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Species clarification of *Isaria* isolates used as biocontrol agents against *Diaphorina citri* (Hemiptera: Liviidae) in Mexico

*Adrien GALLOU**, *María G. SERNA-DOMÍNGUEZ*, *Angélica M. BERLANGA-PADILLA*, *Miguel A. AYALA-ZERMEÑO*, *Marco A. MELLÍN-ROSAS*, *Roberto MONTESINOS-MATÍAS*, *Hugo C. ARREDONDO-BERNAL*

Centro Nacional de Referencia de Control Biológico, Km 1.5 Carretera Tecomán-Estación FFCC, Col. Tepeyac, C.P. 28110, Tecomán, Colima, Mexico

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ABSTRACT

Entomopathogenic fungi belonging to the genus *Isaria* (*Hypocreales: Cordycipitaceae*) are promising candidates for microbial control of insect pests. Currently, the Mexican government is developing a biological control program based on extensive application of *Isaria* isolates against *Diaphorina citri* (Hemiptera: Liviidae), a vector of citrus huanglongbing disease. Previous research identified three promising *Isaria* isolates (CHE-CNRCB 303, 305, and 307; tentatively identified as *Isaria fumosorosea*) from Mexico. The goal of this work was to obtain a complete morphological and molecular characterization of these isolates. Comparative analysis of morphology established that the isolates showed similar characteristics to *Isaria javanica*. Multi-gene analysis confirmed the morphological identification by including the three isolates within the *I. javanica* clade. Additionally, this work demonstrated the misidentifications of three other *Isaria* isolates (CHE-CNRCB 310 and 324: *I. javanica*, formerly *I. fumosorosea*; CHE-CNRCB 393: *I. fumosorosea*, formerly *Isaria farinosa*), underlying the need for a full and correct characterization of an isolate before developing a biological control program. Finally, the inter-simple sequence repeat (ISSR) genotyping method revealed that the CHE-CNRCB 303, 305, and 307 isolates belong to three different genotypes. This result indicates that ISSR markers could be used as a tool to monitor their presence in field conditions.

Introduction

Species within the genus *Isaria* (*Hypocreales*: *Cordycipitaceae*) are entomopathogenic fungi (EPF) with a widespread global distribution (Gams *et al.* 2005). The catalogue of the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) contains more than 1000 *Isaria* strains from numerous countries in North, Central, and South America, Europe, Africa, Australia, and Asia. In addition, *Isaria* strains can infect different insect orders in all developmental stages, and are commonly isolated from soil (D'Alessandro *et al.* 2013). Originally, *Isaria* was considered a subsection within the

* *Corresponding author*. Centro Nacional de Referencia de Control Biológico (Laboratorio de Biología Molecular), Km 1.5, Carretera Tecomán-Estación FFCC, Col. Tepeyac, C.P. 28110, Tecomán, Colima, Mexico. Tel.: +52 (31) 33 24 07 45; fax: +52 (31) 33 24 27 73.
E-mail address: gallou.adrien@hotmail.fr (A. Gallou).

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genus *Paecilomyces* sensu Samson (1974), who divided this genus into two sections: section *Paecilomyces* (thermophilic) and section *Isarioidea* (mesophilic). However, this distinction was based on morphological characteristics that may be highly subjective and lead to ambiguous identifications at the species level. Formal conservation of the generic name *Isaria* was officially accepted in 2005 (Gams *et al.* 2005).

