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Source: Florida Entomologist, 98(1) : 290-297

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.098.0147

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Mexican strains of *Hirsutella* isolated from *Diaphorina citri* (Hemiptera: Liviidae): Morphologic and molecular characterization

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Abstract

Diaphorina citri Kuwayama (Hemiptera: Liviidae), the vector of huanglongbing, has been found in several citrus areas of Mexico, apparently attacked by the fungus *Hirsutella citriformis* Speare (Hypocreales: Ophiocordycipitaceae). As this entomopathogen could represent a potential agent of *D. citri* biological control in the country, we performed this study to characterize 7 strains of the fungus isolated from this insect. For the molecular characterization we used internal transcribed spacer sequences 1 and 2 (ITSs 1 and 2) and 28S rDNA. In the morphological description we determined size of reproductive structures in the strains as well as their blastospore production capacity. BLAST analysis of the ITSs sequences showed less than 91% and 72% of identity and coverage with *Ophiocordyceps sinensis* or *H. citriformis*; meanwhile, gen 28S sequences showed high identity (98-99%) and coverage (98-100%) with *O. elongata* (Petch) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora and *O. coccidiicola* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora. The phylogenetic analysis using 28S sequences demonstrated that the Mexican strains are closely related with *H. citriformis*; however, size of phialide and conidial diameter showed differences with dimensions reported for the species in the original description. Under dark conditions, 6 strains were able to produce mucilaginous colonies that contained blastospores; these structures caused 30.8-41.2% mortality in the target insect. The results suggest that *Hirsutella* strains isolated from *D. citri* in Mexico, correspond to *H. citriformis*. We found dimorphic capacity and variability in size of the reproductive structures of the fungus. This is the first report of ITSs and 28S sequences of *H. citriformis* obtained from strains isolated from *D. citri*.

Key Words: conidia; dimorphism; phialides; phylogenetic analysis

Resumen

Diaphorina citri Kuwayama (Hemiptera: Liviidae), el vector del huanglongbing, ha sido encontrado en varias regiones citrícolas de México, aparentemente atacado por el hongo *Hirsutella citriformis* Speare (Hypocreales: Ophiocordycipitaceae). Debido a que este entomopatógeno puede representar un agente potencial de control biológico contra *D. citri* en el país, realizamos el presente estudio para caracterizar siete cepas de este hongo aisladas de *D. citri*. Para la caracterización molecular se utilizaron las secuencias espaciadoras transcritas internas 1 y 2 (ITSs 1 y 2) y el gen 28S del DNAr. En la descripción morfológica determinamos el tamaño de estructuras reproductivas de las cepas así como su capacidad de producción de blastosporas. El análisis BLAST de las secuencias ITSs mostró menos del 91% y 72% de identidad y cobertura con *Ophiocordyceps sinensis* o *H. citriformis*; mientras que el gen 28S mostró alta identidad (98-99%) y cobertura (98-100%) con *O. elongata* (Petch) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora y *O. coccidiicola* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora. El análisis filogenético con el uso del gen 28S demostró que las cepas mexicanas están fuertemente relacionadas con *H. citriformis*; sin embargo, el tamaño de fiálides y el diámetro de conidios mostraron diferencias con dimensiones reportadas para la especie en la descripción original. Bajo condiciones de oscuridad, seis cepas produjeron colonias mucilaginosas conteniendo blastosporas; estas estructuras causaron 30.8-41.2% mortalidad en el insecto blanco. Los resultados sugieren que las cepas de *Hirsutella* aisladas de *D. citri* en México, corresponden a *H. citriformis*. Encontramos también capacidad dimórfica y variabilidad en el tamaño de estructuras reproductivas del hongo. Este es el primer reporte de secuencias ITSs y del gen 28S de *H. citriformis* obtenidas de cepas aisladas de *D. citri*.

Palabras Clave: conidios; dimorfismo; fiálides; análisis filogenético

Diaphorina citri Kuwayama (Hemiptera: Liividae), the Asian citrus psyllid, is the vector of '*Candidatus* Liberibacter spp.', bacteria recognized as the putative agents of huanglongbing (HLB) or citrus greening (Bové 2006). The detection of this complex in Mexico has led to the development and evaluation of various management strategies, one of the highest of which in importance being to identify entomopathogenic fungi of *D. citri* that could be used as biological control agents of the pest. Worldwide, *Beauveria bassiana* (Bals.-Criv.) Vuill.

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(Padulla & Alves 2009), *Hirsutella citriformis* Speare (Rivero-Aragon & Grillo-Ravelo 2000; Subandiyah et al. 2000; Meyer et al. 2007) and *Isaria fumosorosea* Wize (=*Paecilomyces fumosoroseus*; Subandiyah et al. 2000) are reported infecting *D. citri* and examinations of *D. citri* in different citrus areas of Mexico have yielded observations of *Entomophthora* sp. (Guizar-Guzmán & Sánchez-Peña 2013), *B. bassiana* and *H. citriformis* (Casique-Valdes et al. 2011). *Hirsutella citriformis* has been found in 11 other host species in the world belonging to 8 families of Hemiptera, and a family of Psocoptera (Spear 1920; Mains 1951; Sajap 1993; Hywel-Jones 1997; Boucias et al. 2007) exhibiting the wide host range of this fungal species.

