



Morphological and molecular characterization of a *Hirsutella* species infecting the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), in Florida

Jason M. Meyer *, Marjorie A. Hoy, Drion G. Boucias

Department of Entomology and Nematology, University of Florida, Institute of Food and Agricultural Sciences,
 Building 970, P.O. Box 110620, Gainesville, FL 32611-0620, USA

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Abstract

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, is an invasive pest that vectors citrus greening disease, which recently was detected in Florida. Mycosed adult *D. citri* were collected at four sites in central Florida between September 2005 and February 2006. Observation of the cadavers using scanning electron microscopy revealed that the pathogen had branched synnemata supporting monophyladic conidiogenous cells. A high-fidelity polymerase chain reaction (PCR) assay was used to amplify the 18S rRNA, 28S rRNA and β -tubulin genes of the pathogen for phylogenetic analysis. The morphological and genetic data indicated that the pathogen was a novel isolate related to *Hirsutella citrififormis* Speare. PCR assays using isolate-specific primers designed from the unique putative intron region of the β -tubulin sequence distinguished the psyllid pathogen from five related *Hirsutella* species. The pathogen was maintained *in vivo* by exposing healthy *D. citri* to the synnemata borne on field-collected cadavers. Infected psyllids had an abundance of septate hyphal bodies in their hemolymph and exhibited behavioral symptoms of disease. *In vitro* cultures of the pathogen were slow-growing and produced synnemata similar to those found on mycosed *D. citri*. In laboratory bioassays, high levels of mortality were observed in *D. citri* that were exposed to the conidia-bearing synnemata produced *in vivo* and *in vitro*.

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1. Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), vectors the Gram-negative bacterium *Candidatus Liberibacter asiaticus*, the causal agent of citrus greening disease or Huanglongbing (HLB) (Garnier et al., 2000). Citrus trees infected with *L. asiaticus* are often asymptomatic for years, but they ultimately show signs of disease, including yellowing of leaf veins, leaf mottling, and misshapen and poor-tasting fruit before dying as a result of the infection (da Graça, 1991). *D. citri* rapidly colonized all citrus-growing regions in Florida after it was discovered in

1998 (Halbert, 1998; Knapp et al., 1998; Halbert et al., 2000). In 2005, citrus trees infected with HLB were found near Homestead, FL, and more infected trees have since been detected in multiple counties (Halbert, 2005; Bouffard, 2006; http://www.doacs.state.fl.us/pi/chrp/greening/maps/cgsit_map.pdf). Integrated pest management tactics are needed to slow the spread of HLB to uninfected citrus groves and nurseries.

Currently, suppression of *D. citri* populations is achieved by chemical applications and biological control (Rae et al., 1997; Browning et al., 2006). Foliar insecticides are most effective when the density of *D. citri* is high, particularly during the early phase of each flush (tender new growth) cycle (Browning et al., 2006; Stansly and Rogers, 2006). Natural enemies of *D. citri* in Florida include native lady beetles, lacewings, spiders (Michaud, 2004) and the

* Corresponding author. Fax: +1 352 392 0190.

E-mail address: hessianfly@yahoo.com (J.M. Meyer).

specialist parasitoid, *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae), which was released in a classical biological control program (Hoy et al., 1999; Hoy and Nguyen, 2000; Skelley and Hoy, 2004).

Several species of entomopathogenic fungi, including *Paecilomyces fumosoroseus* (Wize) A.H.S. Brown and G. Smith (Samson, 1974; Subandiyah et al., 2000), *Hirsutella citriformis* Speare (Rivero-Aragon and Grillo-Ravelo, 2000; Subandiyah et al., 2000; Étienne et al., 2001), *Cephalosporium lecanii* Zimm (*Verticillium lecanii*) (Rivero-Aragon and Grillo-Ravelo, 2000; Xie et al., 1988), *Beauveria bassiana* (Bals.) Vuill. (Rivero-Aragon and Grillo-Ravelo, 2000), *Cladosporium* sp. nr. *oxysporum* Berk. and M.A. Curtis (Aubert, 1987) and *Capnodium citri* Berk. and Desm. (Aubert, 1987) have been found to infect *D. citri* worldwide. In most cases, mycosed *D. citri* have been observed during periods of high relative humidity. In Florida, Halbert and Manjunath (2004) noted the occurrence of an unidentified fungal pathogen that attacked *D. citri*, but no information on the pathogen was provided. This research was initiated following the field collection of mycosed *D. citri* between September 2005 and February 2006 in citrus groves in Florida. We report the morphology and biology of this fungal pathogen associated with *D. citri* populations in Florida.

2. Materials and methods

2.1. Insect colony

D. citri were reared according to a method modified from Skelley and Hoy (2004). Small citrus trees, approximately 30–50 cm tall and grown in 15.2 cm diameter pots, were used to maintain *D. citri* in a greenhouse at 20–32 °C with a 16L:8D photoperiod. Twenty trees were pruned each week, fertilized with Peter's 20–20–20 (N–P–K) water-soluble fertilizer (United Industries, St. Louis, MO), and watered as necessary. Approximately 2 weeks after pruning, the trees produced flush and were placed inside wooden-framed mesh cages (0.76 m × 0.91 m × 1.11 m) where adult *D. citri* females were allowed to oviposit. Upon emergence, adult *D. citri* were aspirated and transferred to another cage to initiate the next generation.

