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Isaria poprawskii sp. nov. (Hypocreales: Cordycipitaceae), a new entomopathogenic fungus from Texas affecting sweet potato whitefly☆

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ABSTRACT

Isaria poprawskii is described as a new entomopathogenic species similar to *Isaria javanica* (= *Paecilomyces javanicus*). It was discovered on the sweet potato whitefly, *Bemisia tabaci* biotype B in the Lower Rio Grande Valley of Texas (LRGV), USA. Morphological and DNA examinations indicated the distinctness of *I. poprawskii* from the ex-type isolate of *I. javanica*. *I. poprawskii* produced light yellow young colonies to darker yellow with a grayish-violet center to a taupe or a brownish-gray mature conidial mass; conidia hyaline, one-celled, 3.9 (2.9–4.6) μm long × 1.6 (1.4–2.1) μm wide; colored synnemata, but *I. javanica* ex-type produced white colony, hyaline conidia and no synnemata. A phylogenetic position of *I. poprawskii* was inferred by a nucleotide sequence analysis of β-tubulin along with standard β-tubulin sequences from GenBank. Fifteen unsequenced isolates, including eight from the LRGV, were investigated. The analysis confirmed that *I. poprawskii* could be recovered from LRGV fields, and that both *I. javanica* and *I. poprawskii* are present in the LRGV in sympatry. *I. poprawskii* was shown to be closely related to *I. javanica*; however, it formed its own unique clade, thus confirming its status as a new fungal species.

1. Introduction

Naturally occurring epizootics due to entomopathogens are important regulators of insect populations. Many fungal species are employed as biological control agents of insect pests in crops grown in the field or in greenhouses (Lacey et al. 2001). Fungi are the only entomopathogens able to invade actively through the cuticle of piercing-sucking insects and are the only pathogens affecting whiteflies (Hemiptera: Aleyrodidae).

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An indigenous entomopathogenic fungus has been found causing natural epizootics in the sweet potato whitefly, *Bemisia tabaci* (Gennadius) biotype B (Hemiptera: Aleyrodidae) at the Lower Rio Grande Valley of Texas, USA. In 28 September 2001, infected whiteflies were collected to isolate the fungus, determine its pathogenicity against *B. tabaci* and investigate its thermal characteristics in regards to fungal growth (Cabanillas and Jones, 2009a,b). This entomopathogenic fungus was assumed to be a new species of *Isaria* Pers.

For a species to be considered new, morphological and DNA investigations should be detectable when compared with other closely related species. Although morphological characters may help identifying fungus species, it is evident that for species descriptions, examinations on their morphology and DNA analysis are needed to confirm distinctness (Tigano-Milani et al. 1995; Fargues et al. 2002). Based on recent phylogenetic studies, most of the *Paecilomyces* Bainier species have been reassigned in the genus *Isaria* (Luangsa-ard et al. 2004, 2005; Gams et al. 2005; Hodge et al. 2005) and the newly reclassified *Isaria* species of the order Hypocreales, have been redistributed from the family Clavicipitaceae into three families and placed all of the newly reclassified *Isaria* species in the family Cordycipitaceae (Sung et al. 2007). The purpose of this investigation was to describe a new *Isaria* species based on morphological and DNA examinations.

2. Materials and methods

2.1. Source of fungi

Isolates of the new *Isaria* candidate were obtained from naturally-infected sweet potato whitefly larvae and adults on eggplant leaves in greenhouses at the USDA, ARS Kika de la Garza Subtropical Agricultural Research Center (KSARC) in Weslaco, Texas, in September 2001. Pure cultures were isolated from insect cadavers on Sabouraud dextrose agar with yeast extract (SDAY), Sabouraud maltose agar with yeast extract (SMAY), and water agar (WA) with or without chloramphenicol (5%). Isolates were incubated at 26 °C in a 12:12 h fluorescent light:dark cycle. After three weeks, spores were harvested by rinsing culture plates with sterile water containing 0.02% (v/v) Silwet L-77 (Loveland Industries; Greeley, CO, USA) and storing suspensions at −76 °C (Humber 1997), and on sterile (autoclaved at 125 °C, 20 psi for 1 h) fumed silica crystals (Sigma Chemical Co.; St. Louis, MO) at 4 °C. Vegetative hyphae were also preserved in culture slants on SDAY under a layer of sterile mineral oil at 4 °C. This fungus was deposited in the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, NY) as ARSEF 7028.

To compare ARSEF 7028 with other closely related species, we included the ex-type isolate of a morphologically similar species, *Isaria javanica* CBS 134.22, in all analyses as well as other isolates collected from the LRGV (ARSEF; Humber et al. 2009) or from other geographical localities (Table 1). A commercialized isolate of *Isaria fumosorosea* (PFR-97 = ARSEF 3682) was obtained from ARSEF; all mention of *I. fumosorosea* refers to this isolate. To determine whether ARSEF 7028 could be recovered from LRGV fields, eight ARSEF fungal isolates previously collected from the same area were included in this study (Table 1).

2.2. Morphological studies

For general morphological examinations, ARSEF 7028 was reared on SDAY at 26 °C (±1 °C) for up to 21 days (Table 1). Slide

Table 1 – List of *Isaria* isolates used in this study. Fungal names in parentheses are previous identifications as species of *Paecilomyces* or as previously identified in the ARSEF culture collection.

Fungal isolates[a]	Insect host (order)	Geographic origin
***Isaria fumosorosea* Wize (= *Paecilomyces fumosoroseus* (Wize) Brown & Smith)**		
ARSEF 3682 (deposited as PFR-97)	Aphididae (Hemiptera)	Apopka, Florida, USA
ARSEF 322 (*I. javanica*)	*Litodactylus leucogaster* (Coleoptera)	Gainesville, Florida, USA
I. javanica (Friederichs & Bally) Samson & Hywel-Jones (= P. javanicus (Frieder & Bally) AHS Brown & G Smith)		
CBS 134.22 (ex-type)	*Hypothemus hampei* (Coleoptera)	Java, Indonesia
CBS 174.25	*Brachartona* sp. (Lepidoptera)	Unknown
ARSEF 3573 (*I. farinosa*)	*Bemisia tabaci*[b] (Hemiptera)	McAllen, Texas, USA
ARSEF 3577 (*I. fumosorosea*)	*B. tabaci*[b] (Hemiptera)	McAllen, Texas, USA
ARSEF 3593 (*I. farinosa*)	*B. tabaci*[b] (Hemiptera)	McAllen, Texas, USA
ARSEF 3776 (Paecilomyces sp.)	*B. tabaci*[b] (Hemiptera)	Weslaco, Texas, USA
ARSEF 3778 (Paecilomyces sp.)	*B. tabaci*[b] (Hemiptera)	Mission, Texas, USA
ARSEF 5259 (*I. fumosorosea*)	*B. tabaci*[b] (Hemiptera)	Santa Maria, Texas, USA
ARSEF 5321 (*I. fumosorosea*)	*B. tabaci*[b] (Hemiptera)	Weslaco, TX, USA
ARSEF 5806 (Paecilomyces sp.)	*Panoquina* sp. (Lepidoptera)	Colombia
Isaria poprawskii sp. nov.		
ARSEF 5330 (*I. fumosorosea*)	*B. tabaci*[b] (Hemiptera)	Harlingen, Texas, USA
ARSEF 7028 (ex-type)	*B. tabaci* biotype 'B' (Hemiptera)	Weslaco, Texas, USA
Isaria tenuipes Peck [= P. tenuipes (Peck) Samson]		
CBS 994.73[c]	(Lepidoptera)	Tafo, Ghana
CBS 995.73 (in CBS as *I. javanica*)	(Lepidoptera)	Tafo, Ghana

a ARSEF = USDA-ARS Collection of Entomopathogenic Fungal Cultures; Ithaca, NY. CBS = Centraalbureau voor Schimmelcultures; Utrecht, Netherlands.

b Host possibly *B. tabaci* biotype 'B', which was segregated from *B. tabaci* after the collection of these strains.

c Previously identified as *P. javanicus* (Samson 1974) but reidentified as *I. tenuipes* (Luangsa-ard et al., 2005) based on partial sequence of the TUB2 gene.

mounts in lactophenol-cotton blue were examined with an Olympus® BX50 microscope (Olympus America Inc., Center Valley, PA, USA) fitted for differential interference contrast. Photomicrographs were taken with an Olympus® PM-30 photomicrographic digital camera (Table 1).

