDY and MEA. Fungal cultures were held at 26 °C without light, and conidia were harvested as follows: (i) hyphae + conidia were scraped from plates with a sterilized spatula and suspended in 10 mL sterile water; (ii) the suspension was filtered through Miracloth (Calbiochem, EMD Biosciences Inc., La Jolla, CA) held in a funnel and collected in a glass beaker; (iii) conidia were quantified using a hemocytometer. For *Ifr* AsCP and *Ifr* 97, two replicates each consisting of six SDY and MEA plates (6 cm) were spot-inoculated with 5 μL of 4.5×10^5 conidia + water to measure growth, and 100 μL of 4.5×10^7 conidia/mL to measure conidia production. To test the effect of media on mean growth (diameter) and mean conidia yield for *Ifr* AsCP and *Ifr* 97, the data were subjected to a one-way analysis of variance with PROC GLM, and the least squares means were separated using a probability of a significant divergence of $P \leqslant 0.05$ (SAS Institute, 1996).

Qualitative assays were conducted to determine if the *in vitro* cultures were infective to *D. citri*. Adult *D. citri* from the laboratory colony were grasped with a fine-tipped forceps and touched (20 individuals) or not touched (20 controls) directly to the surface of sporulating cultures of *Ifr* AsCP and *Ifr* 97 grown on quarter-strength SDY media. Inoculated psyllids were then maintained according to the method used to propagate the pathogen on psyllids after exposure to the field-collected cadavers. Mortality due to fungal pathogenesis in these assays was recorded three days after treatment.

2.4. Molecular analyses

DNA was isolated from a single field-collected mycosed adult *D. citri*, from 1-week-old cultures of *Ifr* AsCP initiated from that same cadaver, and from *Ifr* 97 grown on 6-cm plates containing quarter-strength SDY media. All DNA extractions were conducted using PUREGENE reagents (Gentra Systems, Minneapolis, MN) according to the instructions provided by the manufacturer. A portion of the 18S small ribosomal subunit (SSU), the 5′ variable region of the 28S large ribosomal subunit (LSU), and the β-tubulin gene were amplified, cloned and sequenced according to Meyer et al. (2007). DNA sequences were compared to those deposited in GenBank using BLAST (blastn) with the default settings.

DNA fingerprints of *Ifr* AsCP and *Ifr* 97 were produced using amplified fragment length polymorphism (AFLP) analysis, using the methods of Boucias et al. (2000) with the primers shown in Table 1. A negative control was included for each primer that contained all of the reagents for the AFLP procedure but did not have any DNA to ensure that no PCR artifacts confounded the analysis. Amplified DNA was electrophoresed on 1% TAE gels stained with ethidium bromide for visualization under ultraviolet light, and DNA size markers (HyperLadder III and IV, Bioline USA Inc., Randolph, MA) were used to estimate the relative sizes of the amplification products. Amplification products > 0.2 kb and < 2.5 kb were scored for each fungus in the AFLP analysis.

Three polymorphic bands, amplified only from DNA isolated from *Ifr* AsCP, were excised from the gel and homogenized with a sterile blunt-ended pipette tip in 50 μl of sterile water. A 10-μl aliquot of the homogenate was used in a re-amplification reaction using the same AFLP primer and reaction conditions that produced

Table 1
Total number of bands and unique bands (polymorphisms) produced by each primer in the AFLP analysis of DNA isolated from *in vitro* cultures of *Ifr* AsCP and *Ifr* 97

Primer	+3 bases[a]	*Ifr* AsCP		*Ifr* 97	
		Total bands	Poly-morphisms[b]	Total bands	Poly-morphisms
1	GGC	12	2	12	2
2	CAG	8	3	10	5
3	GCC	10	5	9	3
4	AGG	11	0	11	0
5	AGT	7	0	11	4
6	ATA	6	2	8	4
7	ACC	14	3	12	2
8	AGC	11	1	12	3
9	ATT	6	0	11	5
10	AAC	11	3	10	2
11	ACT	10	1	9	0
12	TCG	10	4	10	2
	Totals	116	24	125	32