Molecular phylogenetic studies have resurrected the genus *Isaria* (Luangsa-ard *et al.* 2005; Sung *et al.* 2007). The polyphyletic nature of the genus *Paecilomyces* (i.e., including the sect. *Isarioidea*) has been demonstrated several times previously by analyses of the large and small subunit rRNA genes (Obornik *et al.* 2001; Luangsa-ard *et al.* 2004). However, using the β-tubulin gene and the nuclear ribosomal internal transcribed spacer (ITS) region, Luangsa-ard *et al.* (2005) investigated the phylogenetic relationships of *Paecilomyces* sect. *Isarioidea* species, and established the existence of a monophyletic group named '*Isaria* clade', which includes: *Isaria amoenerosea* Henn., *Isaria cateniannulata* (Z.Q. Liang) Samson & Hywel-Jones, *Isaria cateniobliqua* (Z.Q. Liang) Samson & Hywel-Jones, *Isaria cicadae* Miq., *Isaria coleopterora* (Samson & H.C. Evans) Samson & Hywel-Jones, *Isaria farinosa* (Holmsk.) Fr., *Isaria fumosorosea* Wize, *Isaria ghanensis* (Samson & H. C. Evans) Samson & Hywel-Jones, *Isaria javanica* (Friederichs & Bally) Samson & Hywel-Jones, and *Isaria tenuipes* Peck. More recently, Sung *et al.* (2007) used multilocus sequence typing (MLST) to construct a phylogeny of the clavicipitaceous fungi, distributing the genus *Paecilomyces* among three families of the *Hypocreales* (i.e., *Cordycipitaceae*, *Clavicipitaceae*, and *Ophiocordycipitaceae*). Currently, some species from this genus were excluded from both *Paecilomyces* and *Isaria*, or still await transfer into appropriate genera. For instance, Luangsa-ard *et al.* (2011) showed that *Paecilomyces lilacinus*, placed in the *Ophiocordycipitaceae*, was not related to *Paecilomyces* and proposed the new genus '*Purpureocillium*'. In addition, Kepler *et al.* (2014) recently included in the definition of the genus *Metarhizium* several species previously placed in *Paecilomyces* [i.e., *Metarhizium carneum* (Duché & R. Heim) Kepler, S.A. Rehner & Humber formerly *Paecilomyces carneus* (Duché & R. Heim) A.H.S. Br. & G. Sm.; *Metarhizium marquandii* (Massee) Kepler, S.A. Rehner & Humber formerly *Paecilomyces marquandii* (Massee) S. Hughes; *Metarhizium viride* (Segretain, Fromentin, Destombes, Brygoo & Dodin ex Samson) Kepler, S.A. Rehner & Humber formerly *Paecilomyces viridis* Segretain, Fromentin, Destombes, Brygoo & Dodin].

Diaphorina citri Kuwayama (Hemiptera: Liviidae), the Asian citrus psyllid, is a worldwide citrus pest due to its role as a vector of the phloem-limited bacteria *Candidatus* Liberibacter spp., which causes citrus huanglongbing (HLB) (Halbert & Manjunath 2004). EPF could play an important role in the regulation of *D. citri* populations and are promising microbial candidates for control of phloem feeding pests (Hajek & St. Leger 1994; Goettel *et al.* 2010). Meyer *et al.* (2008) reported the isolation and characterization of an *I. fumosorosea* strain naturally infecting the psyllid in Florida, and Stauderman *et al.* (2012) confirmed their potential in the management of *D. citri*.

Mexico is a megadiverse country that forms part of the Mesoamerican biological corridor that connects North and South America, and is a source of natural enemies to contribute to biological control programs (Williams *et al.* 2013). The 'Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria' (SENASICA), through the 'Dirección General de Sanidad Vegetal' (DGSV) of the Mexican government, has designed a program to develop technology for the biological control of *D. citri*. This program has selected three *Isaria* isolates from Mexico, previously identified as *I. fumosorosea* (i.e., CHE-CNRCB 303, 305, and 307), for extensive applications in the Regional Areas of Control of Asian citrus psyllid (Spanish acronym: ARCOS) (Sánchez *et al.* 2015). Ramírez-Balboa *et al.* (2012) demonstrated the efficacy of these isolates in field condition against *D. citri* (i.e., mycosis between 66 and 81.8 %). Furthermore, to obtain a more highly-virulent mycoinsecticide, the development of highly effective single-spore isolates is in progress (Ayala-Zermeño *et al.* 2015).