2.2. Collection, maintenance, and cultivation of the *D. citri* pathogen

Between September 2005 and February 2006, mycosed *D. citri* were collected at four sites in three counties of Florida on orange and grapefruit trees (Hendry county: 26°20.307'N, 80°54.597'W; Marion county: 28°58.943'N, 81°51.098'W; Polk county: 28°03.656'N, 81°34.937'W; 28°06.295'N, 81°42.895'W). Mycosed psyllids were found during each trip to the field, and they were collected once from Hendry county, twice from Marion county, and four times from Polk county. The cadavers were transported to the laboratory, and the pathogen collected in Polk county and Marion county was maintained by *in vivo* passage con-

ducted at weekly intervals, as follows. Ten to twenty healthy adult *D. citri* were collected from the laboratory 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 conidial-bearing synnematata present on fresh cadavers of mycosed *D. citri*. Treated psyllids were placed individually in 50-mL centrifuge tubes containing a single mature citrus leaf with a water-soaked cotton ball placed under the cap to maintain a relative humidity (RH) of approximately 100% and held at 24–25 °C with a 16L:8D photoperiod.

Cadavers of adult *D. citri* collected in Polk county and Marion county were used to initiate *in vitro* cultures. Single conidia were inoculated on 6-cm diameter plates containing quarter-strength Sabouraud dextrose agar + 1% yeast extract (SDAY). Sub-cultures of the pathogen were maintained by transferring a 1-cm square section from a 2- to 3-week-old culture to a fresh SDAY plate. In order to produce sporulating cultures of the fungus, additional *in vitro* cultures were initiated by inoculating approximately 4 g of autoclaved boiled rice with mycelia produced on the SDAY plates (Sosa Gomez, 1991). Hemolymph samples from surface-sterilized infected *D. citri* were harvested and used to inoculate TNM-FH insect tissue culture media (Sigma, St. Louis, MO) + gentamycin (50 µg/mL) + 5% fetal calf serum to cultivate the hyphal body phenotype. All *in vitro* cultures were maintained in a growth chamber at 26 °C without light. A subculture of the fungus, initiated from an adult *D. citri* cadaver collected in Polk county, was deposited in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF 8315).

2.3. Microscopy

The fungus on the insect host was examined with a dissecting microscope and photographed using the Auto-Montage Pro system using software ver. 5.02 (Synoptics, Frederick, MD). Hemolymph samples from infected adult *D. citri* were examined with differential interference contrast (DIC) microscopy at 360–1000×. For SEM, cadavers of mycosed *D. citri* collected in Polk county were fixed in OsO₄ vapors for 48 h, dehydrated in an ethanol series, critical-point dried using a Bal-Tec 030 critical point dryer, sputter coated with Au/Pt alloy and examined on a Hitachi 4000 FE-SEM operating at 4–6 kV (Quattlebaum and Carner, 1980). Measurements of all digitally captured subjects were made using SPOT software 3.4.3 (Diagnostic Instruments, Sterling Heights, MI).

2.4. Bioassays

Qualitative bioassays were conducted to test the infectivity of the *D. citri* pathogen against a laboratory colony of *D. citri*. Three replicates over time were conducted using adults, each including 20 healthy *D. citri* that were exposed to mycosed psyllids as described above or not treated (control). Third-instar nymphs were also assayed with the fun-

gal pathogen. Tender flush from sour orange trees was used to support nymphal development. Two replicates of ten *D. citri* nymphs were exposed to the mycosed adult psyllids or not treated (control). Mortality due to fungal pathogenesis was monitored daily in the bioassays, which were conducted at 25 °C with a 16L:8D photoperiod. To test the effect of treatment on mean percentage mortality 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 $P < 0.05$ (SAS Institute, 1996).

To determine if the pathogen requires a specific site to initiate infection, the head, thorax or abdomen of five adult *D. citri* was exposed to the synnemata on mycosed adult psyllids and held as described above. Five psyllids were not treated and maintained as the control. Ten adult *D. citri* were exposed to the *in vitro* cultures propagated on rice or not treated (control) and held as described above.

2.5. Molecular analyses

DNA was isolated from synnemata arising from *D. citri* cadavers and also from *in vitro* cultures initiated from the pathogen collected in Polk county and Marion county. Fungal tissue was homogenized and processed using PUREGENE reagents according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN). DNA pellets were air dried for 30 min to remove any remaining EtOH, re-suspended in 25–50 µl sterile water and stored at –70 °C. A high-fidelity polymerase chain reaction (PCR) was used to amplify the 18S small ribosomal subunit (SSU) with primers NS1 (5'-GTAGTCATATGCTTGCTC-3') and FS2 (5'-TAGGNATTCCTCGTTGAAGA-3') (Nikoh and Fukatsu, 2000), the 5' variable region of the 28S large ribosomal subunit (LSU) with primers LS1 (5'-AGTACCCGCTGAACTTAAG-3') and LR5 (5'-CCTGAGGGAACTTCG-3') (Hausner et al., 1993; Rehner and Samuels, 1995), and the β -tubulin gene with degenerate primers betatubF (5'-TGGGCYAARGGYCACTACA CYGA-3') and betatubR (5'-TCAGTGAACCTCATCT CRT CCAT-3') (Tartar et al., 2002). The high fidelity PCR (50 µl) included 50 mM Tris, pH 9.2, 16 mM ammonium sulfate, 1.75 mM MgCl₂, 350 mM dNTPs, 800 pmol of primers, 1 U *Pwo* DNA polymerase and 5 U of *Taq* DNA Poly-