Genetic information and dimensions of reproductive structures of *H. citriformis* strains associated with *D. citri* are scarce. There are sequences of some loci (ITSs, 18S and 28S rDNA, and β-Tubulin gen) from strains of *H. citriformis* obtained from diverse insect species that have been used to establish the identity of *Hirsutella* strains collected from *D. citri* (Subandiyah et al. 2000; Meyer et al. 2007; Casique-Valdes et al. 2011). ITS1 and ITS2 are frequently used to establish the possible identity of fungi (Singh et al. 2010; Lindner & Banik 2011); such sequences have been scarcely employed in *H. citriformis* isolated from *D. citri* to establish the relationship with other species of the genus. Regarding size of phialides and conidia from strains of *H. citriformis* from *D. citri,* they are very diverse according to the original species description (Speare 1920). It is expected that new strains of this fungus species could show variability in the extent of their structures.

In Mexico, the presence of *H. citriformis* infecting *D. citri* was reported in Tamaulipas state by Casique-Valdes et al. (2011) based on analysis of the 28S gene (sequence absent in GenBank) and characteristics of reproductive structures developed in culture media; however, the *Hirsutella* genus on *D. citri* was reported earlier in citrus growing areas of the country (López-Arroyo et al. 2008), including a strain of *Hirsutella* sp. isolated from *D. citri* in the state of Tabasco, Mexico, (INIFAP-Hir-1) that was able to develop mycelial colonies as well as colonies with mycelial growth and sectors of mucilaginous appearance in culture media (Hernández-Luna et al. 2010). These sectors contained blastospores, like in *Metarhizium flavoviridae* (Fargues et al. 2002), *I. fumosorosea* (Avery et al. 2009) and *B. bassiana* (Lane et al. 1991), which are capable of causing infection in *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae), *D. citri,* and *Nephotettix virescens* (Distant) (Hemiptera: Cicadellidae), respectively. Recently, we have shown that this strain of *Hirsutella* can produce abundant blastospores in liquid fermentation (Romero-Rangel et al. 2012). The objective of this research was to characterize 7 *Hirsutella* Mexican strains isolated from *D. citri* using ITSs and 28S gene sequences, morphology of phialides and conidia, production capacity of blastopores and their pathogenicity to *D. citri* under laboratory conditions. Such information will contribute to increase the knowledge about this species that has been neglected for use in pest biological control.

Materials and Methods

ISOLATION OF STRAINS

Hirsutella strains were isolated from mycosed adults of *D. citri* with presence of synnemata; the insect specimens were collected in diverse states of Mexico (Table 1). Monoconidial strains were obtained directly from sporulated synnemata or from conidia produced in colonies developed from fragments of these structures. The strains remained active in potato dextrose agar medium (PDA, Difco) and stored for long period in mineral oil and water (Rodríguez & Gato 2010).

Table 1. Source of *Diaphorina citri* mycosed by *Hirsutella* strains from 7 states of Mexico.

Strain	Locality	Collection date	Host plant
INIFAP-Hir-1	Huimanguillo, Tabasco	August 2008	Valencia orange
INIFAP-Hir-2	Mocochá, Yucatán	September 2011	Mandarin
INIFAP-Hir-5	Nuevo Israel, Quintana Roo	January 2010	Orange jasmine
INIFAP-Hir-6	Edzná, Campeche	September 2011	Orange jasmine
INIFAP-Hir-7	Tapachula, Chiapas	December 2011	Orange jasmine
IB-Hir-1	Tlapacoyan, Veracruz	September 2011	Persian lime
IB-Hir-2	Tecomán, Colima	September 2011	Persian lime

GENETIC CHARACTERIZATION

The strains were grown in potato dextrose broth, incubated at 25° C for 15 days with stirring at 250 rpm. The mycelium was washed twice with 1X TE. A portion of mycelia was transferred to a 1.5 ml conical tube, added 250 µL of lysis buffer (200 mM Tris pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and macerated with pestle, and 250 µL of lysis buffer and 500 µL of phenol/chloroform/isoamyl alcohol (25:24:1) were added. The DNA was precipitated with sodium acetate (3M, pH 7) and isopropanol, and washed with 70% ethanol. The DNA was resuspended in 1X TE buffer and RNase was added.

The ITS-1-5.8S-ITS-2 region was amplified by PCR using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGAATATGC-3') primers. PCR was performed with 3.5 mM of $MgCl_2$, 4 ng μL^{-1} of each primers, 200 µM dNTPs, 1U Taq DNA polymerase, reaction buffer to 1X, 1 µL (500 ng) of DNA and water. The PCR consisted of one cycle of denaturation at 95 °C for 5 minutes, 35 cycles at 94 °C for 30 seconds, 60 °C for 45 seconds and 72 °C for 90 seconds, followed by one final extension step at 72 °C for 8 minutes (Robles-Yerena et al. 2010). An amplification of a partial-28S ribosomal gene was performed using the primers LS1 (5'-AGTACCCGCTGAACTTAAG-3') and LR5 (5'-CCTGAGGGAAACTTCG-3') as well as the amplification conditions described by Meyer et al. (2007).

The amplification products were purified with the kit from Bio Basic Inc. (EZ-10 spin column BS354) according to manufacturer's instructions. Bidirectional sequencing was performed at the National Autonomous University of Mexico. Sequences were aligned using the program BioEdit Sequence Alignment Editor (Copyright© 1997-2007 Tom Hall) (Hall 1999). The consensus sequences were analyzed on the page of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) and deposited in NCBI-GenBank with accession numbers indicated in the obtained phylogenetic trees.

Sequences of both ITSs and 28S gene of *Hirsutella* species were searched in the NCBI and independent phylogenetic analysis with sequences of Mexican *Hirsutella* strains were performed. Sequences were aligned using the MUSCLE algorithm in MEGA 5.1 program (Tamura et al. 2011) and withdrew from the nucleotide alignment that were at the ends, due to the lack of these sequences in some species. The resulting alignment was subjected to phylogenetic analysis using the Neighbor-Joining method based on the Kimura 2 parameter model, with the option of pairwise deletion of gaps in MEGA. To evaluate the stability of the trees the bootstrap method with 1,000 replicates was used.