For a biological control program to be successful, knowledge of the exact identities of the pest and biological control agent species is crucial. For this reason, the three *Isaria* isolates were characterized morphologically as *I. fumosorosea* prior to its consideration within a biological control program. However, the change in status of the genus *Isaria* pointed out the need to use molecular methods (Luangsa-ard *et al.* 2005; Sung *et al.* 2007) to characterize the three *Isaria* isolates. Likewise, Cabanillas *et al.* (2013), using molecular techniques, determined that 12 out of 16 *Isaria* or *Paecilomyces* isolates listed in the ARSEF or 'Centraalbureau voor Schimmelcultures' (CBS; Utrecht, Netherlands) collections were misidentifications. Therefore, the purpose of this study was to obtain a complete morphological and molecular characterization of the *Isaria* isolates (i.e., 303, 305, and 307) being used as biological control agents against *D. citri* in the Mexican ARCOS.

Materials and methods

Fungal isolates

In this study, isolates provisionally identified as *Isaria fumosorosea* (i.e., CHE-CNRCB 303, 305, 307, 310, and 324) or *Isaria farinosa* (CHE-CNRCB 393) by morphological examination (Table 1), were obtained from the 'Colección de Hongos Entomopatógenos' of the 'Centro Nacional de Referencia de Control Biológico'. Isolate 393 (EH-402) was previously obtained from the culture collection of fungal pathogen strains of the 'Laboratorio de Micología Básica' (Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México).

Morphological analysis

For morphometric evaluation, microcultures were grown on quarter-strength Sabouraud dextrose agar medium with yeast extract (SDAY/4; 10 g L^{-1} dextrose, 2.5 g L^{-1} peptone, 2.5 g L^{-1} yeast extract, and 15 g L^{-1} agar), and incubated at 27 °C (±2 °C) for 7–14 d. Slides were prepared with lactophenol/blue cotton (10:1), and examined with phase contrast optics on an optical microscope AXIO Scope A1 (Carl Zeiss, Microscopy GmbH, Gottingen, Germany) using the 100× objective. Images were photographed digitally with an AxioCam ICc 1 camera (Carl Zeiss) using the AxionVision SE64 Release 4.9.1 software (03-2013). At least, 30 independent measurements of conidia

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Table 1 – Fungal isolate codes, species name, locality of collection, host, GenBank accession numbers, and conidial measurements in culture in quarter-strength Sabouraud dextrose agar medium with yeast extract (SDAY/4).

Isolate	Species	Locality	Isolate source	GenBank number			Conidia (µm)		Phialides (µm)	
				ITS	β-tub	TEF exon	Length	Width	Length	Width
CHE-CNRCB 303 (Pf15*)	*Isaria javanica* (*Isaria fumosorosea***)	Armería, Colima, Mexico	*Bemisia tabaci* (Hemiptera: Aleyrodidae)	KM234212	KT225604	KT225598	4.59 ± 0.35^{b}	1.59 ± 0.16^{cd}	5.15 ± 0.41^{b}	2.15 ± 0.25^{b}
CHE-CNRCB 305 (Pf17)	*Isaria javanica* (*Isaria fumosorosea*)	Armería, Colima, Mexico	*Bemisia tabaci* (Hemiptera: Aleyrodidae)	KM234214	KT225605	KT225599	4.34 ± 0.29^{c}	1.54 ± 0.15^{d}	5.43 ± 0.84^{a}	1.86 ± 0.24^{c}
CHE-CNRCB 307 (Pf21)	*Isaria javanica* (*Isaria fumosorosea*)	Armería, Colima, Mexico	*Bemisia tabaci* (Hemiptera: Aleyrodidae)	KM234217	KT225606	KT225600	4.24 ± 0.26^{c}	1.64 ± 0.12^{cd}	5.53 ± 0.40^{ab}	1.92 ± 0.26^{c}
CHE-CNRCB 310 (Pf27)	*Isaria javanica* (*Isaria fumosorosea*)	France	*Spodoptera litoralis* (Lepidoptera: Noctuidae)	KT225592	KT225601	KT225595	4.76 ± 0.38^{ab}	1.84 ± 0.14^{b}	5.44 ± 0.49^{ab}	2.30 ± 0.15^{ab}
CHE-CNRCB 324 (Pf42)	*Isaria javanica* (*Isaria fumosorosea*)	Minatitlán, Colima, Mexico	NA	KT225593	KT225602	KT225596	4.92 ± 0.31^{a}	1.68 ± 0.16^{c}	5.73 ± 0.52^{a}	2.39 ± 0.18^{a}
CHE-CNRCB 393 (EH-402)	*Isaria fumosorosea* (*Isaria farinosa*)	NA	NA	KT225594	KT225603	KT225597	3.48 ± 0.31^{d}	2.11 ± 0.17^{a}	4.81 ± 0.43^{c}	2.18 ± 0.21^{b}