merase (Roche Molecular Biochemicals, Indianapolis, IN) (Barnes, 1994; Hoy et al., 2001). The PCR cycling parameters included three linked temperature profiles: (i) 1 cycle consisting of denaturation at 94 °C for 2 min; (ii) 10 cycles, each consisting of denaturation at 94 °C for 10s, annealing at 50 °C for 30s, and elongation at 68 °C for 1 min; and (iii) 25 cycles, each consisting of denaturation at 94 °C for 10s, annealing at 50 °C for 30s, and extension at 68 °C for 1 min plus an additional 20s for each consecutive cycle (Hoy and Jeyaprakash, 2005). PCR products were separated on 1% agarose TAE gels, stained with ethidium bromide, and visualized with ultraviolet light. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and cloned into the pCR2.1 TOPO plasmid (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated from overnight cultures of randomly picked *Escherichia coli* colonies using Qiagen Plasmid Mini columns (Valencia, CA), and the size of the inserts was confirmed with *EcoRI* digestion followed by gel electrophoresis. Three clones of each gene were bidirectionally sequenced using an ABI Prism DNA Sequencer at the Interdisciplinary Center for Biotechnology Research Core Facility at the University of Florida, Gainesville, FL. DNA sequences were compared to those deposited in GenBank with BLAST (blastn) using the default settings. The deduced amino acid sequences of DNA sequences were obtained using the translate tool of the Expert Protein Analysis System (ExpASY) provided on the Proteomics Server (Swiss Institute of Bioinformatics).

2.6. Phylogenetic analysis

The LSU and β -tubulin sequences from the *D. citri* pathogen were used for phylogenetic analysis. Sequences from seven *Hirsutella* species that had both the LSU and β -tubulin genes available in GenBank were included in the analysis (Table 1). *Beauveria bassiana*, which belongs to the phylum Ascomycota, class Sordariomycetes, order Hypocreales and family Clavicipitaceae, was used as the outgroup taxon in the analysis. The DNA sequences were aligned with CLUSTAL X v. 1.83 (Thompson et al., 1997), and the LSU and β -tubulin sequences were combined according to species using MacClade 4.0 software (Maddison and Maddison, 2000). The putative intron region of the β -tubulin gene was removed from the data matrix for each taxon because the

Table 1
Collection data and GenBank Accession Nos. for taxa used in phylogenetic analysis

Taxon	Collection data		GenBank Accession No.	
	Host	Origin	LSU	β -Tubulin
<i>B. bassiana</i>	Orthoptera: Gryllotalpidae	Brazil	DQ075680	DQ079603
<i>H. citrififormis</i>	Hemiptera: Delphacidae	Indonesia	DQ075678	DQ079601
<i>H. guyana</i>	Hemiptera: Cicadellidae	Philippines	DQ075676	DQ079598
<i>H. homalodisciae</i>	Hemiptera: Cicadellidae	FL, USA	DQ075674	DQ079600
<i>H. kirchneri</i>	Acari: Eriophyidae	UK	AY518382.1	DQ079597
<i>H. nodulosa</i>	Lepidoptera: Pyralidae	MI, USA	DQ075675	DQ079596
<i>H. repens</i>	Hemiptera: Delphacidae	Korea	DQ075679	DQ079602
<i>H. thompsonii</i>	Acari: Eriophyidae	FL, USA	DQ075673	DQ079595
Florida <i>Hirsutella</i> isolate from <i>D. citri</i>	Hemiptera: Psyllidae	FL, USA	EF363707	EF363706

Table 2

Putative intron sequences for the Florida *Hirsutella* isolate from *D. citri* ARSEF 8315 (GenBank Accession EF363706) and *H. citrififormis* ARSEF 2346 (GenBank Accession DQ079601)

Species	Size (bp)	β -tubulin intron sequence
Florida <i>Hirsutella</i> isolate from <i>D. citri</i> ARSEF 8315	81	<u>628</u> <u>GTACGTGCCGCGGGCCCTCGAACCGGCCTTCCATAGCCCCTCCGCTCCCCCTCGAGCCTGTGCTGACGAAGGTGGATAG</u>
<i>H. citrififormis</i> ARSEF 2346	83	<u>571</u> <u>GTACGTGACGCCATGATGGCTCGAGGCCGCTCGCTCCCATTGCCCCCTTGCTCGAGCTCCTACTGACGATGGCCACCCACAG</u>