MORPHOLOGIC CHARACTERIZATION

PDA fragments of about 1 cm^2 were cut, placed on glass slides and deposited in sterile Petri dishes with filter paper moistened with sterile water. Each strain was inoculated at each end of the PDA fragment, covered with a sterile coverslip and incubated at 25 ± 2 °C until the fungal growth with its characteristic synnemata (4 weeks) appeared. Posteriorly,

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the coverslips were removed and placed on a slide containing lactophenol cotton blue. A fragment of agar from the glass slide was also detached; then, in the place that it was occupying a drop of lactophenol cotton blue was applied and a new coverslip was positioned. Slides were observed under the microscope where we performed measurements of phialides (n = 15), conidia (n = 10) and mucus surrounding the conidia (n = 10); measurements were obtained in microns. Dimensions were compared with the description of *H. citriformis* reported by other authors.

PRODUCTION CAPACITY OF BLASTOSPORES

The blastospore production capacity by 7 *Hirsutella* strains was determined using the process described by Hernández-Luna et al. (2010). INIFAP-Hir-1 strain was taken as a reference. Five fragments (approximately 3 mm^2) from the edge of a mycelial colony were distributed in Petri dishes (4 in the periphery and one in the middle) containing PDA. Each Petri dish was considered a repetition and 6 replicates were placed under dark condition at 26° C for 5 days. The experiment was established in a completely randomized design. In each replication the percentage of fragments with mucilaginous colonies and the occurrence of blastospores (formed by budding cells) were determined. For analysis, data were transformed using arc sine of the square root of percentage values. In the results, we presented original data means.

BLASTOSPORE BIOASSAYS

We evaluated effects of blastospores from 3 *Hirsutella* strains (INIFAP-Hir-1, INIFAP-Hir-6, IB-Hir-2) sprayed against *D. citri*; in such test we included an untreated control; we used a complete randomized design with 4 treatments and 7 replications/treatment. Blastospores were obtained inoculating potato dextrose broth (PDB) with four 1 cm^2 mycelial colony pieces of the strains indicated above; posteriorly, they were incubated during 11 days at 25 °C and agitation at 250 rpm. Blastospores produced were adjusted at 1 x 10^6 blastospores mL^{-1} for use in bioassay. *D. citri* adults with a healthy appearance were anesthetized using cotton impregnated with 80 µl of chloroform. Anesthetized insects were placed on absorbent paper in a plastic tray and were sprayed with the blastospore suspension at approximately 15 cm distance for 3 consecutive times; the insects in the untreated control were sprayed with PDB sterile medium. Fifteen inoculated insects (per treatment replication) were placed on one tender leaf of Valencia orange. The experimental arena consisted of a plastic container with a sponge saturated with sterile distilled water where the leaf with the 15 *D. citri* specimens was placed. Treatments were kept under constant conditions at 26 ± 1 °C, 16:8 h L:D. Insect mortality was recorded each 3 or 4 days after inoculation, during 27 days. All dead insects were placed in a humid chamber to verify mycelial growth by the fungal strains evaluated. For data analysis, we proceeded as in the previous section.

DATA ANALYSIS

Data of blastospore production and mortality were subjected to one way ANOVA; comparisons among treatment means were performed using Tukey test (P = 0.05) (Experimental Design Program ver. 1.0, Faculty of Agriculture, Universidad Autónoma de Nuevo León; Olivares 2012).

Results

GENETIC CHARACTERIZATION

We found in GenBank sequences for 30 *Hirsutella* species; 4 species have sequences of the 18S ribosomal gene, 14 species the 28S ribosomal gene, 9 species the β-tubulin gene, while 19 species have ITS1 and ITS2 sequences including the 5.8S gene. Table 2 shows *Hirsutella* species sequences deposited in GenBank and used in the phylogenetic analyzes performed in this research. The PCR products from 7 *Hirsutella* Mexican strains isolated from *D. citri* ranged from 864-914 bp for the 28S gene and from 522-596 bp for the ITS-1 and ITS-2 region. BLAST analysis in NCBI showed that ITSs sequence of the *Hirsutella* Mexican strains have high identity with sequences of 5.8S gene and ITS2 of *Ophiocordyceps* and *Cordyceps*; whereas, the analysis of the 28S gene sequences exhibited high identity with *O. elongata* and *O. coccidiicola*.

Table 2. Accessions of *Hirsutella* species listed in the Genbank and used to establish their relationships with *Hirsutella* Mexican strains isolated from *D. citri*.

No.	Species	ITSs	28S
1	*H. aphidis*	EF194156.1	
2	*H. citriformis*	JF906754.1 JF894156.1	AY518376.1 DQ075678.1 EF363707.2 KJ803256.1
3	*H. gigantea*		JX566977.1
4	*H. gregis*	EF194155.1	
5	*H. guyana*	DQ345595.1	DQ075676.1
6	*H. homalodisca*		DQ075674.1
7	*H. huangshanensis*	EF689043.1	
8	*H. illustris*		AY518380.1
9	*H. kirchneri*	EF194154.1	AY518382.1
10	*H. liboensis*	FJ957892.1	
11	*H. longicolla*	FJ973071.1	
12	*H. minnesotensis*	DQ078757.1	
13	*H. necatrix*		AY518381.1
14	*H. nodulosa*	EF194146.1	AY518379.1 DQ075675.1
15	*H. proturicola*	AB378557.1	AB378557.1
16	*H. rhossiliensis*	EF546655.1	EF546655.1
17	*H. rostrata*	EF194150.1	
18	*H. sinensis*	AJ243980.1	
19	*H. stilbelliformis*		GQ866966.1 GQ866967.1
20	*H. strigosa*		AY518377.1
21	*H. subulata*		AY518378.1
22	*H. thompsonii*	FJ973072.1	DQ075673.1 AY518375.1 AF339528.1
23	*H. tunicata*	JN247824.1	
24	*H. uncinata*	AY365468.1	
25	*H. vermicola*	DQ345592.1	
26	*H. versicolor*	DQ345594.1	