For more detailed morphological comparisons of the conidia and conidiogenous cells of the fungi, cultures were grown on quarter-strength SDAY (SDAY/4; 2.5 g/L peptone, 10 g/L dextrose, 2.5 g/L yeast extract, 15 g/L agar), incubated at 16 °C and a 12 h:12 h light:dark cycle for 5–7 d until sporulation was evident. Small quantities of mycelia and spores were mounted in 50% acetic acid/cotton blue (or, as noted, in lactic acid cotton blue), and examined with phase contrast optics on an Olympus BX51 microscope (Olympus America Inc.) at 1000×. Images were photographed digitally with a Jenoptik ProgRes CFscan camera (Jenoptik Laser, Optik, Systeme GmbH; Jena, Germany) at 4080 × 3072 pixels. A total of 30 independent measurements of conidia and conidiogenous cells were made for each isolate from prints (adjusted to 2100 × 1581 pixels) of these images (Table 2). The measurement data were analyzed using SAS Software (SAS Institute 2004), PROC GLIMMIX, with a log-normal response function, variance components covariance structure, and Tukey-adjusted comparisons of means among the eight isolates, or a single comparison between pooled measurements of two isolates assigned via molecular methods to the clade including ARSEF 7028 versus pooled measurements across six isolates assigned to the *javanica* clade (Table 2).

To compare growth rates and colony coloration between ARSEF 7028, *I. javanica* CBS 134.22 and *I. fumosorosea*, 5 mm diameter disks of unsporulating mycelia from five day-old cultures were placed upside down in the center of SDAY plates and incubated at 26 °C in a 12:12 h fluorescent light:dark cycle. Four replicate plates were prepared for each fungus. Surface radial growth was recorded using two cardinal diameters previously drawn on the bottom of each dish, and the growth average (mm-diam.) was estimated and/or the colony color was determined at 7, 9, 11, 14 and 21 d (Table 3; Fig. 1A).

2.3. *Molecular, phylogenetic and identification analyses*

2.3.1. *DNA isolation*

For extraction of total genomic DNA, fungi were grown on SDAY at 26 °C for 4–7 d under a 14:10 h fluorescent light:dark cycle. DNA isolation was performed by the method of Murray et al. (2005). Twenty (±5) mg of mycelia were vortexed together with approximately 100 mg of 0.5 mm diameter glass beads (BioSpec Products, Inc; Bartlesville, OK, USA), and 200 μl STE buffer (Aronstein et al. 1995) in a 1.5 ml microfuge tube for 5 min. This mixture was incubated at 95 °C for 5 min and vortexed again 1 min. Samples were then centrifuged at 16,000 *g* for 2 min and the supernatant (≥100 μl) containing the template DNA was collected for use in PCR.

2.3.2. *Amplification and DNA sequencing of the β-tubulin (TUB2) partial gene*

The TUB2 gene from the fungal isolates was amplified as described in Luangsa-ard et al. (2005), utilizing the Bt2a and Bt2b primers (Glass and Donaldson 1995). Reactions were performed in a final volume of 20 μl with the following components: 1X PCR buffer [50 mM KCl, 20 mM Tris–HCl (pH 8.4), 2.0 mM $MgCl_2$, and 0.01% gelatin], 0.25 mM deoxynucleotide triphosphates, 0.25 μM primer set, 1.0 μl of stock genomic DNA and 2.0 Units of *Taq* DNA Polymerase (New England Biolabs; Beverly, MA USA). The cycling parameters were as follows: 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 30 s. For subcloning purposes, a final extension step was performed for 15 min. Amplification products were subcloned with the TOPO Cloning Kit (Invitrogen Life Technologies; Carlsbad, CA USA). Plasmid minipreps were prepared by the QIAprep Spin Miniprep Kit (Qiagen Inc; Valencia, CA USA), and sequencing in both directions was performed by Davis Sequencing (Davis, CA USA) as previously described (de León et al. 2006, 2008; Triapitsyn et al. 2008). The forward primer nucleotide sequence (24-bp) was trimmed from each of our selected fungal isolates in order to perform the phylogenetic analysis since the downloaded GenBank sequences did not contain this sequence. The TUB2 fragment sizes are therefore reported in the current study without the forward primer sequence. GenBank accession numbers for TUB2 fungal sequences of our selected fungal isolates are shown in Table 4.

2.3.3. *DNA sequence analysis*

The DNA sequencing software program Sequencher (Gene Codes Corp.; Ann Arbor, MI, USA) was utilized to process the raw sequences as described in previous works (de León et al. 2006, 2008; Triapitsyn et al. 2008). The alignment program ClustalX (Thompson et al. 1997) and the phylogenetic program PAUP version 4.0b10 for Macintosh (Swofford 2002) were utilized for alignment, bootstrapping (as percentage of 1000 replications) (Felsenstein 1985), and reconstruction of trees. Phylogenetic trees were constructed using both distance and maximum parsimony methods. For the distance analysis, the neighbor-joining algorithmic method was performed utilizing the uncorrected 'p' genetic distance parameter (Saitou and Nei 1987). For the parsimony analysis, heuristic searches for the most parsimonious tree were conducted using closest stepwise addition and the branch-swapping algorithm by tree bisection-reconnection. The bootstrap method (1000 replications) with fast-heuristic search was conducted for this analysis. In all analyses, all characters were unordered and unweighted; gaps were treated as missing data, and constraints were not enforced. The β-tubulin standard or reference sequences for related fungal species from this study were downloaded from GenBank and the accession numbers are shown in Table 4. A BLASTN search (Zhang et al. 2000) was used to compare TUB2 sequences generated in this study to TUB2 sequences obtained from GenBank.

2.3.4. *PCR-RFLP assays*

For polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays, fungal DNA was extracted utilizing the DNeasy® Plant Mini Kit (Qiagen Inc, Valencia, CA USA). About 100 mg of fungal mycelium were mixed with approximately 500 mg of 0.5 mm glass beads to disrupt the fungi in the kit's lysis buffer (400 μl). The mixtures were vortexed (Vortex Genie 2 with attachment for 1.5 ml centrifuge tubes; Scientific Industries; Bohemia, NY USA) for a total of 30 min,

Table 2 – Measurements (in μm ± SD) of conidia and conidiogenous cells of *Isaria poprawskii* sp. nov. (ARSEF 7028 and 5330) and six other *Isaria* isolates collected from infected whiteflies (*Bemisia* spp) in fields of the Lower Rio Grande Valley of Texas, USA and cultured on SDAY/4 medium. Fungi were mounted in 50% acetic acid cotton blue and measured from photographs taken at 1000×.