The 5'-GTA and AG-3' eukaryotic consensus boundaries are underlined at each end, and the putative stop codon in the *H. citrififormis* ARSEF 2346 sequence is in boldface (sequence position number 584–586). The isolate-specific reverse PCR primer (reverse complement shown in boldface: sequence position number 688–707) was nested in the unique putative intron sequence of the Florida *Hirsutella* isolate from *D. citri* and used to discriminate it from five other *Hirsutella* species.

high degree of variability in that region prevented an acceptable DNA sequence alignment. The final data matrix was composed of 1128 total characters (505 bp of LSU, 623 bp of β -tubulin). The incongruence length difference (ILD) test was used to measure incongruence between the LSU and β -tubulin datasets (Farris et al., 1995). Maximum likelihood (ML) and maximum parsimony (MP) analyses were conducted using heuristic searches implemented in PAUP* 4.0b4a. For ML, the MODELTEST 3.7 program (Posada and Crandall, 1998) was executed on the data matrix to select the best-fit nucleotide substitution model for the alignment. The substitution rate-matrix parameters and shape parameter (α) were estimated via ML. Support for each branch in the ML tree was generated by the bootstrap method (100 replicates) in PAUP*. For MP, the number of parsimony-informative characters, tree length, consistency index and retention index were obtained in PAUP*.

2.7. Isolate-specific PCR

Isolate-specific PCR primers were designed based on the unique putative intron region of the β -tubulin sequence of the Florida *Hirsutella* isolate from *D. citri*. The forward primer (betatub_intF: 5'-GGCTTCCAGATCACCC-ACTC-3') was designed at the 5' portion of the β -tubulin sequence (5' = position 71), and the reverse primer (betatub_intR: 5'-TATCCACCTTCGTCAGCACA-3') was nested within the unique putative intron region (5' = position 707) (Table 2). The specificity of the primers was tested on DNA isolated from *in vivo* and *in vitro* cultures of the Florida *Hirsutella* isolate from *D. citri* and on DNA samples from *in vitro* cultures of five related *Hirsutella* species (*H. citrififormis* ARSEF 2346, *H. guyana* ARSEF 878, *H. homalodiscae*, *H. nodulosa* ARSEF 5473, and *H. thompsonii*). A 0.9–1.0 kb portion of the β -tubulin gene was amplified from each DNA sample using degenerate primers (see above) to control for template quality. PCRs (25 μ l) included 5 units of *Taq* DNA Polymerase, 1 \times PCR buffer (Bioline USA, Inc., Randolph, MA), 350 mM dNTPs, and 800 pmol of primers. Standard 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 58 °C for 30 s, and extension at 68 °C for 1 min. A final extension of 5 min at 68 °C concluded the assay. PCR products were analyzed by gel electrophoresis as described above.

3. Results

3.1. Collection, maintenance and cultivation of the *D. citri* pathogen

Between September 2005 and January 2006, a total of 365 mycosed adult *D. citri* were collected from orange and grapefruit trees at four sites in central Florida. No mycosed *D. citri* nymphs were detected during the field collection. Mycosed adult *D. citri* (Fig. 1A) were attached to the undersurface of leaves, the stems of leaves, or on the undersurface of branches by a brown-colored mycelial mat underlying the ventral portion of the head and thorax.

The pathogen was propagated *in vivo* by exposing healthy adult and immature psyllids to the synnemata borne on the field-collected cadavers. One week post-inoculation at 24–25 °C, topically treated adult psyllids displayed changes in behavior. Disease symptoms included trembling legs and antennae, wing flicking, reduced flight and a darkening of the cuticle on the head and thoracic regions. After succumbing to the pathogen, adult *D. citri* had pale-white hyphae emanating from the intersegmental regions of the legs, thorax, head, and from the tip of the abdomen. This “early” phenotype was not observed in the field-collected cadavers. Mycosed adult psyllids were situated in a feeding position with their head down and secured to the leaf or side of the centrifuge tube by a mycelial mat. Synnemata were first observed protruding from the head and abdomen of *D. citri* cadavers 14 and 16 days after inoculation, respectively, when held at 24–25 °C. The distal regions of the synnemata were covered with small drops of mucus. Cadavers of *D. citri* remained infectious for at least 10 weeks in the laboratory when held at 24–25 °C in the centrifuge tube, but infectivity after 10 weeks was not assessed. Mycosed immature *D. citri* (Fig. 1B) had white hyphae that emerged initially from the intersegmental regions of the legs and then developed to cover the entire dorsal surface of the insect.

In vitro cultures propagated on SDAY media were slow growing at 26 °C, with a diameter measuring 1.3 ± 0.1 (standard deviation) cm after 7 days, 2.3 ± 0.1 cm after 14 days and 3.5 ± 0.1 cm after 21 days ($N=3$). These cultures appeared white to light-gray 2 weeks post-inoculation and did not sporulate during the 5 weeks that they were maintained. An *in vitro* culture grown on rice produced multiple synnemata on a single rice grain 6 weeks post-inoculation. The morphology and number of these synnemata was similar to the synnemata produced on a single adult *D. citri*.