Phylogenetic analysis with ITSs of 19 *Hirsutella* species and the Mexican strains was performed using an alignment spanning 606 bp. The results showed that the strains from Mexico are highly similar to each other and share a node in common with sequences labeled as *H. citriformis* in GenBank (accession JF906754 and JF894156) (Fig. 1). Percentage of variation among the sequences of the Mexican isolates was 0.00-0.21%. In the case of the *Hirsutella* species variation was from 0.00% in related taxa (i.e. *H. kirchneri* with *H. gregis*) to 28.89% in disparate taxa (i.e. *H. longicolla* with *H. guyana*). Values among the monophyletic Mexican isolates and *H. citriformis* (accession JF906754 and JF894156) were 15.45-15.73%, which show the poor relationship among the *Hirsutella* Mexican strains isolated from *D. citri* and *H. citriformis* sequences deposited in GenBank from *Oliarus dimidiatus* Berg (Hemiptera: Cixiidae) (accession JF894156) and *Ectopsocus* sp. (Psocodea: Ectopsocidae) (accession JF906754).

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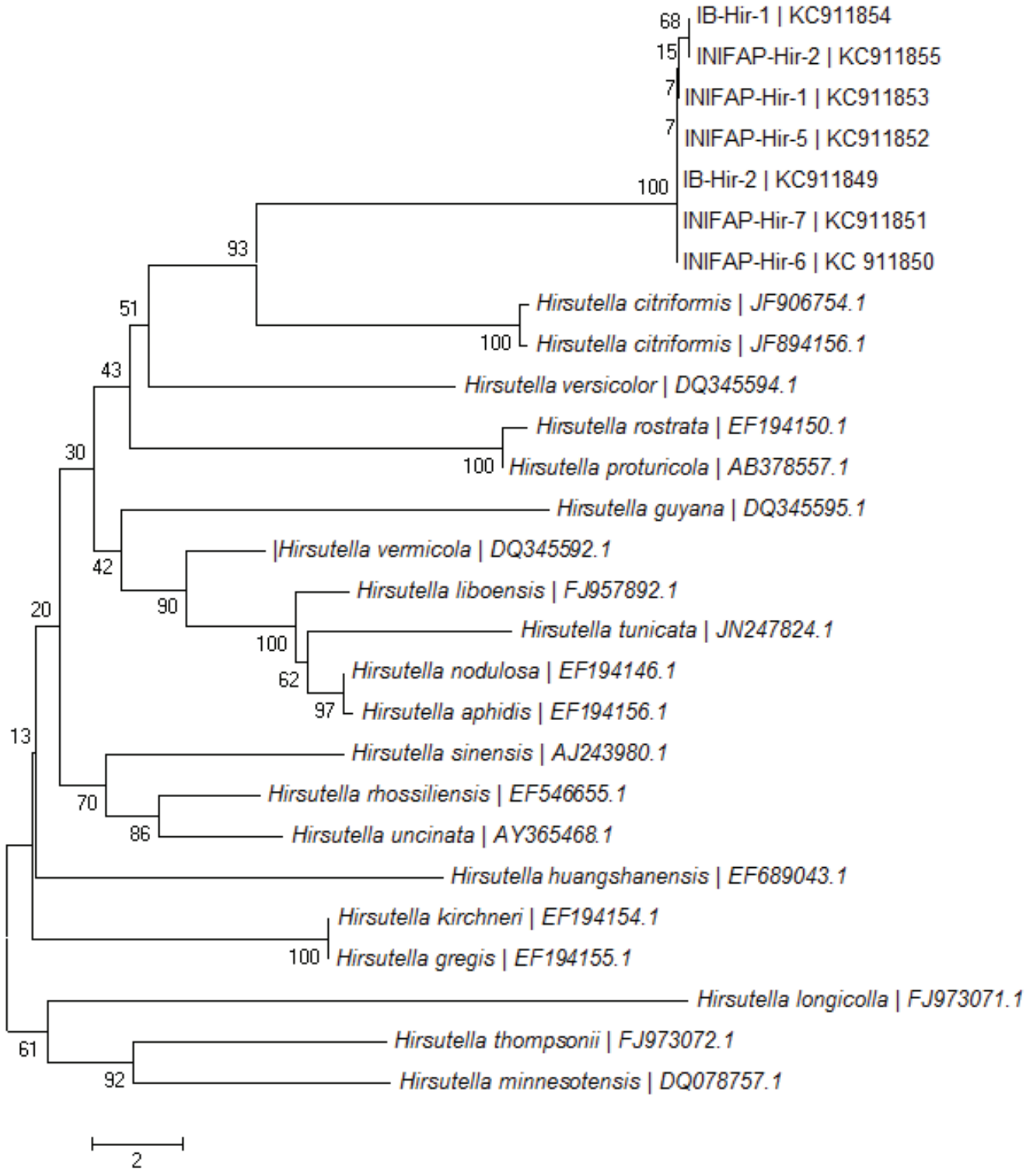

Fig. 1. Phylogenetic tree constructed with sequences of ITS1-5.8S-ITS2 of 19 *Hirsutella* species enlisted in the NCBI and 7 Mexican strains isolated from *D. citri* in Mexico. Percentages from bootstrap analysis that support branches in the tree are shown in the respective nodes. Scale represents the number of substitutions/100 nucleotides.