Isaria species ARSEF no.	Conidia			Conidiogenous cells			Collection data
	Length[a]	Width	L/W	Length	Width	L/W	Collector Host plant; city, date
poprawskii 7028	3.90 ± 0.43 a	1.60 ± 0.17 ab	2.46 ± 0.36 ab	5.88 ± 0.86 b	2.37 ± 0.25 ab	2.51 ± 0.46 b	HE Cabanillas Eggplant; Weslaco, Sept 2001
poprawskii 5330	3.85 ± 0.49 ab	1.62 ± 0.16 a	2.39 ± 0.34 ab	6.84 ± 1.29 a	2.33 ± 0.27 abc	2.97 ± 0.63 a	TJ Poprawski Okra; Harlingen, Sept 1994
javanica 3573	3.54 ± 0.52 bc	1.54 ± 0.15 ab	2.31 ± 0.36 ab	4.81 ± 0.68 d	2.15 ± 0.29 c	2.25 ± 0.34 bc	TJ Poprawski Cabbage; McAllen, Feb 1992
javanica 5259	3.42 ± 0.38 c	1.53 ± 0.13 ab	2.25 ± 0.33 b	4.72 ± 0.81 de	2.46 ± 0.26 a	1.93 ± 0.33 d	SP Wraight Broccoli; Santa Maria, Nov 1992
javanica 5321	3.69 ± 0.33 abc	1.51 ± 0.12 b	2.46 ± 0.22 ab	5.64 ± 0.68 bc	2.38 ± 0.31 ab	2.40 ± 0.38 bc	SP Wraight Broccoli; Weslaco, Nov 1992
javanica 3577	3.62 ± 0.35 abc	1.58 ± 0.12 ab	2.32 ± 0.31 ab	5.04 ± 0.64 d	2.37 ± 0.29 ab	2.15 ± 0.37 cd	TJ Poprawski Cabbage; McAllen, Feb 1992
javanica 3776	3.79 ± 0.34 abc	1.52 ± 0.13 ab	2.50 ± 0.31 a	4.93 ± 0.47 d	2.23 ± 0.31 bc	2.24 ± 0.32 bc	SP Wraight Poinsettia; Weslaco, Aug 1992
javanica 3778	3.61 ± 0.33 abc	1.53 ± 0.12 ab	2.37 ± 0.26 ab	5.13 ± 0.74 cd	2.27 ± 0.31 abc	2.30 ± 0.44 bc	SP Wraight (Not known); Mission (TX) March 1992
F, *P*[b]	$F = 4.45$, $P = 0.0001$	$F = 2.47$, $P = 0.0182$	$F = 2.33$, $P = 0.0255$	$F = 23.3$, $P < 0.0001$	$F = 3.85$, $P = 0.0006$	$F = 15.0$, $P < 0.0001$	
I. poprawskii clade	3.87 ± 0.46 a	1.61 ± 0.16 a	2.43 ± 0.35 a	6.36 ± 1.19 a	2.35 ± 0.26 a	2.74 ± 0.59 a	
I. javanica clade	3.61 ± 0.39 b	1.54 ± 0.13 b	2.37 ± 0.31 a	5.05 ± 0.73 b	2.31 ± 0.31 a	2.21 ± 0.39 b	
F, *P*[c]	$F = 15.0$, $P = 0.0001$	$F = 12.8$, $P = 0.0004$	$F = 1.23$, $P = 0.2676$	$F = 98.6$, $P < 0.0001$	$F = 1.05$, $P = 0.3062$	$F = 55.9$, $P < 0.0001$	

a Mean values ($n = 30$ for individual isolates; $n = 60$ for the *I. poprawskii* clade and $n = 180$ for the *I. javanica* clade) within a column followed by the same letter are not significantly different.
b Degrees of freedom = 7, 232 for tests of variation among isolates.
c Degrees of freedom = 1, 238 for tests of variation between clades.

Table 3 – Morphological features of *Isaria poprawskii* on SDAY at 26 °C and its comparison with characteristics of related *Isaria* species.

Species	Conidiallength (μm)	Conidial shape	Color of conidia/colony	Conidiophore and other key characters
Isaria poprawskii	2.9–4.6	Cylindrical to fusiform	Hyaline/colony white to yellow with a grayish-violet in the center to brownish-gray	Smooth-walled, colorless; distinct tan to yellowish-brown synnemata
I. javanica[a]	2.1–4.3	Cylindrical to fusiform	Hyaline/colony white to yellow to grayish-violet; reverse uncolored to yellow	Smooth-walled, colorless; few or no synnemata
I. tenuipes[b]	3.0–7.5	Cylindrical, mostly curved	Hyaline, 1(-2)-celled, colony white-cream, buff near vinaceous buff or pinkish buff; reverse cream to yellow	Smooth-walled, hyaline; conspicuous synnemata
I. fumosorosea[b,c]	3.0–4.0	Cylindrical to fusiform, long ovoid	Hyaline to pink/colony rosy-tan to smoky-pink (or gray)	Smooth-walled, hyaline; pinkish-tan synnemata
I. farinosa[b,c]	2.0–3.0	Ellipsoidal to fusiform, short fusoid to lemon-shaped	Hyaline/colony white, bright yellow to cream; reverse cream to yellow	Smooth-walled, hyaline; yellow synnemata

a Brown and Smith (1957).
b Samson (1974).
c Humber (1997).

during which time the mixtures were incubated on ice for 3 min at 10 min internals. DNA was eluted from the kit columns with 50 μl of elution buffer. Amplification of TUB2 was performed as described above; except that the concentration of the primer set (Bt2a/2b) was decreased (0.1875 μM) to reduce the formation of primer-dimers. The amplification products were gel purified using the Wizard® SV and PCR Clean-Up System (Promega Corporation; Madison, WI, USA). The amount of amplification product was estimated based on the DNA Mass Ladder (Invitrogen Life Technologies). In a total volume of 20 μl, 60 ηg of gel-purified amplification product were mixed with 1X bovine serum albumin (2 μg), 1X NEBuffer #1 (for *HpyCH41V*; *I. poprawskii*-specific clade) or NEBuffer #4 (for XmnI; *I. javanica*-specific clade), and 5 Units of the appropriate restriction enzyme (New England BioLabs). The reactions were incubated for 2 and 2.5 h at 60 °C and 37 °C for Hpy*CH41V* and Xmn*I*, respectively. The digests were loaded onto 3% agarose 1X TBE (90 mM Tris-borate, 2 mM EDTA) gels in the presence of 0.2 μg/ml ethidium bromide. The digests were loaded onto 3% agarose 1X TBE (90 mM Tris-borate, 2 mM EDTA) gels in the presence of 0.2 μg/ml ethidium bromide and submitted to electrophoresis at 110 V for 4 and 8 h for Hpy*CH4*I*V* and Xmn*I*, respectively. The TUB2 amplification products were of original size, and the forward primer sequences (24-bp) were not removed for these assays. The original product size was 356-bp for each *I. javanica* and *I. poprawskii*.

2.3.5. *ISSR-PCR DNA fingerprinting*

Inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) amplifications were performed as described in previous works (de León et al. 2006, 2008; de León and Morgan 2007; Triapitsyn et al. 2008). The reactions were performed with the 5′-anchored primer HVH $(TG)_7T$ (Zietkiewicz et al. 1994) (notations: H = A/T/C, and V = G/C/A). Briefly, reactions were performed in a final volume of 20 μl with the following components: 1X PCR buffer (50 mM KCl, 20 mM Tris–HCl [pH 8.4], 1.5 mM $MgCl_2$, and 0.01% gelatin), 0.25 mM deoxynucleotide triphosphates, 0.25 μM ISSR primer, 1.0 μl of stock genomic DNA and 0.05 U/μl *Taq* DNA Polymerase (New England Biolabs; Beverly, MA, USA). The cycling parameters were as follows: 1 cycle at 94 °C for 2 min followed by 45 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min. Amplification products were loaded onto 2% agarose gels and separated by electrophoresis in 1X TBE buffer. The molecular weights of the DNA bands were calculated with Quantity One® Software v. 4.6.3 utilizing the Point to Point Molecular Weight Calculation Method (Bio-Rad; Hercules, CA, USA) based on the 1.0 kb DNA ladder. Faint bands were excluded from the analyses.