3.2. Microscopy

A total of 9 ± 3 (mean \pm standard deviation) synnemata were observed extending from the body of field-collected mycosed *D. citri* ($N=10$). The synnemata, measuring 0.8 ± 0.6 mm in length ($N=90$) and 50.0 ± 10.0 μ m in diameter ($N=90$), were simple or possessed short lateral branches (Fig. 1A). Monophyladic conidiogenous cells arose laterally and terminally from the distal portions of the synnemata (Fig. 1C–D). Conidiogenous cells measured

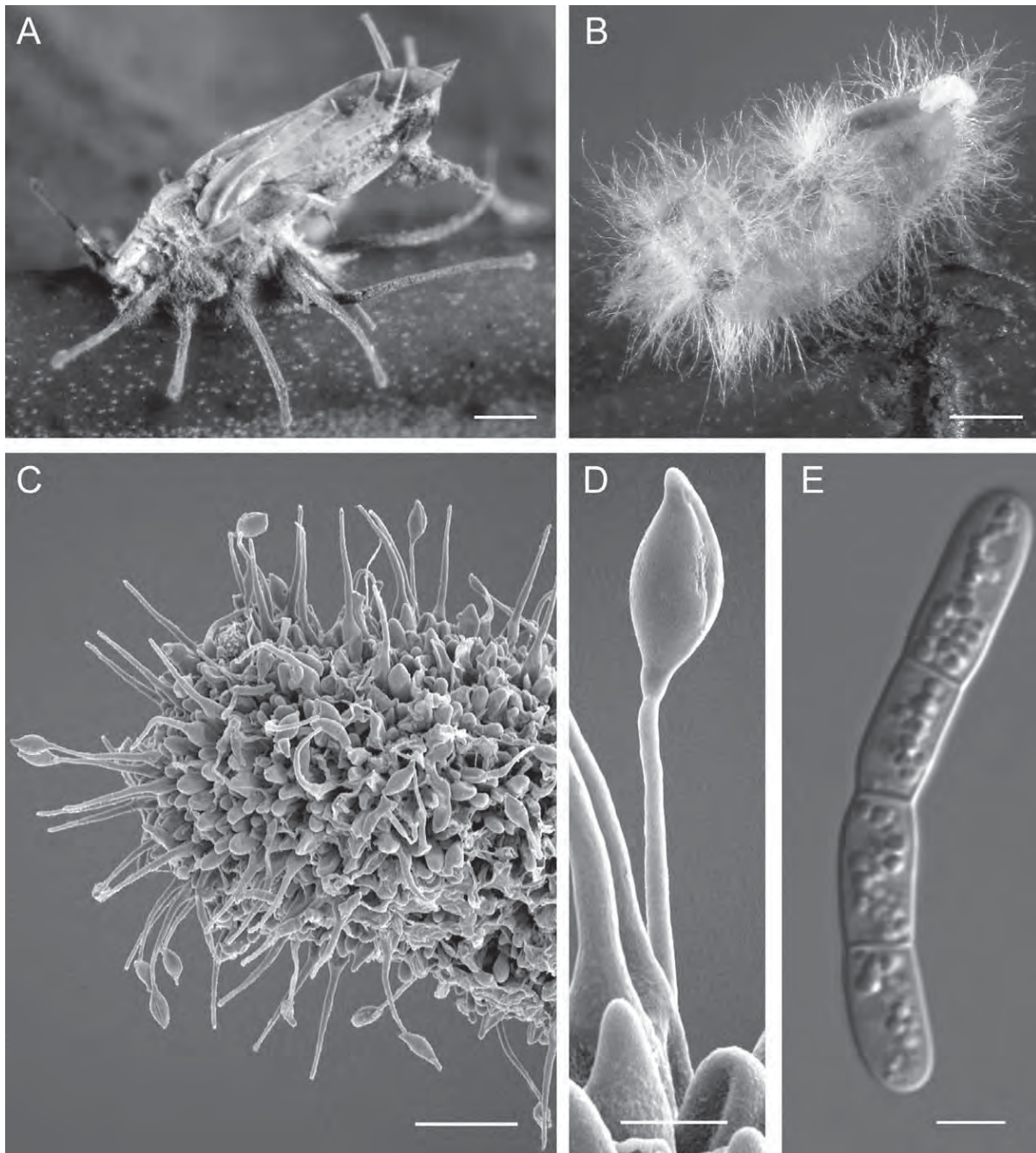


Fig. 1. Light and electron micrographs of the Florida isolate of *Hirsutella* from *D. citri*. (A) synnemata borne on mycosed adult *D. citri*, (B) deceased immature *D. citri* bearing fungal hyphae, (C) apex of synnemata showing mononematious philades, (D) high magnification of the philade and conidia, and (E) septate hyphal body isolated from hemolymph of infected adult *D. citri*. Scale bars: (A) 0.5 mm, (B) 0.4 mm, (C) 15.0 μ m, (D) 3.0 μ m, and (E) 5.0 μ m.

17.5 ± 1.9 µm in length ($N=16$) and had an ellipsoid base with a diameter of 2.6 ± 0.5 µm that tapered to 0.6 ± 0.1 µm at the conidial apex ($N=15$). Each phialide produced a single, smooth-walled, aseptate, fusiform or ellipsoidal conidium averaging 5.9 ± 0.8 µm ($N=17$) in length and 2.6 ± 0.3 µm in diameter ($N=16$) (Fig. 1C–D).