Amplification products between 0.2 and 2.5 kb were included for the analysis.
[a] Indicates the terminal 3 nucleotides added to the *Eco*RI adaptor sequence. (GACTGCGTACCAATTC+) of AFLP primers.
[b] Polymorphisms refers to bands unique to the isolate.

each polymorphism. Re-amplified PCR products were cloned and sequenced as described above. Isolate-specific PCR primers were designed based on each AFLP polymorphism sequence with the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 2). The forward primers included the *Eco*RI specificity site at the ends of each AFLP polymorphism sequence, and the reverse primers were nested in the middle portion of each sequence. Primers designated with an "A" or "B" were designed to include the *Eco*RI specificity site at the 5′ or 3′ end of the sequence, respectively. The annealing temperature was optimized for each psyllid pathogen-specific PCR primer pair using the temperature gradient feature of the MyCycler thermal cycler (Bio-Rad, Hercules, CA). The PCR reactions (25 μl) included 2.5 units of *Taq* DNA polymerase and 1X stock PCR buffer (Bioline USA, Inc, Randolph, MA), 350 mM dNTPs, and 800 pmol of primers. The PCR cycling parameters included an initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at the optimized temperature for each primer pair for 30 s, and extension at 68 °C for 1 min. A final extension at 68 °C for 5 min concluded the reaction.

The specificity of each psyllid pathogen-specific PCR primer pair was tested in a standard PCR assay using DNA isolated from *in vitro* cultures of *Ifr* AsCP and *Ifr* 97 and from psyllids killed by *Ifr* AsCP and *Ifr* 97. The samples from psyllids killed by each pathogen included two replicates each consisting of dead individual adults and nymphs. As a control for DNA template quality, a 0.9–1.0 kb portion of the β-tubulin gene was amplified from each DNA sample, and a negative control (no DNA) was included for each isolate-specific primer pair tested. PCR products were analyzed by gel electrophoresis as described above.

3. Results

3.1. Collection of psyllid cadavers and description of the fungus

During field sampling in September and October 2005, a total of six mycosed adult *D. citri* were collected from the undersurface of foliage on orange trees in Polk county, FL (Fig. 1A). These dead psyllids were attached to the foliage by mycelia and situated in a feeding position. The dorsal surface of the field-collected cadavers was covered with fungal growth. During field sampling, no mycosed nymphs or eggs of *D. citri* were observed.

SEM of the psyllid cadavers revealed that *Ifr* AsCP had conidiophores bearing whorls of divergent, mononematous phialides that terminated in smooth-walled conidia (Fig. 1B). The flask-shaped phialides ($N = 6$) averaged 5.2 ± 1.1 μm (standard deviation) in length and 1.8 ± 0.3 μm in diameter at the swollen basal region. Each phialide ($N = 8$) tapered to a distinct neck averaging 0.4 ± 0.1 μm in diameter that interfaced with the cylindrical or fusiform conidia. Mature conidia harvested from a mycosed psyllid averaged 4.1 ± 0.5 μm in length and 2.0 ± 0.2 μm in diameter ($N = 21$). Vegetative hyphae were hyaline, smooth-walled, and had an average diameter of 1.5 ± 0.3 μm ($N = 21$). Together, these morphological characteristics were consistent with those of entomopathogenic fungi in the genus *Isaria* (=*Paecilomyces*) (Samson, 1974; Humber, 1998).