3. Results

3.1. *Taxonomy*

Isaria poprawskii Cabanillas, de León, Humber, Murray & Jones, sp. nov.

Figs. 1–6.

MycoBank no.: MB 518019.

Isaria poprawskii has a yellow to taupe conidial mass, colored synnemata and forms a separate phylogenetic clade, which differentiates from *I. javanica* and other related *Isaria* species.

Holotype: CUP 67573 (Plant Pathology Herbarium, Cornell University), infected larvae and adults of *B. tabaci* on cotton leaf, *Gossypium hirsutum* L., collected from its original place of discovery and the fungus produced the same characteristic structures (29 Oct 2002).

Paratype: CUP 67574, infected larvae and adults of *B. tabaci* biotype B on a tomato leaf, *Solanum lycopersicum* L., collected from its original place of discovery and the fungus produced the same characteristic structures (02 July 2004).

Ex-type culture: ARSEF 7028, derived from holotype collection.

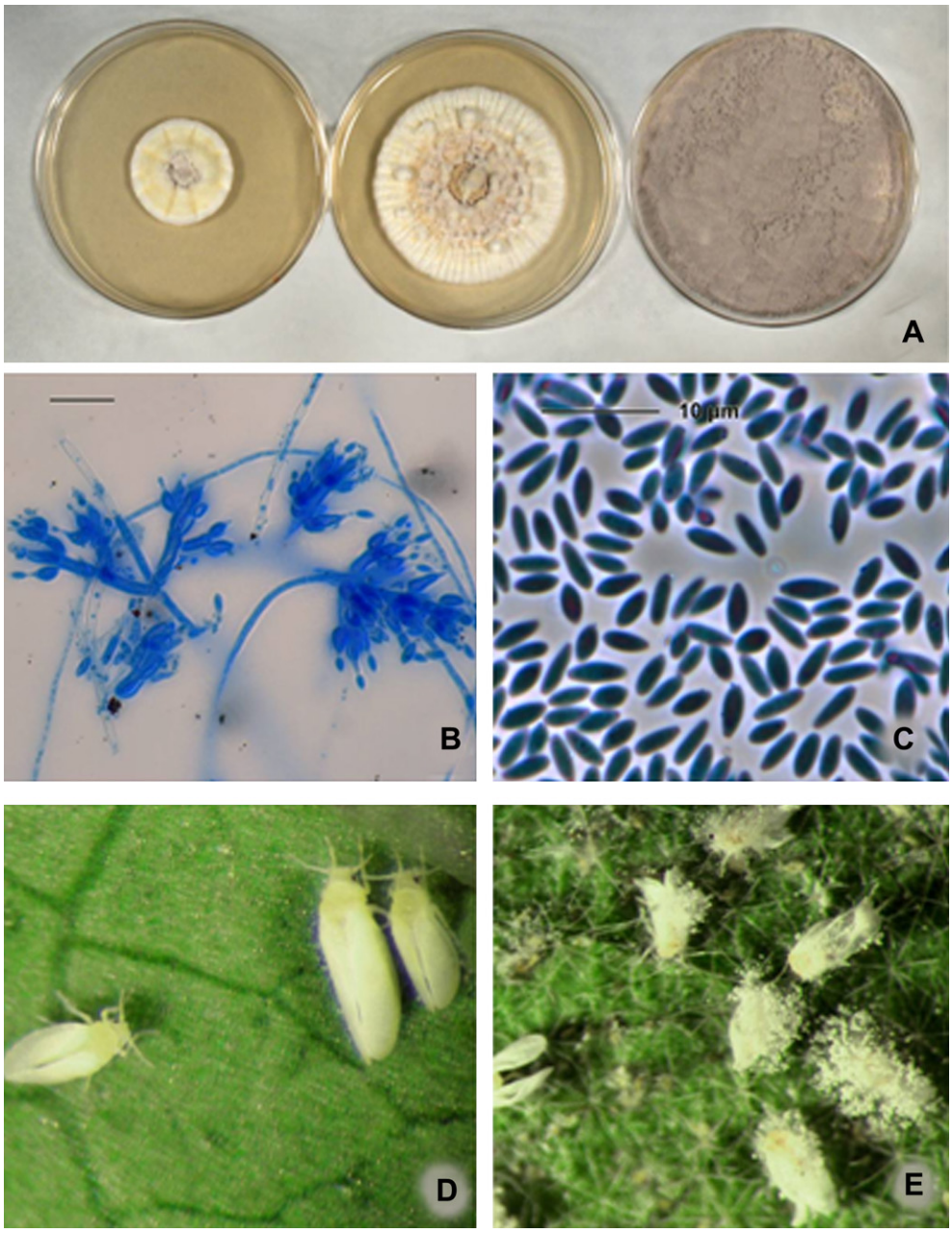

Fig. 1 – *Isaria poprawskii*. A: Growth on SDAY at 26 °C at 7, 14, and 21 d (left to right, respectively). Note profuse sporulation at 21 days. B: Conidiophores, conidiogenous cells in whorls. C: Individual stained conidia. D: Healthy adult whiteflies. E: Fungus-infected adult whiteflies. Note individual conidiophores and profuse sporulation of *I. poprawskii* on cadavers. *Bars* B, C 10 μm.

Etymology: *poprawskii*, in memory of Tadeusz J. Poprawskii, an important colleague and scientific researcher who worked on the affected target hosts in the fungus' type locality, and a tireless champion worldwide for insect pathology and microbial control.

Teleomorph: Unknown.

Young colonies (SDAY at 26 °C) with a white basal felt becoming light yellow to dark yellow with grayish-violet in the center, with conidial mass taupe or brownish-gray (Fig. 1A); older colonies grayish-violet with yellow patches; reverse distinct yellow to brownish-gray. The underside (reverses) of colonies showed a distinct yellow to brownish-gray, wheel-like growth with spokes radiating from the center. Vegetative hyphae smooth, septate, hyaline, 0.5–2.2 μm diam. Conidiophores erect and mononematous or often synnematous, or prostrate with solitary phialides borne directly; erect conidiophores fertile distally, usually bearing verticillate clusters of branches each bearing clusters of two or more conidiogenous cells or with verticillate clusters of conidiogenous cells arising directly on conidiophore apices (Figs. 1B, 2A); synnemata (height × width; $n = 20$), 5 (2.0–7.0) × 6 (4.0–8.0) mm, simple or dichotomously or

Table 4 – GenBank accession numbers for β-tubulin-like gene (TUB2) sequences. (A). The standard or reference DNA sequences from Luangsa-ard et al. (2005) were downloaded from GenBank. (B). Sequences originated from this study. (C). Outgroups included in this study, also from Luangsa-ard et al. (2005).