One week post-inoculation, microscopic examination of the hemolymph isolated from diseased adult *D. citri* revealed an abundance of septate hyphal bodies measuring 26.7 ± 6.2 µm long and 4.8 ± 0.4 µm in diameter ($N=20$) (Fig. 1E). Observations using DIC microscopy confirmed that the *in vitro* cell phenotype derived from hemolymph samples closely resembled the structure of the hyphal bodies isolated from the hemolymph of infected *D. citri*.

3.3. Bioassays

No sign of disease was observed in the control psyllids throughout the bioassays, and no mortality was observed in immature and adult psyllids exposed to the pathogen until days 5 and 6, respectively. The mean time after inoculation until death was 7.4 ± 0.7 (standard deviation) days in adults ($N=60$) and 5.3 ± 0.6 days in immatures ($N=20$) at 25 °C and approximately 100% RH. After 6 days, there was 8.3% mortality in adult psyllids exposed to the pathogen, which was significantly greater than the 1.7% mortality in the controls ($F=8.0$; $df=1, 4$; $P<0.05$). After 9 days, 100% mortality was observed in adult psyllids exposed to the pathogen, which was significantly greater than the 1.7% mortality in the controls ($F=3481$; $df=1, 4$; $P<0.0001$).

Inoculations targeting specific tagma (head, thorax or abdomen) of adult *D. citri* all resulted in infections that caused mortality, while no mortality was observed in the controls. The adult *D. citri* exposed to synnemata produced *in vitro* on rice all succumbed to the pathogen between 9 and 10 days after they were inoculated at 25 °C and approximately 100% RH. These psyllids exhibited the same behavioral symptoms of disease as previously described, and no mortality was observed in the controls.

There was 75% mortality in immature psyllids exposed to the pathogen after 5 days, which was significantly greater than the 5% mortality in the controls ($F=98$; $df=1, 2$; $P=0.01$). After 7 days, 100% mortality was observed in immature psyllids, which was significantly greater than the 5% mortality in the controls ($F=361$; $df=1, 2$; $P=0.003$). The synnemata structure found commonly on adults formed on only one of the twenty immature *D. citri* exposed to the pathogen in the bioassays.

3.4. Molecular analyses

DNA prepared from the synnemata of a *D. citri* cadaver collected in Polk county and from three asporogenous *in vitro* cultures was subjected to PCR amplification with gene-specific primers. The three *in vitro* cultures used in the analysis included two cultures initiated from mycosed adult *D. citri* from Polk county (propagated on SDAY media)

and one culture initiated from hyphal bodies found in the hemolymph of infected adult *D. citri* inoculated by exposure to a cadaver from Polk county (grown in TNM-FH insect tissue culture media). Sequence data from the four DNA preparations were obtained for the SSU (1521 bp, GenBank Accession No. EF363708), LSU (896 bp, GenBank Accession No. EF363707) and β -tubulin (964 bp, GenBank Accession No. EF363706) genes, and these sequences were 100% identical for each gene analyzed in each DNA preparation. This indicated that the *in vitro* isolates were identical to the isolate that infected *D. citri*. An additional β -tubulin gene sequence was amplified from DNA extracted from an *in vitro* culture (grown on SDAY) initiated from a *D. citri* cadaver collected in Marion county. This sequence was 100% identical to the β -tubulin sequence of the pathogen collected in Polk county, which suggested that the same isolate was infecting *D. citri* at each location.

A BLAST search of the SSU, β -tubulin, and LSU gene sequences produced significant alignments to these sequences from species in the phylum Ascomycota and class Sordariomycetes. The most significant alignment for both the SSU and β -tubulin genes was to sequences from *H. citrififormis* Speare, in the order Hypocreales and family Clavicipitaceae. The next most significant alignments for the SSU gene were to SSU sequences of various *Paecilomyces* and *Cordyceps* species, all classified in the Clavicipitaceae. The next most significant alignments for the β -tubulin gene were to β -tubulin sequences in the family Chaetosphaeriales. The most significant alignments of the LSU gene were to LSU sequences from various *Cordyceps* species in the Clavicipitaceae.

The SSU sequence was 99% identical (1516/1521 bp) to the SSU sequence of *H. citrififormis* isolated from *D. citri* in Indonesia (GenBank Accession No. AB032476) (Subandiyah et al., 2000). The LSU sequences were 97% identical to the available LSU sequences of the *H. citrififormis* isolates ARSEF 532 (750/766 bp: GenBank Accession No. AY518376) and ARSEF 2346 (585/600 bp: GenBank Accession No. DQ075678), which were 100% identical to each other in the 600 bp available for comparison. There were no sequences available in GenBank for the SSU of *H. citrififormis* isolates ARSEF 532 or ARSEF 2346.