3.2. Laboratory propagation of the psyllid disease

We tested if *Ifr* AsCP could be propagated on healthy *D. citri* from a laboratory colony in qualitative experiments. Adult *D. citri* ($N = 20$) were either topically exposed to a field-collected cadaver or not exposed (control). After 72 h at 24–25 °C and approximately 100% RH, all of the adult *D. citri* from the laboratory colony that were exposed to the field-collected cadaver died, but no mortality was observed in the control. In order to characterize the progression of disease in *D. citri*, we will briefly describe the infection phenotype. Inoculated psyllids displayed disease symptoms, including twitching of legs and antennae, 2–3 days after they were exposed to the field-collected cadavers. Immediately prior to death, in-

Table 2
Primers designed from AFLP polymorphisms of *Ifr* AsCP and details relevant for use in the PCR

Polymorphism #	Primers (F/R)	Primer sequence[a]	Primer position[b]	GenBank Accession No.	AFLP size (bp)	Product size (bp)	Annealing temperature (°C)
1	*Ifr* AsCP-1A-F	5′-TCATATTGGCCGGATATATACAA-3′	5	EF429307	429	245	63
	Ifr AsCP-1A-R	5′-GAGGCTGCAAGTAAGGGCTATC-3′	249				
1 (complement)	*Ifr* AsCP-1B-F	5′-CAATTCATAGCAAAAGACAAAAGA-3′	429	EF429307	429	178	63
	Ifr AsCP-1B-R	5′-TCTCTCTCCTGTCCCACCAT-3′	252				
2[c]	*Ifr* AsCP-2A-F	5′-CAATTCACCCCTCCTTCTAG-3′	1	EF429308	1161	235	60
	Ifr AsCP-2A-R	5′-TACCCTATAGCAGCGGCATT-3′	235				
3	*Ifr* AsCP-3A-F	5′-TCACTGTAGATGGTGCCATTG-3′	5	EF429309	924	151	63
	Ifr AsCP-3A-R	5′-TGTGCAAGAAGCAGCTGAAG-3′	155				
3 (complement)	*Ifr* AsCP-3B-F	5′-CAATTCACTGGTGAAGAGCTTG-3′	924	EF429300	924	203	63
	Ifr AsCP-3B-R	5′-AATGCCAGCATTTTCTCTGG-3′	722				

AFLP polymorphisms 1–3 were produced by primers 6, 7, and 11, respectively.
[a] The *Eco*RI specificity sites included in the forward PCR primers are underlined, and the reverse primers are nested in the DNA sequence at the designated position.
[b] Sequence positions of the forward and reverse primers are relevant to the 5′ and 3′ ends of the DNA sequence, respectively.
[c] Only the AFLP recognition site at the 5′ end of AFLP polymorphism number 2 was used for primer design.

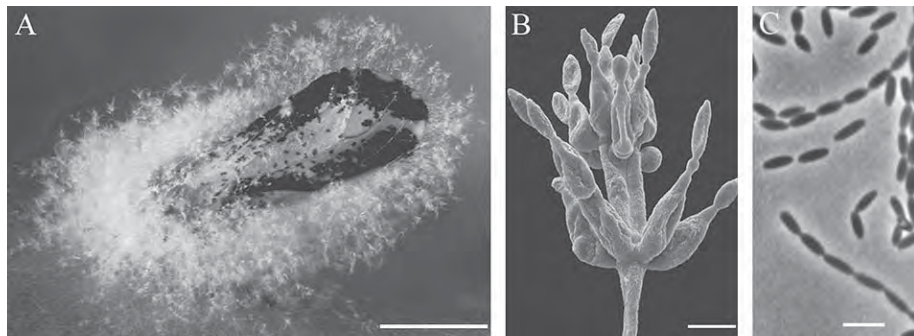

Fig. 1. Light and electron micrographs of *Ifr* AsCP. (A) dead adult *D. citri* killed by *Ifr* AsCP, (B) high magnification of *Ifr* AsCP showing a whorl of mononematous phialides terminating in conidia, (C) tape-mount of *Ifr* AsCP grown on SDY media showing chains of conidia. Scale: (A) 1 mm, (B) 3 μm and (C) 5 μm.

fected psyllids had fungal hyphae emerging from the tarsi and intersegmental regions of the legs. No fungal cells were observed in the hemolymph of diseased adult *D. citri* ($N = 10$), examined approximately 64–72 h after exposure to the pathogen. The dead adult psyllids were lightly fastened to the leaf or side of the centrifuge tube by white fungal mycelia. One week post-mortem, nearly the entire external surface of adult *D. citri* was covered by the fungus, which was similar to the phenotype of diseased psyllids collected in the field. The fungus appeared light gray, dry and powdery after it sporulated on the dead insect. Mortality was also observed in nymphs and eggs of *D. citri* exposed similarly to *Ifr* AsCP (data not shown).