Fungal isolate	Accession number
(A) Luangsa-ard et al. (2005)	
Isaria japonica BCC 2808	AY624241
I. japonica BCC 2821	AY624242
I. amoeneseus CBS 107.73	AY624207
I. amoeneseus 729.73	AY624208
I. cateniannulatus CBS 152.83	AY624211
I. cateniobliquus CBS 153.83	AY624212
I. coleopterorum CBS 102.73	AY624215
I. coleopterorum CBS 110.73	AY624216
I. farinosus CBS 541.81	AY624218
I. farinosus CBS 262.58	AY624217
I. fumosoroseus CBS 107.10	AY624222
I. fumosoroseus CBS 244.31	AY624220
I. fumosoroseus CBS 375.70	AY624221
I. ghanensis CBS 105.73	AY624223
I. javanicus CBS 134.22 ex-type	AY624224
I. tenuipes CBS 994.73	AY624225[a]
I. tenuipes ARSEF 5135	AY624234
I. tenuipes CBS 997.73	AY624233
(B) Current study	
I. poprawskii ARSEF 7028	EF585303
I. javanica CBS 134.22	EF585304
I. fumosorosea ARSEF 322	EF585301
I. javanica CBS 174.25	EF585302
I. tenuipes CBS 995.73	EF585305
I. javanica ARSEF 5806	EF585306
I. fumosorosea PFR-97	EF585300
I. tenuipes CBS 994.73	EF585307
I. javanica ARSEF 3577	FJ899914
I. javanica ARSEF 5259	FJ899918
I. javanica ARSEF 5321	FJ899919
I. poprawskii ARSEF 5330	FJ899920
I. javanica ARSEF 3573	FJ899913
I. javanica ARSEF 3593	FJ899915
I. javanica ARSEF 3776	FJ899916
I. javanica ARSEF 3778	FJ899917
(C) Outgroups	
Paecilomyces marquandii CBS 182.27	AY624229
Metarhizium flavoviride BBC 7672	AY624248
Nomuraea rileyi CBS 806.71	AY624250
Mariannaea camptospora CBS 209.73	AY624245
Nectria mariannaeae CBS 132.41	AY624243

a Previously referred to as *P. javanica* (Samson 1974).

irregularly branched (Fig. 2B) with sterile bases and fertile apices becoming increasingly colored tan to yellow-brown with age and accumulation of conidia. Conidiogenous cells with cylindrical to bottle-shaped base tapering to thin, distinct neck, overall dimensions 5.4–5.6 × 2.4–2.6 μm (Fig. 2A); conidiogenous cells on host insects with bases more inflated or subglobose. Conidia in chains, smooth-surfaced, hyaline, one-celled (Fig. 1B, C), from host 3.5 (3.0–4.0) × 1.5 (1.2–1.8) μm ($n = 20$); on SDAY, cylindrical to fusiform, 3.9 (2.9–4.6) × 1.6 (1.4–2.1) μm (Fig. 1C; Tables 2 and 3). Chlamydospores unknown. On insects, the fungus usually produced a white powdery felt, with numerous conidiophores; usually inflated with globose phialides (Figs. 1E, 2A).

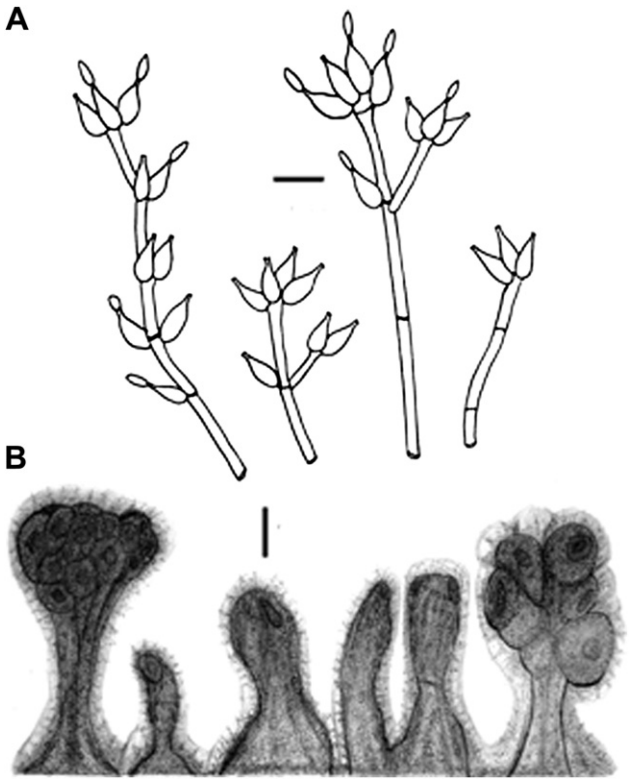

Fig. 2 – *Isaria poprawskii*. A: Conidiophores and conidiogenous cells flask-like (with swollen base and a distinct neck), borne singly or clustered on swollen vesicles. B: Synnemata rising from culture on SDAY. Apex of synnemata bear clumps of conidia borne on irregular, somewhat concentric lobes. *Bars* A: 10 μm; B: 1 mm.

3.2. *Morphological studies*

The data on measurements of conidia and conidiogenous cells of *I. poprawskii* and six other *Isaria* isolates collected from whiteflies in fields of the Lower Rio Grande Valley of Texas are summarized in Table 2. The morphological features of *I. poprawskii* and its comparison with characteristics of related species are summarized in Table 3 and illustrated in Figs. 1 and 2.

Growth and colony coloration (SDAY, 26 °C) between *I. poprawskii*, *I. javanica* ex-type and *I. fumosorosea* were different. For example, *I. poprawskii* showed colored colonies, initially white to light yellow to a darker yellow with a grayish-violet center, finally to a taupe or a brownish-gray of a mature conidial mass (Fig. 1A). The undersides (reverses) of colonies showed a distinct yellow to brownish-gray, wheel-like growth with spokes radiating from the center. In contrast, colonies of *I. fumosorosea* developed the characteristic rosy-tan to grayish-pink pigmentation due to the pigmented conidial mass, while the *I. javanica* ex-type culture was white on SDAY. However on SDAY/4, all of the *I. javanica* isolates became colored with the same rosy-tan color usually seen for *I. fumosorosea* (Table 2).

The morphological similarities between *I. poprawskii* and *I. javanica* did not allow for unambiguous morphological differentiation between these two species, although measurements