The most similar β -tubulin gene sequence deposited in GenBank was a 915-bp sequence from *H. citrififormis* isolate ARSEF 2346 (GenBank Accession No. DQ079601). However, when these two sequences were aligned, the β -tubulin sequence for the Florida *Hirsutella* isolate from *D. citri* was 57 nucleotides longer at the 5' end, and the *H. citrififormis* sequence had an additional 6 nucleotides at the 3' end. The β -tubulin sequences were 95% identical (508/534 bp) between bases 94 and 627 (bases 37–570 of the *H. citrififormis* isolate ARSEF 2346) and 95% identical (242/256 bp) between bases 709 and 964 (bases 654–909 of ARSEF 2346). These sequences were different between bases 58 and 93 (bases 1–36 of the *H. citrififormis* isolate ARSEF 2346), and 628 and 708 (bases 571–653 of the *H. citrififormis* isolate ARSEF 2346). There were no sequences available in Gen-

Bank for the β -tubulin gene of *H. citriformis* isolate ARSEF 532 or the *H. citriformis* isolate collected on *D. citri* in Indonesia (Subandiyah et al., 2000).

The β -tubulin gene sequence of the Florida *Hirsutella* isolate from *D. citri* is unique because it does not contain a stop codon within a putative intron, whereas all other available β -tubulin sequences from *Hirsutella* isolates contain a stop codon in this region (Boucias et al., 2006). The 5'-GTA and AG-3' eukaryotic consensus boundaries were detected at position 628 and 708 of the β -tubulin sequences from the Florida *Hirsutella* isolate from *D. citri* and at 571–653 of *H. citriformis* isolate ARSEF 2346, respectively, that flanked putative introns (Table 2). The putative intron sequences of the Florida *Hirsutella* isolate from *D. citri* (81 bp) and *H. citriformis* (83 bp) are larger than the putative intron sequences of other *Hirsutella* species, which range from 53–59 bp (Boucias et al., 2006). When the putative introns and the unique sequences at the 5' region were removed from the β -tubulin sequences of the Florida *Hirsutella* isolate from *D. citri* and from *H. citriformis* isolate ARSEF 2346, the deduced amino acid sequences were 99% identical (261/263 amino acids). However, it has not been demonstrated that the putative introns of the β -tubulin mRNA are actually spliced out, so the intron-free deduced amino acid sequences may not reflect the actual amino acids of the β -tubulin proteins *in vivo*.

3.5. Phylogenetic analyses

The β -tubulin and LSU DNA sequences from the *D. citri* pathogen, seven related *Hirsutella* species, and the outgroup *Beauveria bassiana* were used to compile a data matrix for ML and MP analysis (Table 1). The GTR + G nucleotide substitution model (Yang et al., 1994) was selected by MODELTEST for the data matrix and used for ML analysis. The topologies of the ML and MP (154 parsimony-informative characters, tree length = 487, consistency index = 0.6920, retention index = 0.4643) trees were identical. The consensus tree shown in Fig. 2 was rooted with the outgroup *B. bassiana*. However, the bootstrap method did not support separation of *H. thompsonii* from the outgroup. The remaining *Hirsutella* species were clustered in a monophyletic clade supported by the bootstrap method (66%) that consisted of two groups. Group I had five taxa including a clade that paired *H. guyana* and *H. homalodiscae* (bootstrap 100%), a second clade linking *H. repens* with *H. kirchneri* (bootstrap 99%), and a third clade including *H. nodulosa*. Group II consisted of a single clade linking *H. citriformis* isolate ARSEF 2346 with the Florida *Hirsutella* isolate from *D. citri*, and the pairing was strongly supported by the bootstrap method (99%). The ILD test indicated that the data partitions for the intron-free β -tubulin and LSU sequences were heterogeneous ($P=0.01$), so the datasets were analyzed separately by MP. In these analyses, the clades that paired *H. guyana* with *H. homalodiscae* and the *D. citri* pathogen with *H. citriformis* remained as sister taxa, respectively, but rearrangements in the topology were

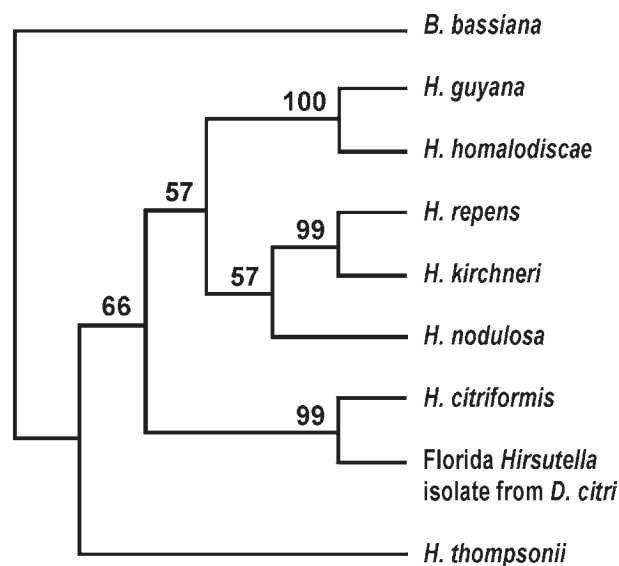


Fig. 2. Consensus phylogenetic tree using ML and MP for the Florida *Hirsutella* isolate from *D. citri* and related fungal species.

observed for some of the other *Hirsutella* species, as was previously reported by Boucias et al., 2006.