3.3. *In vitro* culturing

The colony morphology of *Ifr* AsCP was characterized using *in vitro* cultures maintained on quarter-strength SDY and MEA media and compared to that of the reference strain, *Ifr* 97. After 1 week on SDY, cultures of *Ifr* AsCP were floccose, powdery and appeared white to light gray (Fig. 2A), while older cultures that were sporulating abundantly turned gray-pale pink. The undersides of these cultures appeared smooth and pale yellow. On MEA, cultures were similar (Fig. 2B) except the undersides were white. Overall, the colony characteristics of *Ifr* AsCP were similar to *Ifr* 97; however, the following differences were noted. The edges of *Ifr* 97 cultures grown on both quarter-strength SDY and MEA media were not smooth (Fig. 2C–D). Also, the underside of *Ifr* 97 cultures grown on quarter-strength SDY media were pale yellow with dark yellow circular and linear regions indicating signs of radiating growth, and the underside of *Ifr* 97 grown on MEA media was pale yellow. Tape-mounts of *Ifr* AsCP and *Ifr* 97 prepared from 1-week-old cultures grown on quarter-strength SDY media showed that the conidia of both *Ifr* AsCP (Fig. 1C) and *Ifr* 97 (not shown) were cylindrical to fusiform, smooth-walled and formed in chains on mononematous conidiophores, as described by Samson (1974) for *Ifr* (=*P. fumosoroseus*).

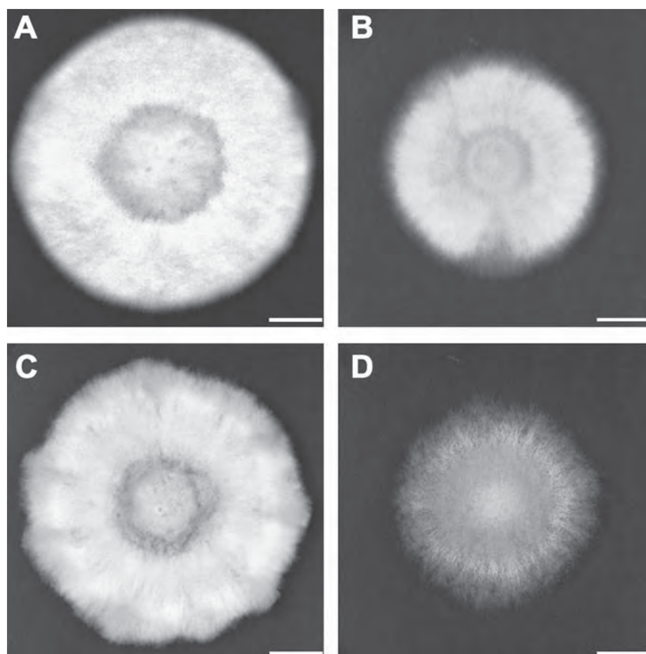

Fig. 2. *In vitro* cultures of *Ifr* AsCP and *Ifr* 97 on quarter-strength SDY and MEA media photographed 1-week post-inoculation. Cultures were maintained without light at 26 °C. (A) *Ifr* AsCP on quarter-strength SDY, (B) *Ifr* AsCP on MEA, (C) *Ifr* 97 on quarter-strength SDY and (D) *Ifr* 97 on MEA. Scale: 0.5 mm.