Phylogenetic analysis of the 28S gene was performed with an alignment of 495 bp, and the phylogenetic tree (Fig. 2) showed that Mexican *Hirsutella* strains are identical to each other and 2 *H. citriformis* sequences deposited in GenBank (accessions AY518376 and DQ075678) obtained from strains isolated from *Nilaparvata lugens* (Hemiptera: Delphacidae). Other two 28S gene sequences of *H. citri-*

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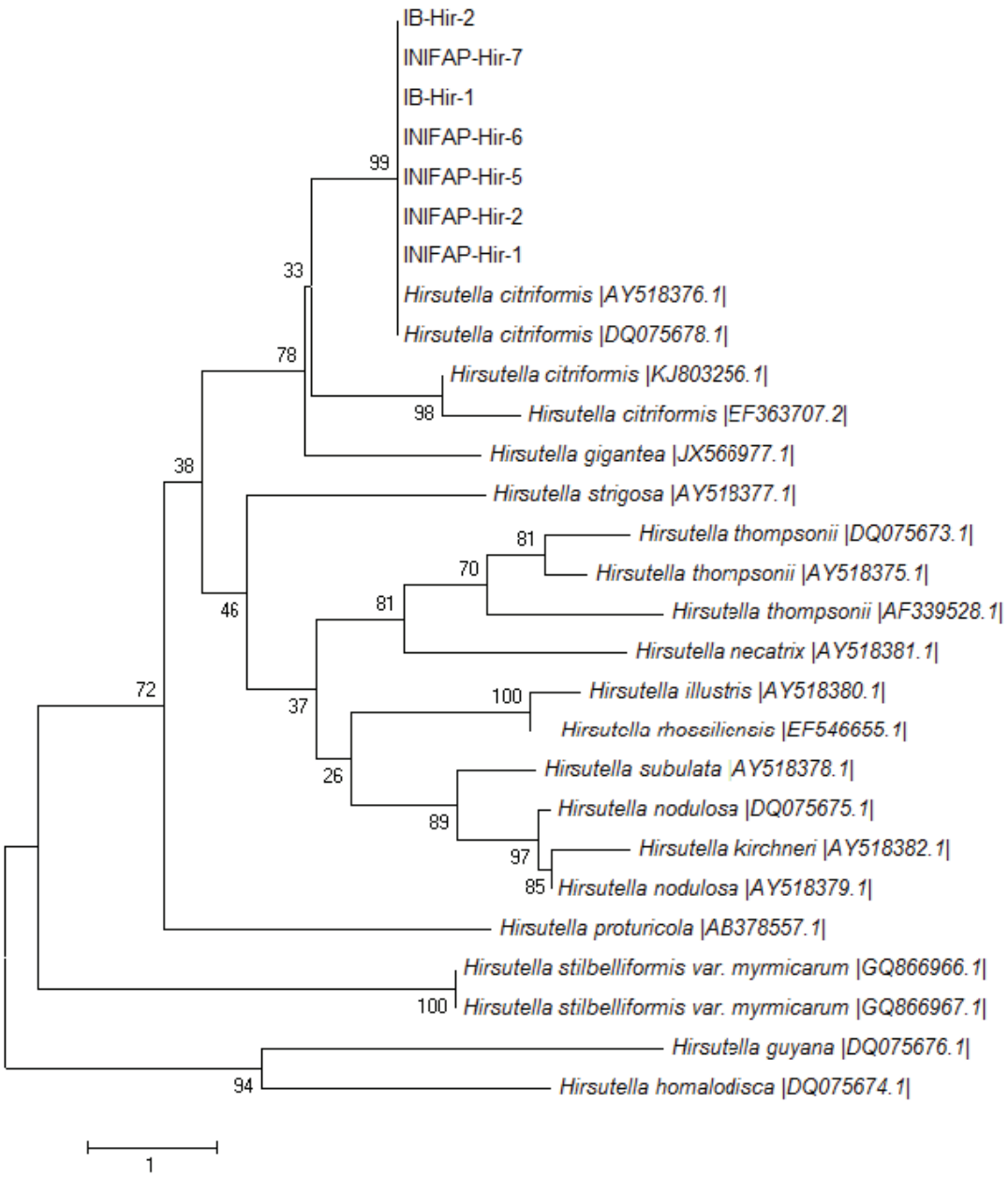

Fig. 2. Phylogenetic tree constructed with sequences of the 28S ribosomal gene of 14 *Hirsutella* species enlisted in the NCBI and 7 Mexican strains isolated from *D. citri* in Mexico. Percentages from bootstrap analysis that support branches in the tree are shown in the respective nodes. Scale represents the number of substitutions/100 nucleotides.

formis isolated from *D. citri* (accession EF363707) and *O. dimidiatus* (accession KJ803256, ARSEF878 strain) are separated from all of the above and between themselves. The variation between the sequences of the species of *Hirsutella* was 0.62% in closely related taxa (i.e. *H. illustris* with *H. rhossiliensis*) to 9.62% in disparate taxa (i.e *H. homalodisca* with *H. kirchneri*). Phylogenetic analysis of 28S gene sequence showed that Mexican *Hirsutella* strains are closely related to *H. citriformis*.

MORPHOLOGIC CHARACTERIZATION

The results showed high variation in the size of structures of the 7 strains grown in microculture (Table 3). The total length of phialides ranged 26-42 µm, the bulbous base of phialides 4.0-8.5 µm and sterigma 20.0-38.0 µm. Conidium dimensions varied 5.4-6.3 µm in length and 1.6-2.0 µm in diameter. Conidia surrounded with mucus varied 5.6-6.2 µm in diameter. Length and diameter range of the mucus conidia are within the description of *H. citriformis* type material (Mains 1951).