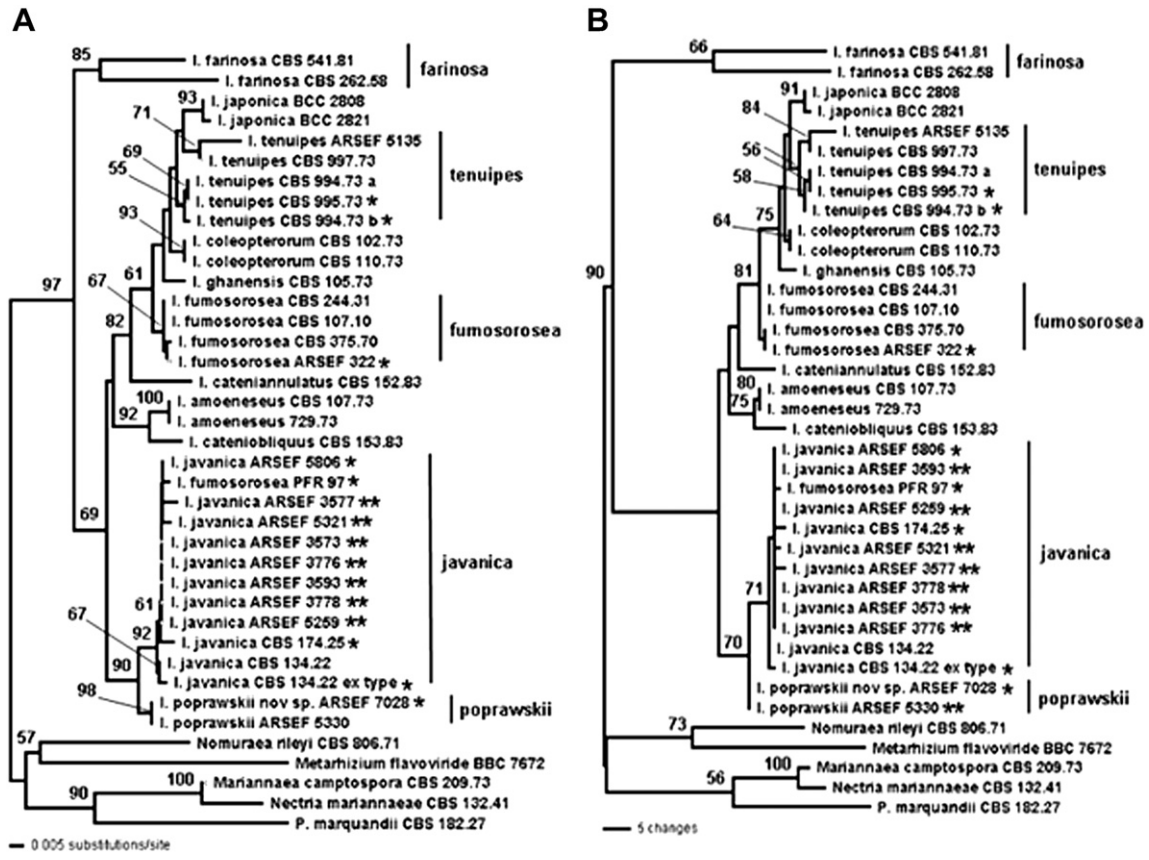

Fig. 3 – Phylogenetic relationships among the selected fungal isolates along with the standard fungal species [Luangsa-ard et al. (2005)] inferred from TUB2 sequence data. A: Neighbor-joining distance analysis. B: Parsimony analysis: bootstrap 50% majority-rule consenses tree based on 88 parsimony-informative characters. Tree length = 273 steps; consistency index = 0.696; and retention index = 0.814. Bootstrap support values (as a percentage of 1000 replications) are displayed at the nodes. The asterisks (*) after the names are the isolates sequenced in the current study. The field-collected strains [two asterisks (**)] are described in Table 1.

pooled across two *I. poprawskii* isolates indicated that *I. poprawskii* had longer conidia and conidiogenous cells than did six pooled *I. javanica* isolates (Table 2).

3.3. *Molecular phylogenetic analyses and identification*

3.3.1. *Phylogenetic relationships of* I. poprawskii *and other selected* Isaria *isolates inferred from β-tubulin-like gene sequences*

To confirm the species status of *I. poprawskii* and to verify the classifications of selected *Isaria* isolates resembling this new species, we compared TUB2 sequence data generated from a series of field-collected *Isaria* isolates from the LRGV (Table 1) to a range of other ARSEF isolates of morphologically similar and distinct *Isaria* species, and also considered the GenBank standard sequences for β-tubulin that were studied by Luangsa-ard et al. (2005) (Table 4). A total of 34 in-group isolates were analyzed (Table 4A, B) and rooted using out-group isolates shown in Table 4C. The 16 isolates for which we generated TUB2 sequences (Table 4B) yielded DNA fragments of 331 to 332-bp. Our sequencing of TUB2 (GenBank accession no. EF585304; Table 4B) from *I. javanica* CBS 134.22 (the ex-type isolate) differed by 3-bp in size and 5-bp overall from the ex-type sequence reported by Luangsa-ard et al. (2005). The same size DNA fragment (332-bp) was observed in TUB2 for both *I. poprawskii* (ARSEF 7028) and *I. javanica* CBS 134.22 (GenBank EF585304), although the two species differ consistently at four base pair positions within this fragment (Table 6). A 3-bp size difference and an overall 9-bp difference were seen in TUB2 between *I. poprawskii* and the *I. javanica* ex-type isolate (AY624224) reported in Luangsa-ard et al. (2005). A BLASTN search (Zhang et al. 2000), using the *I. poprawskii* ARSEF 7028 TUB2 sequence as the query, showed the *I. javanica* ex-type strain (AY624224) sequence as the one most similar to *I. poprawskii* (Total score = 575 and *E*-value = 5e-161). The sequences of these two species differed by 3% (Max Identity = 97%). This analysis also confirmed that the fungus described here belongs in the Cordycipitaceae (Hypocreales) and is, therefore, an *Isaria* species. The molecular data allowed correction of the identifications of several isolates whose TUB2 sequences are congruent with the ex-type isolate of *I. javanica*; the earlier identifications of these isolates are given in Table 1.

A neighbor-joining distance tree revealed that all of the 16 sequenced fungal isolates clustered within four clades representing three described species (*I. javanica*, *Isaria tenuipes*,

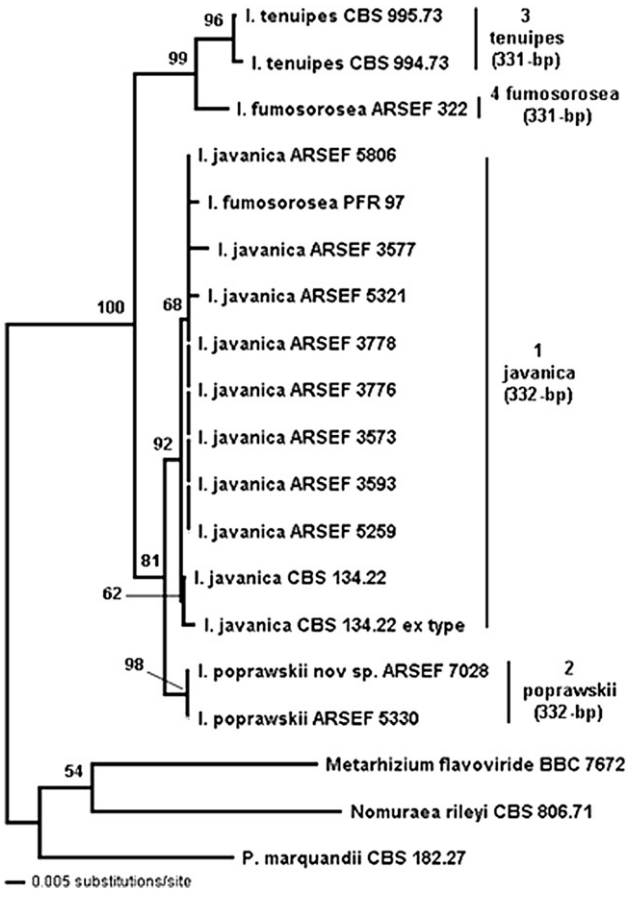

Fig. 4 – Neighbor-joining distance phylogram inferred from TUB2 sequence data of 16 selected fungal isolates analyzed separately. Bootstrap support values (as a percentage of 1000 replications) are displayed at the nodes. Clade labels are listed in Table 6.

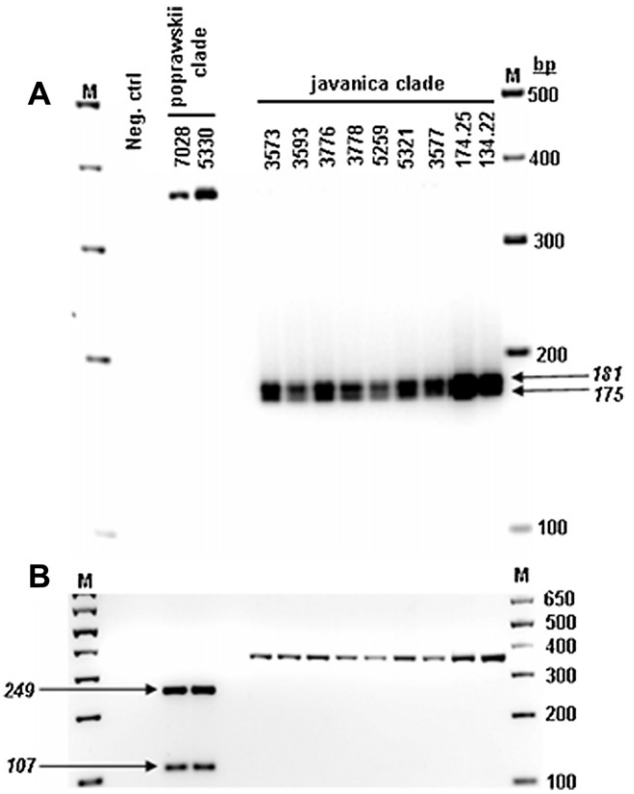

Fig. 5 – Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays using the β-tubulin (TUB2) partial gene of fungal isolates belonging to the *I. poprawskii* and *I. javanica* clades. Refer to Table 6 and Fig. 4 for assignments. A: Digestion with the *javanica*-specific restriction enzyme XnmI produced two DNA fragments: 175- and 181-bp. B: Digestion with the *poprawskii*-specific enzyme HypCH41V generated two band fragments: 107- and 249-bp. For the *poprawskii* clade: *I. poprawskii* ARSEF 7028 and ARSEF 5330; for the *javanica* clade: *I. javanica* ARSEF 3573, ARSEF 3593, ARSEF 3776, ARSEF 3778, ARSEF 5359, ARSEF 5321, ARSEF 3577, CBS 174.25, and CBS 134.22 ex-type. Neg. ctrl, negative control (no template DNA). M, 1.0 kb DNA ladder.