After 1 week at 26 °C, there were differences observed in the growth rate and conidia yield between *Ifr* AsCP and *Ifr* 97 cultures grown on quarter-strength SDY and MEA media (Fig. 2A–D). On quarter-strength SDY media, cultures of *Ifr* AsCP grew to an average diameter of 3.0 ± 0.1 cm (standard deviation) which was significantly larger than the diameter of *Ifr* 97 cultures, which averaged 2.6 ± 0.1 cm ($F = 355.8$; df = 2; $P < 0.001$). The average diameter of cultures of *Ifr* AsCP grown on MEA media was 2.1 ± 0.1 cm, which was also significantly larger than the 1.9 ± 0.1 cm average diameter of *Ifr* 97 cultures ($F = 54.9$; df = 2; $P < 0.001$). On quarter-strength SDY and MEA media, the average number of conidia harvested from *Ifr* 97 cultures ($4.5 \times 10^8 \pm 3.7 \times 10^7$; $2.0 \times 10^8 \pm 2.3 \times 10^7$, respectively) was significantly greater than the conidia harvested from cultures of *Ifr* AsCP ($1.1 \times 10^8 \pm 1.6 \times 10^7$; $9.9 \times 10^7 \pm 1.4 \times 10^7$, respectively) ($F = 866.3$; df = 2; $P < 0.0001$; $F = 184.8$; df = 2, $P < 0.0001$, respectively).

Qualitative assays were conducted to test if *in vitro* cultures of *Ifr* AsCP and *Ifr* 97 were infective to *D. citri*. All adult psyllids ($N = 20$) exposed to *Ifr* AsCP or *Ifr* 97 cultures on quarter-strength SDY media were killed within 3 days, but no sign of fungal pathogenesis was observed in the untreated control. After 1 week, these cadavers had the same phenotype as the dead psyllids collected in the field.

3.4. Molecular analyses

To substantiate identification of the psyllid pathogen and to compare it with *Ifr* 97, the 18S rRNA (SSU), 28S rRNA (LSU) and β-tubulin gene sequences were obtained from these fungi. For *Ifr* AsCP, DNA was isolated from a single field-collected cadaver and from an *in vitro* culture initiated from the same source, and DNA was isolated from an *in vitro* culture of *Ifr* 97. For *Ifr* AsCP, the sequences from the SSU (1520 bp) (GenBank Accession No. EF429302), LSU (890 bp) (EF429301), and β-tubulin (933 bp)

(EF429303) genes were 100% identical, according to gene, obtained from the cadaver and *in vitro* culture. For *Ifr* 97, the SSU (1520 bp) (EF429305), LSU (890 bp) (EF429304), and β-tubulin (933 bp) (EF429306) genes were 99% (1519/1520 bp), 100% (890/890) and 99% (932/933) identical to these sequences of *Ifr* AsCP, respectively. When comparing the β-tubulin genes of *Ifr* AsCP and *Ifr* 97, the nucleotide substitution was at position 103 (T in *Ifr* AsCP and C in *Ifr* 97), but this did not create differences in the deduced amino acid sequences.

BLAST searches of the SSU, LSU and β-tubulin sequences yielded related sequences from various Ascomycete fungi in the class Sordariomycetes, order Hypocreales, and family Cordycipitaceae. The SSU sequence of *Ifr* AsCP was 100% identical (1520/1520 bp: *E* value = 0) to the SSU sequence of a *Ifr* isolate from *D. citri* in Indonesia (AB032475) (Subandiyah et al., 2000). Unfortunately, there were no available sequences for the LSU or β-tubulin genes from the Indonesian *Ifr* isolate for further comparison. Also, there were no other deposited *Ifr* sequences containing a significant homologous portion of the LSU sequence for comparison.