PRODUCTION OF BLASTOSPORES

There were significant differences among the strains in the ability to develop mucilaginous colonies containing blastospores (P = 0.01) (Fig. 3). The INIFAP-Hir-1 strain (control) developed mucilaginous colonies producing blastospores in darkness in 100% of fragments; in contrast, IB-Hir-1 strain from Veracruz, Mexico, was unable to produce mucilaginous colonies (Fig. 4) even though it was isolated and cultured

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Table 3. Dimensions (µm) of morphological structures of *Hirsutella* strains isolated from *D. citri* in Mexico, and comparison with the description of *H. citriformis* (Mains 1951) and from other sources.

	Phialide length			Conidium		
Strain (Source)	Total	Base	Sterigma	Length	Diameter	Mucus diameter
INIFAP-Hir-1/Tabasco (CM*)	37.83 ± 0.50	4.88 ± 0.38	32.9 5 ± 0.72	5.90 ± 0.089	1.74 ± 0.19	5.99 ± 0.13
INIFAP-Hir-2/Yucatán (CM)	36.96 ± 0.09	8.09 ± 0.56	28.87 ± 0.12	5.96 ± 0.15	1.96 ± 0.05	5.92 ± 0.08
INIFAP-Hir-5/Q. Roo (CM)	36.50 ± 0.23	5.08 ± 0.49	31.42 ± 0.19	5.83 ± 0.14	1.84 ± 0.15	5.87 ± 0.14
INIFAP-Hir-6/Campeche (CM)	40.90 ± 0.31	5.10 ± 0.48	35.8 ± 0.40	5.84 ± 0.26	1.94 ± 0.11	5.90 ± 0.13
INIFAP-Hir-7/Chiapas (CM)	32.00 ± 0.09	5.12 ± 0.23	26.88 ± 0.19	6.04 ± 0.05	1.99 ± 0.03	5.91 ± 0.09
IB-Hir-1/Veracruz (CM)	32.09 ± 0.53	5.82 ± 0.26	26.27 ± 0.58	6.11 ± 0.10	1.97 ± 0.05	5.96 ± 0.08
IB-Hir-2/Colima (CM)	30.70 ± 0.46	6.00 ± 0.48	24.7 ± 0.84	5.91 ± 0.14	1.79 ± 0.03	5.86 ± 0.13
Speare 1920 (H)	**			5.5-8.5	1.5-1.8	
Mains 1951 (H)	36.0-54.0	6.0-14.0	30.0-40.0	5.0-8.0	2.0-2.5	5.0-10.0
Hywel-Jones 1997 (H)	18.5-52.0			3.5-5.0	1.0-1.5	
Alvarez et al. 2003 (H)			16.8-23.6	6.8-9.1	1.5-2.3	
Meyer et al. 2007 (H)			17.5 ± 1.9	5.9 ± 0.8	2.6 ± 0.3	
Toledo et al. 2013; ARSEF 8378 (H)	35.6-55.4		28.7-47.5	5.9-7.9	2.0-3.0	
Toledo et al. 2013; ARSEF8679 (H)	22.4-34.7		16.8-28.0	5.6-7.8	2.2-2.8	
Subandiyah et al. 2000 (CM)	27.5-62.3	5.1-9.4	22.4-52.9	6.4-7.6	2.1-2.8	
Casique-Valdes et al. 2011 (CM)				6.8-7.0	1.5-2.0	

*(CM) = Culture media; (H) = Host. ** = No data.

in the same year as the other contrasted strains; in these strains the capacity for blastospore production was variable from 56.7 to 100% of the fragments.

BLASTOSPORE BIOASSAY

D. citri mortality caused by *H. citriformis* blastospores began 6 days after inoculation. After 27 days, mortality varied 30.8 to 41.2% in treated insects; in the control mortality was 17.6% (Fig. 5). Number of mycosed dead insects was low.

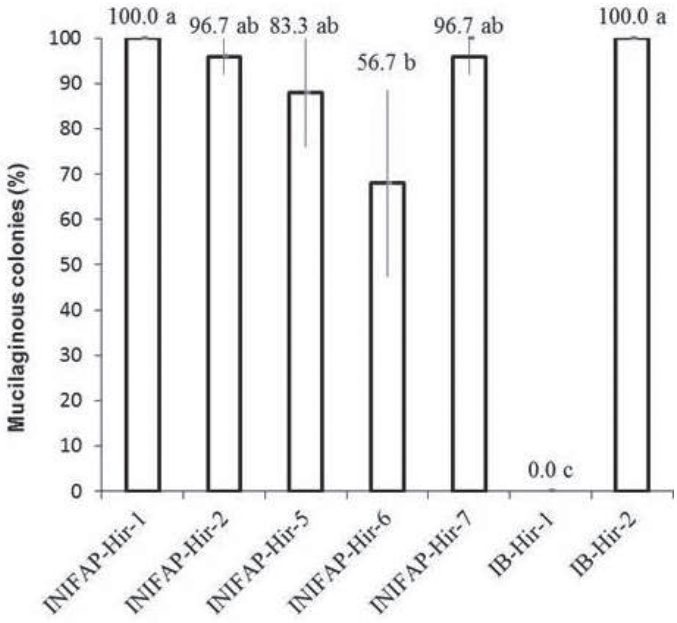

Fig. 3. Percentage of mucilaginous colonies developed from fragments of mycelial colonies of 7 *Hirsutella* Mexican strains after 5 days under dark conditions. Values followed by the same letter did not differ statistically (Tukey; *P* = 0.05). Lines in the bars indicate SD.