3.6. Isolate-specific PCR

PCR assays conducted using the isolate-specific PCR primers designed based on the β -tubulin gene produced a 0.6-kb product with template DNA extracted from both *in vivo* and *in vitro* cultures of the Florida *Hirsutella* isolate from *D. citri*. No amplification products were detected in PCR assays that included DNA extracted from a healthy adult psyllid or DNA prepared from *in vitro* cultures of five other related *Hirsutella* isolates. However, amplification of a 0.9–1.0 kb portion of the β -tubulin gene using degenerate primers was observed in all samples, demonstrating that each DNA template was adequate for the PCR and contained the β -tubulin gene sequence.

4. Discussion

This research resulted in the morphological and molecular characterization of a *Hirsutella* isolate related to *H. citriformis* found infecting *D. citri* in Florida citrus groves during 2005 and 2006. The structure of the synnemata, phialades, and conidia formed on mycosed *D. citri* were similar to the characteristics of *Hirsutella citriformis* described on the brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) (Brady, 1979). The measurements of the conidia were also consistent with this description, but there were distinct differences in length of the phialades (30–40 μm) and diameter of the synnemata (200–300 μm) produced on *N. lugens* (Brady, 1979) in comparison to those structures found on mycosed *D. citri* (phialade length = $17.5 \pm 1.9 \mu\text{m}$, synnemata diameter = $50.0 \pm 10.0 \mu\text{m}$). The β -tubulin gene sequence of the Florida *Hirsutella* isolate from *D. citri* was novel and different from the

β -tubulin gene sequence from *H. citriformis* isolate ARSEF 2346. Boucias et al. (2006) also reported that size and nucleotide differences were found in the putative intron region of the β -tubulin gene among other *Hirsutella* species. The isolate-specific PCR primers that were based on the unique putative intron region of the β -tubulin sequence distinguished the Florida *Hirsutella* isolate from *D. citri* from five other related *Hirsutella* species, and will be useful to identify the pathogen on *D. citri* cadavers in future studies addressing its frequency and distribution.

The origin of the Florida *Hirsutella* isolate from *D. citri* is unknown, but it may have accompanied *D. citri* to Florida. This *Hirsutella* isolate is not the same as the Indonesian isolate of *H. citriformis* from *D. citri* due to differences in the SSU sequence (Subandiyah et al., 2000). However, this pathogen could represent an as-of-yet unidentified Asian *H. citriformis*. Alternatively, an indigenous isolate of *Hirsutella* may have adapted to *D. citri* after it became established in Florida. *Hirsutella citriformis* was previously identified in Florida (Mains, 1951), and it is known to attack *D. citri* in Cuba and Guadeloupe (Rivero-Aragon and Grillo-Ravelo, 2000; Étienne et al., 2001) and a variety of other hemipteran insects including the leucaena psyllid (Mains, 1951; Rombach and Roberts, 1987; Sajap, 1993; Hywel-Jones, 1997; Villacarlos and Robin, 1999).

The studies of the pathogen–host interaction were made possible using cultures of the pathogen maintained in the laboratory. Conidia produced *in vivo* and *in vitro* were pathogenic to psyllids and produced a phenotype similar to that observed in field-collected cadavers, thus fulfilling Koch's postulates. We do not yet know if the pathogen overwhelmed *D. citri* during its vegetative stage, killed the psyllids with toxins such as hirsutellins, hirsutides and hirsutatins (Mazet and Vey, 1995; Liu et al., 1995; Isaka et al., 2005; Lang et al., 2005), or used a combination of these effects. The susceptibility of immature psyllids to the pathogen was consistent with that reported by Étienne et al. (2001), in which immature *D. citri* were killed by *H. citriformis* in Guadeloupe.

In the field, survival and transmission of this pathogen were likely enhanced by the production of synnemata, which emanated laterally from the cadavers near the ventral portion of the head and thorax and from the tip of the abdomen. The synnemata could provide a slow release of infectious conidia because only a limited number of phylades produced conidia simultaneously, while the vast majority of phylades remained undifferentiated. The pathogen could have been protected from exposure to sunlight because the cadavers were usually found secured to the underside of citrus foliage. Similarly, cadavers of the sugarcane leaf hopper killed by *H. citriformis* were found on the undersurface of sugarcane leaves (Singaravelu et al., 2003). Further investigation addressing the transmission mechanisms of the pathogen is needed to characterize the spatial and temporal patterns of the pathogen–host interaction.

Additional research also is needed to evaluate the potential for using the Florida *Hirsutella* isolate from *D. citri* as

part of an integrated pest management program to suppress *D. citri* populations. The fastidious nature of the fungal pathogen, coupled with the slow development of cultures *in vitro*, may limit the likelihood of developing it as a microbial insecticide. However, studies are needed to investigate additional culturing methods aimed at increasing sporulation of the pathogen *in vitro* and to develop and test a formulation of fragmented mycelia, as was conducted with *H. thompsonii* to control citrus rust mite (McCoy et al., 1971). An augmentative biological control approach, in which live infected *D. citri* are released or mycosed psyllids are manually dispersed, might also be used to increase the prevalence of the pathogen in *D. citri* populations in Florida, or elsewhere.

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