The β-tubulin sequence of *Ifr* AsCP (EF429303) was most similar to that of *Ifr* ARSEF 3590 (DQ079604: *E* value = 0). When aligned, the *Ifr* AsCP β-tubulin sequence was 24 and 26 bases longer than the *Ifr* 3590 sequence at the 5′ and 3′ ends, respectively. The *Ifr* AsCP sequence was 97% identical (587/604 bp) to the *Ifr* ARSEF 3590 sequence from bases 25–627 (bases and 1–604 of *Ifr* ARSEF 3590) and 96% identical (221/230 bp) from bases 678–906 (665–894 of *Ifr* 3590). A 5′-GTA and AG-3′ eukaryotic intron consensus boundary was detected between bases 628–677 of *Ifr* AsCP and bases 605–664 of *Ifr* ARSEF 3590 that flanked different 50-bp and 60-bp putative intron sequences, respectively. When the introns and additional 5-′/3-′ *Ifr* AsCP nucleotides were excluded from the aligned sequences, the deduced amino acid sequences were 98% identical (271/277 amino acids).

These molecular data support identification of the psyllid pathogen as *I. fumosorosea* Wize (*Ifr*). A voucher culture of the fungus was deposited in the USDA-ARS Collection of Entomopathogenic Fungal Cultures: accession ARSEF 8316.

An AFLP assay was conducted to further differentiate *Ifr* AsCP and *Ifr* 97 because the SSU, LSU and *β-tubulin* gene sequences of these isolates were nearly 100% identical, yet there were clear differences in their *in vitro* growth characteristics. A total of 21% (24/116) and 26% (32/125) of the bands produced in the AFLP assay using 12 different primers were unique to *Ifr* AsCP or *Ifr* 97, respectively (Table 1). Only primer 4 did not produce any polymorphisms between the two fungi. Three bands that were unique to *Ifr* AsCP following amplification with primer 6, 7, and 11 are shown by the arrows in Fig. 3, and these polymorphisms were designated as AFLP-1–3. These bands were excised from the gel, re-amplified, cloned and sequenced. The sequences obtained for AFLP-1 (EF429307), AFLP-2 (EF429308), and AFLP-3 (EF429309) were 429, 1161, and 924 bp in length, respectively (Table 2), and each sequence was flanked by the adapter sequence and *Eco*RI restriction site, as expected following amplification with each AFLP primer (Table 1). No significant alignments to other fungal sequences were retrieved from GenBank following a BLAST search (blastn) of the AFLP-1–3 sequences.

PCR primers were developed based on the AFLP-1–3 polymorphisms to develop molecular markers specific to *Ifr* AsCP. Using the isolate-specific primer pairs *Ifr* AsCP-1A-F/R and *Ifr* AsCP-2A-F/R, PCR products were detected in a sample containing DNA isolated from an *in vitro* culture of *Ifr* AsCP and in samples of DNA isolated from psyllids killed by *Ifr* AsCP (two samples each from individual adult and immature *D. citri*) but not from DNA isolated from an *in vitro* culture or four dead psyllids killed by *Ifr* 97 (two cultures each from individual adult and immature *D. citri*) (Fig. 4A and C). Amplification products were observed in both *Ifr* AsCP and *Ifr* 97 samples using the primers *Ifr* AsCP-1B-F/R (Fig. 4B), *Ifr* AsCP-3A-F/R (Fig. 4D) and *Ifr* AsCP-3B-F/R (Fig. 4E). Thus, amplification was specific to *Ifr* AsCP with primers designed from the 5′ end of AFLP-1 but not at the 3′ end, which indicated that a polymorphism was likely present only at the 5′ end of this region. Amplification of *Ifr* AsCP and *Ifr* 97 with primers designed based on both the 5′ and 3′ ends of AFLP-3 was surprising, and indicated that AFLP-3 was likely to be an artifact. No amplification products were detected in the negative control for each *Ifr* AsCP-specific primer pair, as expected.