I. fumosorosea) and the new species, *I. poprawskii* (Fig. 3A, B). *I. poprawskii* formed its own unique clade with very strong support (98%), demonstrating that it is a new species (Fig. 3A). Interestingly, *I. fumosorosea* ARSEF 5330, field-collected in the LRGV in 1994, clustered with the new species, but the standard *fumosorosea* clade falls elsewhere, indicating that *I. fumosorosea* ARSEF 5330 (Table 1) is also *I. poprawskii* (Fig. 3A). In fact, both of these isolates have the exact (100%) same TUB2 sequence. This finding demonstrates that *I. poprawskii* is present and persistent in the fields of the LRGV. However, most of the fungal isolates collected from the LRGV clustered within the *javanica* clade (Fig. 3A). As further evidence of isolate misidentification, the isolate *I. javanica* CBS 995.73 clustered within the *tenuipes* clade. Finally, isolate *I. javanica* ARSEF 322 (Gainesville, Florida, USA) clustered within the *fumosorosea* clade. A single most parsimonious tree (Fig. 3B) showed a very similar topology to that of the neighbor-joining tree.

The 16 fungal isolates selected for separate analyses clustered within the same four clades identified in the general phylogenetic analyses (Fig. 3A, B) when they were analyzed separately. In a neighbor-joining distance tree (Fig. 4), *I. poprawskii* again clustered into a unique clade closely related to the *javanica* clade. The two clades received strong support [98% for clade 2 (*poprawskii* clade) and 92% for clade 1 (*javanica* clade)]. A single most parsimonious tree [tree length = 146 steps; consistency index (CI) = 0.842; and retention index (RI) = 0.783] showed the same tree topology as the neighbor-joining tree, with 72% bootstrap support for the *poprawskii* clade (not shown). Further support for this close relationship was seen in the size of the TUB2 fragments (Fig. 5). All individuals within clades 1 and 2 were observed with a TUB2 size fragment of 332-bp. Clades 3 (*tenuipes* clade) and 4 (*fumosorosea* clade) were observed with a TUB2 size fragment of 331-bp each.

Levels of genetic divergence in the TUB2 fragment among the 16 selected fungal isolates analyzed separately were determined by calculating the pairwise estimates for genetic distance (Table 5). The percentage sequence divergence (%D) shows a variance of (0.0–1.2%) among the 11 fungal isolates within clade 1 (*javanica* clade). However this intra-clade

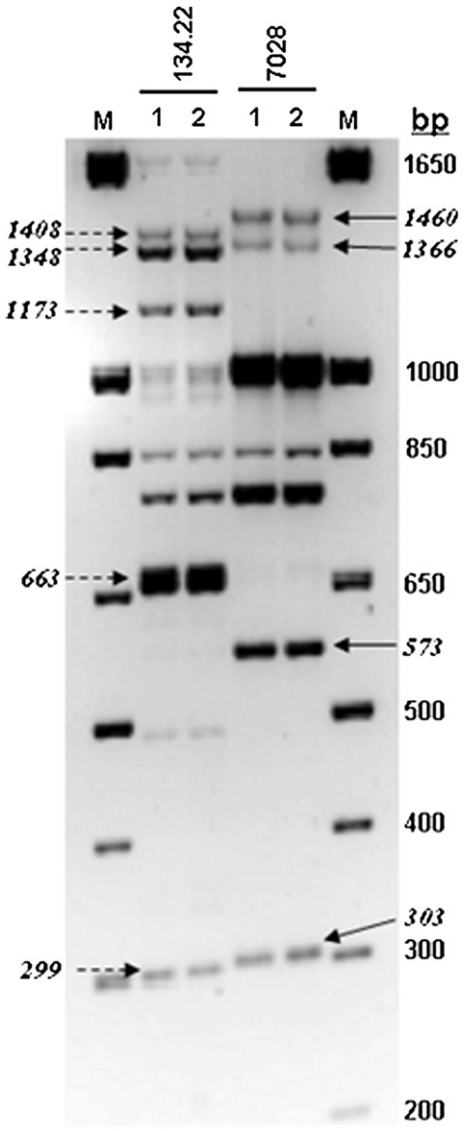

Fig. 6 – ISSR-PCR DNA fingerprinting of the ex-type cultures of *Isaria javanica* CBS 134.22 and *I. poprawskii* ARSEF 7028, respectively. Broken arrows represent *I. javanica* CBS 134.22-specific DNA fragments, and solid arrows represent *I. poprawskii* type ARSEF 7028-specific fragments. Molecular weights of species-specific bands are shown in italics. M, 1.0 kb DNA ladder.

variance was smaller than the inter-clade variance (1.5–2.1%) between clades 1 (*javanica* clade) and 2 (*poprawskii* clade). These results are in agreement with the phylograms shown in Figs. 3 and 4. Compared to clade 1, clades 3 and 4 had greater genetic distances, 4.7–6.1 and 4.7–5.4%, respectively. Since several field-collected fungal isolates were included in the analysis, a pattern of clade-specific or diagnostic nucleotides were identified that allowed the discrimination of individual isolates or species (Table 6). For example, at nucleotide position #84, all the isolates within the *poprawskii* clade contained a cytosine (C), whereas, all the isolates within the *javanica* clade contained a thymine (T). In all, four diagnostic nucleotides were uncovered between the two clades.

Table 5 – Levels of genetic divergence (%D) (range) among the 16 selected fungal isolates analyzed separately. Refer to Fig. 4 for clade assignments.

Fungal isolate assigned clades	Clade 1[a] (*javanica* clade)	Clade 2 (*poprawskii* clade)	Clade 3 (*tenuipes* clade)	Clade 4 (*fumosorosea* clade)
Clade 1	0.0–1.2			
Clade 2	1.5–2.1	0.0–0.0		
Clade 3	4.7–6.1	4.7–5.1	0.0–0.3	
Clade 4	4.7–5.4	4.4–4.4	2.2–2.5	0.0–0.0

a The alignment program ClustalV (Higgins et al. 1994) from DNAStar (DNAStar Inc.; Madison, WI, USA) was used to calculate percentage divergence, measured as a function of genetic distance.

3.3.2. *Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of specimens from the I.* javanica *and I.* poprawskii *clades*

A restriction map analysis of the TUB2 sequences of all isolates within the *poprawskii* and *javanica* clades was performed utilizing the program NEBcutter V2.0 from New England BioLabs (http://tools.neb.com/NEBcutter2/index.php). The results demonstrated that the clade-specific nucleotide differences in TUB2 sequences led to changes in restriction enzyme sites between the two clades. The following restriction enzymes were specific to *I. poprawskii*: Bsp1286*I*, HpyCH4*IV*, BstN*I*, and PspG*I*, and the following were specific to *I. javanica*: XmnI, Tsp509*I*, and ApoI. Two restriction enzymes (*I. poprawskii*-specific HpyCH4*IV* and *I. javanica*-specific XmnI) were selected to discriminate the *Isaria* isolates

Table 6 – Clade-specific or diagnostic nucleotides identified in the TUB2 gene fragment between isolates of the *Isaria poprawskii* and *I. javanica* clades. Refer to Fig. 4.