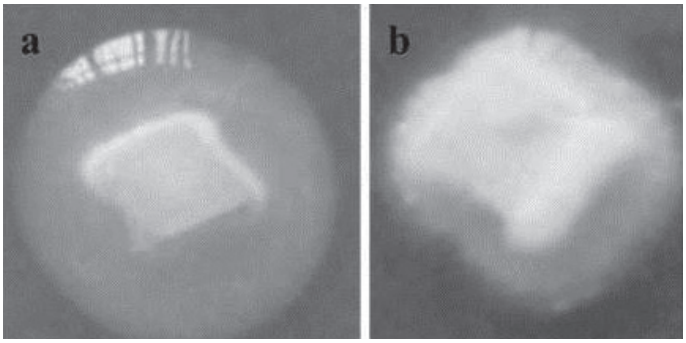

Fig. 4. Production of mucilaginous colonies by *Hirsutella citriformis* Mexican strains under dark conditions. Mucilaginous colony produced by strain INIFAP-Hir-1 (left) and colonies without mucilage in strain IB-Hir-1 (right), both after 5 days of culture under dark conditions.

Discussion

H. citriformis is the only species of the genus *Hirsutella* that has been found infecting *D. citri* (Hall et al. 2012; Casique-Valdes et al. 2011; Meyer et al. 2007; Subandiyah et al. 2000). In our study, we observed that dimensions of the phialide and conidia diameter reported by Mains (1951) differ from the size that we obtained in the *Hirsutella* strains isolated from *D. citri* in Mexico. In addition, the extent of reproductive structures of *H. citriformis* acquired directly from the host (Meyer et al. 2007; Alvarez et al. 2003; Hywel-Jones 1997) or from those developed in culture medium (Casique-Valdes et al. 2011; Subandiyah et al. 2000) were also different from the species original description. Previously, Mains (1951) noted that the dimensions of the *H. citriformis* synnemata on their hosts were variable and that this could be due to the size of insects where the fungus developed. In our study, it was expected that the size of reproductive structures of the *Hirsutella* strains grown in culture media could be more similar to each other; however, the results and the aforementioned reports show the high variability of this fungus species.

BLAST analysis showed that the sequences of 5.8S and ITS-2, and 28S of the *Hirsutella* Mexican strains were related with species of the

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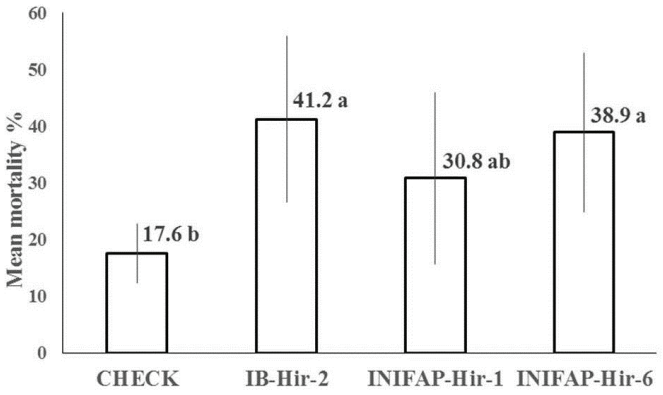

Fig. 5. Mortality of *Diaphorina citri* caused by *Hirsutella citriformis* blastospores. Values followed by the same letter did not differ statistically (Tukey; *P* = 0.05). Lines in the bars indicate SD.

genus *Ophiocordyceps*; such results were expected because *Hirsutella* is considered the anamorph stage of the teleomorph stage *Ophiocordyceps* (Evans et al. 2011; Seifert & Boulay 2004). Due to the ITS-1 low relationship with genera of entomopathogenic fungi, as it was observed by Toledo et al. (2013) with ITSs sequences of *Hirsutella* sp., we revised the presence of the ITS1 primer in the strains under study; its occurrence was confirmed (anchor in the 18S ribosomal gene) and such finding indicates that the results from BLAST analysis of the Mexican *Hirsutella* strains sequences were reliable. It was expected that the sequences of the Mexican strains of the 28S gene in BLAST analysis had similarity with EF363707 accession of *H. citriformis* isolated from *D. citri* and deposited by Meyer et al. (2007); we confirmed that our strains matched such accession.

Phylogenetic analysis of ITSs showed that the Mexican strains share a node with sequences JF906754 (ARSEF 8679, strain isolated from psocids) and JF894156 (ARSEF 8378, strain isolated from cixiids) accessions deposited in GenBank as *H. citriformis*. However, Toledo et al. (2013) state that the sequences of these accessions are undefined species of *Hirsutella*. Our results confirm that the strains isolated from *D. citri* in Mexico represent a distinct species to the reported by those authors. In the other hand, the 28S gene showed high relationship of the *Hirsutella* Mexican strains with sequences of the accessions AY518376 (Liu et al. 2005) and DQ075678 (Boucias et al. 2007) isolated from delphacids in Indonesia and submitted to GenBank as *H. citriformis*. In the case of accessions KJ803256 and EF3633707 submitted to GenBank as *H. citriformis,* they have no close relationship with Mexican strains of this entomopathogenic fungus, suggesting that both sequences belong to different species of *Hirsutella*. Phylogenetic analysis with ITSs and 28S sequences displayed that the strain ARSEF8378 (accessions JF894156 and KJ803256) is separated from the Mexican strains which have high similarity between each other; also, it shows that it is possible to separate the species from other taxa of the genus. Relationship of the *Hirsutella* Mexican strains with sequences of *Cordyceps* and *Ophiocordyceps* species in the BLAST analysis demonstrates the need to conduct a thorough phylogenetic and morphological study that allows to establish with greater certainty the relationship between these teleomorphs and *Hirsutella* species. In GenBank there are also sequences from other DNA loci (18S, β-tubulin) of *H. citriformis* strains (Subandiyah et al. 2000); nonetheless, its use for purposes of establishing relationships between most of the genus species is limited by the scarce number of taxa that have these reported sequences.