4. Discussion

Two fungal pathogens were found infecting *D. citri* in central Florida during the fall of 2005 which now have been identified as *H. citriformis* (Meyer, 2007; Meyer et al., 2007) and *I. fumosorosea* (this study), using both morphological and molecular genetic data. We cannot exclude the possibility that *Ifr* AsCP is the same as the Indonesian *Ifr* isolate from *D. citri* because the SSU sequences were 100% identical, and, unfortunately, the LSU and β-tubulin gene sequences from the Indonesian isolate were not available for further comparison. Subandiyah et al. (2000) conducted a phylogenetic analysis using the SSU sequence from the Indonesian *Ifr* isolate, which was 100% identical to the SSU of *Ifr* AsCP, so this was not repeated here. *Ifr* AsCP was closely related but distinguishable from another local isolate, *Ifr* 97. The *Ifr* AsCP-specific PCR primers will be useful to confirm the identity of cultures maintained long-term *in vitro* and to identify cadavers collected in field collections or

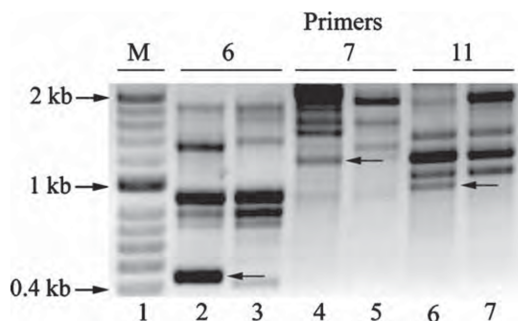

Fig. 3. Representative results of AFLP analysis using DNA isolated from *Ifr* AsCP and *Ifr* 97. Lane (1) DNA size marker, lanes (2, 4 and 6) AFLP of *Ifr* AsCP with primers 6, 7 and 11, respectively, lanes (3, 5 and 7) AFLP of *Ifr* 97 with primers 6, 7 and 11, respectively. DNA bands marked with arrows were excised from the gel, sequenced, and used to design isolate-specific PCR primers for *Ifr* AsCP.

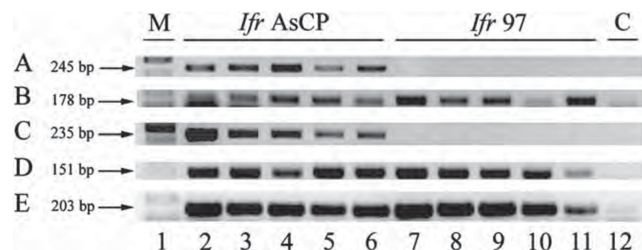

Fig. 4. PCR amplification of DNA isolated from psyllids killed by *Ifr* AsCP and *Ifr* 97 and from *in vitro* cultures of *Ifr* AsCP and *Ifr* 97 using AFLP primers. (A) primers *Ifr* AsCP-1A-F/R, (B) primers *Ifr* AsCP-1B-F/R, (C) primers *Ifr* AsCP-2A-F/R, (D) primers *Ifr* AsCP-3A-F/R and (E) primers *Ifr* AsCP-3B-F/R. Lane (1) DNA size marker, (2) *in vitro* culture of *Ifr* AsCP (grown on quarter-strength SDY media), (3–4) *D. citri* adults killed by *Ifr* AsCP, (5–6) *D. citri* nymphs killed by *Ifr* AsCP, (7) *in vitro* culture of *Ifr* 97 (grown on quarter-strength SDY media), (8–9) *D. citri* adults killed by *Ifr* 97, (10–11) *D. citri* nymphs killed by *Ifr* 97, (12) negative control (no DNA).

field trials evaluating the pathogen as a microbial insecticide. In support of this, the primers *Ifr* AsCP-1A-F/R and *Ifr* AsCP-2A-F/R were used to confirm that *D. citri* nymphs were killed by *Ifr* AsCP in a pilot field trial using *Ifr* AsCP as a microbial insecticide in Florida (Hoy et al. unpublished). Clearly, follow-up studies are needed to evaluate the specificity of these markers against a wider spectrum of *Ifr* isolates. A variety of molecular approaches have been utilized to assess genetic diversity among multiple *Ifr* isolates (Tigano-Milani et al., 1995; Cantone and Vandenberg, 1998; Oborník et al., 2001; Fargues et al., 2002; Luangsa-ard et al., 2004, 2005; Dalleau-Clouet et al., 2005; Inglis and Tigano, 2006), and these tools could be used to ascertain the relatedness of these isolates to *Ifr* AsCP in future research.