Isaria isolate	Nucleotide position			
	084	155	215	251
***I. poprawskii* clade 2**				
I. poprawskii ARSEF 7028 ex-type	C	T	T	C
I. poprawskii ARSEF 5330	C	T	T	C
***I. javanica* clade 1**[a]				
I. javanica CBS 134.22 ex-type[b]	T	A	C	T
I. javanica CBS 174.25	T	A	C	T
I. javanica ARSEF 5806	T	A	C	T
I. javanica ARSEF 3776	T	A	C	T
I. javanica ARSEF 3778	T	A	C	T
I. fumosorosea ARSEF 3682 (PFR-97)	T	A	C	T
I. javanica ARSEF 5259	T	A	C	T
I. javanica ARSEF 5321	T	A	C	T
I. javanica ARSEF 3577	T	A	C	T
I. javanica ARSEF 3573	T	A	C	T
I. javanica ARSEF 3593	T	A	C	T

a With the exception of three isolates (*I. javanica* CBS 134.22 ex-type [Java, Indonesia]); *I. javanica* CBS 174.25 (unknown origin); and *I. fumosorosea* ARSEF 3682 (PFR-97; Apopka, Florida), isolates within this clade were field-collected from the LRGV of south Texas (see Table 2).

b *Isaria javanica* CBS 134.22 ex-type isolate (GenBank accession no. EF585304; see Table 4B) as sequenced in the current study.

from the two clades. The restriction sites for these two enzymes are not present in the *I. fumosorosea* or *I. tenuipes* TUB2 sequences. The restriction digest with the enzyme XmnI digested only the isolates belonging to the *javanica* clade, producing band fragments of 175- and 181-bp (Fig. 5A) and the fungal isolates belonging to the *poprawskii* clade were not digested. Likewise, the restriction digest with HpyCH4IV digested only the isolates belonging to the *poprawskii* clade, generating bands of the expected sizes, 107- and 249-bp (Fig. 5B). Fungal isolates *I. fumosorosea* PFR-97 and *I. javanica* ARSEF 5806 are not shown, but each restricted with only the *javanica*-specific restriction enzyme, as predicted.

3.3.3. *ISSR-PCR DNA fingerprinting of the ex-type isolates of* I. javanica *and* I. poprawskii

The two ex-type cultures *I. javanica* CBS 124.22 and *I. poprawskii* ARSEF 7028, each of which was obtained from its respective holotype collection, were subjected to ISSR-PCR DNA fingerprinting. ISSR-PCR uncovered fixed species-specific DNA banding patterns that allowed the discrimination of the two ex-type fungal species. Five and four species-specific bands were generated in *I. javanica* CBS 134.22 and *I. poprawskii* ARSEF 7028, respectively (Fig. 6). The resulting bands ranged in size from ca. 300 to more than 1650 base pairs.

3.4. *Ecology*

The Lower Rio Grande Valley of Texas is a semi-arid subtropical region that receives about 600–700 mm of rainfall annually. Temperatures in summer and early fall are rather constant in the mid 30's (°C) during the daytime and mid 20's at night (NOAA 2004). The GPS coordinates for the location where greenhouse epizootics occurred are 26° 09.401′N, 97° 57.735′W, and the altitude is about 27 m above mean sea level. *I. poprawskii* showed a consistently high ability to persist in the greenhouse environment during the presence or absence of whiteflies at temperatures ranged as high as 42 °C.

4. Discussion

Isaria poprawskii can be separated from *I. javanica* and other related *Isaria* species by morphological and DNA characters. Morphologically, the shape (cylindrical to fusiform) and the length of the conidia of *I. poprawskii* (2.9–4.6 μm) are similar to *I. javanica* (2.1–4.3 μm), but they are different when compared to other characteristics. For example, the colony color and shape of *I. poprawskii* in young colonies is light yellow and in older colonies become darker yellow with a grayish-violet center, then the color of the mature conidial mass is taupe or a brownish-gray (Fig. 1A). In contrast, colonies of *I. javanica* ex-type culture (CBS 134.22) show a uniform white color regardless of colony age on SDAY. However, on SDAY/4, all of the *I. javanica* isolates (ARSEF isolates, Table 2) show colored colonies with the same rosy-tan color usually seen for *I. fumosorosea*. Furthermore, *I. poprawskii* develops synnemata, typical of members of the *Isaria* species, with distinct tan to yellowish-brown coloration with age; but *I. javanica* ex-type produces no synnemata. It should be noted that many *Isaria* cultures form synnemata for some time after being isolated but eventually lose this ability. However, morphometrics alone does not separate all eight *Isaria* isolates originally from the LRGV (Table 2) into one of these two species (*I. poprawskii* or *I. javanica*), underscoring the need to include genome-based characters and developmental data to characterize fungal taxa.

Our molecular examinations clearly support the classification of *I. poprawskii* as a distinct species. A neighbor-joining distance tree revealed that all of the 16 sequenced fungal isolates clustered within four clades representing three described species (*I. javanica*, *I. fumosorosea* and *I. tenuipes*) and the new species, *I. poprawskii* formed its own unique clade (Fig. 3A, B).

Interestingly, we found that fungal isolates collected from the fields of the LRGV and deposit in the ARSEF are misidentified as *Paecilomyces* species (Table 1), which indeed are either *I. poprawskii* or *I. javanica*; thus indicating that both fungi live in this region in sympatry. For example, *I. fumosorosea* ARSEF 5330 is indeed *I. poprawskii*; but most of the LRGV isolates including *I. fumosorosea* (ARSEF 3577, ARSEF 5259, ARSEF 5321), I. *farinosa* (ARSEF 3573, ARSEF 3593), *Paecilomyces* sp. (ARSEF 3776, ARSEF 3778, ARSEF 5806) clustered within the *javanica* clade (Fig. 3A).

More interesting are the findings of fungal isolates collected from geographic locations other than the LRGV of Texas, which also are misidentified as *Paecilomyces* or *Isaria* spp. For example, the Florida (USA) – collected isolate *Paecilomyces fumosoroseus* ARSEF 3682 (currently being used as a commercial product in biocontrol against insect pests as 'PFR-97') clustered within the *I. javanica* clade. Furthermore, *I. javanica* CBS 994.73 from Ghana and *Paecilomyces javanicus* CBS 995.73 from Ghana, which was previously identified as *P. javanicus* by Samson (1974); but reidentified as *I. tenuipes* by Luangsa-ard et al. (2005) are clustered as *I. tenuipes*. Further molecular studies are being performed on similar fungal isolates to elucidate their taxonomic identification and develop diagnostic markers (PCR-RFLP) as to discriminate the four main *Isaria* species.

Isaria poprawskii appears to be naturally selected for biological control of whiteflies at high temperatures. This fungus was found naturally infecting nymphs, pupae and adults of *B. tabaci* in greenhouses on several whitefly plant hosts at the LRGV of Texas. Its potential as a candidate for biocontrol of harmful insects in agriculture is based on its pathogenic capacity (Cabanillas and Jones 2009a); it can be easily cultured in common solid media for *Isaria* and *Paecilomyces* (Cabanillas and Barker 1989) and has the ability to survive high temperatures (Cabanillas and Jones 2009b). All these attributes indicate that *I. poprawskii* (TX) is a unique potential biocontrol agent for insect pests. The strain of *I. poprawskii* described here is called the TX strain after Texas.

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