The results demonstrate that for the separation of taxa within the genus *Hirsutella,* ITSs sequences provide a higher resolution than those of the 28S gene within the genus *Hirsutella*. This is supported by the high variation expressed in substitutions per 100 nucleotides observed in the ITSs sequences in comparison with those of 28S gene. Based on *H. citriformis* being the only registered species of *Hirsutella* attacking *D. citri* and the sequence similarity of the Mexican strains with the 28S gene from sequences described as this species (Boucias et al. 2007; Liu et al. 2005), it is considered that the *Hirsutella* strains isolated from *D. citri* in Mexico correspond to the species *H. citriformis*. We believe that ITSs sequence analysis of the strains ARSEF 532 and ARSEF2346 (accessions AY518376 and DQ075678 in GenBank, respectively) could establish with greater certainty the relationship of our strains with *H. citriformis* attacking *D. citri* and other hemipterans in the world.

In the present research, we confirm in the Mexican strains of *H. citriformis* the development under dark conditions of colonies with mucilaginous appearance that contained blastospores. Such spores showed different shapes and sizes like the ones observed previously by our research group (Hernández-Luna et al. 2010). Occurrence of colonies showing different phenotype is known in diverse fungal species including human pathogens like *Candida albicans* (Robin) Berkhout (Molero et al. 1998) and *Histoplasma capsulatum* Darling (Maresca & Kobayashi 1989) and plant pathogens such as *Ustilago maydis* (DC.) Corda (Bolker 2001) and *Microbotryum violaceum* (Pers.) G. Deml and Oberw. (Hughes & Perlin 2005). Fungi with capacity to develop colonies showing different phenotype and cell structures are known as dimorphic fungi. Dimorphism in these fungi commonly is due to diverse environmental and nutritional factors (Maresca & Kobayashi 1989) and it has an important role in pathogenesis and survivorship (Shahid et al. 2012; Bolker 2001; Lo et al. 1997). The results that we obtained suggest that culturing *H. citriformis* from micelial colonies under dark conditions it is possible to retrieve a colony phenotype of mucilaginous appearance containing blastospores.

In this research we demonstrated that *H. citriformis* blastospores cause mortality in *D. citri*, which was variable depending on the strain (Fig. 4). Considering that blastospores of other entomopathogenic fungi has been found infecting insect species (Avery et al. 2009; Fargues et al. 2002; Lane et al. 1991), and that *H. citriformis* blastospores develop in high quantity and at very short time in liquid cultures (Romero-Rangel et al. 2012), additional studies aimed to identify the conditions for development of the epizootic when blastospores are applied need to be conducted to determine its biological control potential.

The first record of *Hirsutella* genus infecting *D. citri* in Mexico was made during 2008; posteriorly, it was registered in other citrus growing areas of the country (López-Arroyo et al. 2008). Presence of *D. citri* was noted in the country during 2002 (López-Arroyo et al. 2004). There is not an early record of *H. citriformis* incidence in any host close to the *D. citri* first report of occurrence in Mexico. The finding of this entomopathogenic fungus in this pest and its extended distribution in the Mexican citrus areas, lead to the hypothesis that perhaps *H. citriformis* was already present years before in the citrus plantations. According to the diverse list of hosts where it have been found in the world (Toledo et al. 2013; Casique-Valdes et al. 2011; Boucias et al. 2007; Hywel-Jones 1997; Sajap 1993; Mains 1951; Speare 1920), it could be infecting hemipterans in the neighborhood of the groves, maybe in grasses or other plant species that met temperature and relative humidity conditions for the fungus and epizootic development (Hall et al. 2012). In any case, its presence in Mexico contributes to increase the potential arsenal of biological control agents that could be used against the vector of huanglongbing. Studies to determine its capacity in the control of Asian citrus psyllid in the field remains as a priority.

We concluded that the fungal strains isolated from *D. citri* found in 7 different states of Mexico correspond to *H. citriformis* Speare. We based the diagnosis on the host where they were isolated (Hall et al. 2012; Casique-Valdes et al. 2011; Meyer et al. 2007; Subandi-

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yah et al. 2000), the similarity of the 28S rDNA gene sequence with those of strains isolated from Indonesian delphacids (Boucias et al. 2007; Liu et al. 2005), and morphometric characteristics of reproductive structures (Mains 1951). In addition, we have demonstrated that *H. citriformis* comprises dimorphic strains that grow as yeasts under dark conditions, and that these are capable of causing mortality in *D. citri*. Yeasts in mucilaginous colonies are like the hyphal bodies found by Meyer et al. (2007) in hemolymph of *D. citri* insects infected with *Hirsutella*.

This is the first study in which the characterization of *Hirsutella* strains isolated from *D. citri* has been performed based on the size of reproductive structures in microculture conditions, sequence analysis of 28S rDNA and ITSs, as well as in its production capacity and pathogenicity of blastospores.

Acknowledgments

The authors thank CONACYT for the funding of this study as part of the project FONSEC-SAGARPA-CONACYT 2009-108591. We are deeply thankful to many technicians of the diverse Mexican State Committees for Plant Health, who enthusiastically helped in the collection of infected specimens. O. Pérez-González also thanks CONACYT for fellowship support.

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