The phenotype of mycosed adult psyllids produced in the laboratory was the same as that observed in the field, thus fulfilling Koch's postulates. Pathogenesis of *D. citri* by *Ifr* AsCP was different than that observed for *H. citriformis*; *Ifr* AsCP killed *D. citri* 4–6 days faster than *H. citriformis* tested under the same conditions (Meyer et al., 2007). The efficiency by which *Ifr* AsCP causes mortality to *D. citri* in the laboratory is an attractive attribute in terms of its potential use for microbial control. Interestingly, the behavioral symptoms of disease were similar in adults of *D. citri* infected by both pathogens. Surprisingly, no evidence of fungal cells of *Ifr* AsCP was found in the hemolymph of infected *D. citri* adults during the two days preceding mortality. By contrast, psyllids infected with *H. citriformis* had abundant hyphal bodies in the hemolymph (Meyer et al., 2007). This also contrasted with other studies, where other isolates of *Ifr* were found occupying the hemocoel of the glasshouse whitefly (Gökçe and Er, 2005), diamondback moth, and fall armyworm (Altre and Vandenberg, 2001). *Ifr* AsCP may be necrotrophic, killing *D. citri* with toxins and then utilizing the dead insect for development. The toxin dipicolinic acid was found in an *Ifr* isolate that killed immature whiteflies (Asaff et al., 2005), and another toxin was found in *Isaria tenuipes* (=*Paecilomyces tenuipes*) (Nam et al., 2001); however, this has not yet been demonstrated in *Ifr* AsCP. Further investigation is warranted to provide a detailed account of the infection mechanisms used by *Ifr* AsCP and *H. citriformis* in *D. citri*.

Currently, there are no fungal pesticides registered for *D. citri* management in Florida. There is potential for developing *Ifr* AsCP as a microbial insecticide because conidia, which are infective to all life stages of *D. citri*, can be readily produced *in vitro*. In this study, we have completed the first steps toward the use of a microbial control agent, as reviewed by Montesinos (2003), including sampling, culturing, identification, and characterization by molecular, morphological and biological assays. Immature *D. citri* were killed by *Ifr* AsCP during a pilot field trial (Hoy et al., unpublished), so additional field experiments are warranted to evaluate *Ifr* AsCP for psyllid management and to compare its efficacy with the commercially available formulation of *Ifr* 97 (Vidal et al., 1998; Faria and Wraight, 2001). *Ifr* has been successfully used against insecticide-resistant whitefly populations, particularly in glasshouses (reviewed by Smith, 1993), and in orchard field trials to control pear psylla (Puterka, 1999).

The utility of *Ifr* AsCP for citrus IPM depends on costs and multiple factors related to the development of a microbial insecticide. In general, *Ifr* species have a broad host range (Smith, 1993), so the effect of the pathogen on non-target species such as the psyllid parasitoids and other natural enemies should be investigated under laboratory and field conditions (Pell and Vandenberg, 2002). The use of *Ifr* AsCP to control *D. citri* in Florida may be limited because copper is frequently applied to control plant pathogens in Florida citrus, which may negatively influence *Ifr* AsCP (Timmer et al., 2006). A quantitative survey to characterize the distribution, abundance, and seasonality of the naturally occurring interaction between *D. citri* and *Ifr* AsCP in Florida could provide information about the role of this entomopathogen in the population dynamics of *D. citri* and offer clues on how to maximize the effect of the pathogen in an IPM program.

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