All fungal isolates were obtained from the Colección de Hongos Entomopatógenos of the Centro Nacional de Referencia de Control Biológico (CHE-CNRCB; Mexico). At least 30 independent measurements of conidia ($n = 30$) and phialides ($n = 30$) were made for each isolate. Values of each morphological measurement in a column followed by a different letter differed significantly at $P \leq 0.05$ (ANOVA and Tukey's multiple comparison test). Data not available (NA).

*Original code registration of the isolates in the CHE-CNRCB culture collection.

**Previous species identification of the isolates in the CHE-CNRCB culture collection.

($n = 30$) and phialides ($n = 30$) were made for each isolate. The data were analysed using SPSS software for Windows, version 17 (SPSS Inc., Chicago, IL, USA, 2008), by an analysis of variance (ANOVA) ($\alpha = 0.05$). Tukey's multiple comparison test was conducted to identify significant differences ($P \leq 0.05$).

DNA extraction

For extraction of total genomic DNA, the isolates were initially grown on SDAY with yeast extract at 27 °C (±2 °C) for 15 d. Afterward each of the isolates was cultivated in 50 mL of liquid medium (40 g L^{-1} glucose, 10 g L^{-1} polypeptone, and 10 g L^{-1} yeast extract) in a 250 mL Erlenmeyer flask on a shaker (150 rpm) at 27 °C (±2 °C) for 3 d. The fungal biomass was then collected by filtration, rinsed various times in deionized sterile water, transferred to serological bottles of 10 mL, and frozen at −50 °C. Immediately, the samples were lyophilized with the FreeZone 4.5 Liter Cascade Benchtop Freeze Dry System (Labconco Corp., Kansas City, USA) over 6 h, and then stored at 4 °C until DNA extraction. Genomic DNA was extracted from 10 mg of powered tissue, ground in liquid nitrogen using a mortar and pestle, with the DNeasy Plant Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. Purity of genomic DNA was determined in a BioTek Epoch spectrophotometer (BioTek, Winooski, USA) using the A260/A280 absorbance ratio. DNA concentration and quality were evaluated using the Fragment Analyzer™ Automated CE system associated with the High Sensitivity Genomic DNA Analysis Kit, as recommended by the manufacturer's protocol (Advanced Analytical Technologies Inc., Ames, USA).

PCR amplification and sequencing

The genes used in this study were the exon region of translation elongation factor 1-α (TEF exon), β-tubulin (β-tub), and ITS region. The TEF exon (1000 bp) was amplified with the 983F and 2218R primers, and sequenced with the amplification primers and two additional internal primers (1577F and 1567R) (Rehner & Buckley 2005). The β-tub (1500 bp) was amplified with the T1 and T22 primers (O'Donnell & Cigelnik 1997), and sequenced with the amplification primers and three additional internal primers (Bt1F, Bt1R, and Bt2R) (Bischoff *et al.* 2009). The ITS (600 bp) was amplified and sequenced with the universal primers ITS5 and ITS4 (White *et al.* 1990). PCR amplifications were performed in a total volume of 25 µL using the GoTaq® G2 Flexi DNA Polymerase Kit (Promega, Madison, USA) following the manufacturer's instructions, and 1–40 ng of genomic DNA. PCR for TEF exon and β-tub was performed using a touchdown PCR procedure (Don *et al.* 1991). The PCR amplifications were initiated by denaturation at 94 °C for 2 min. The annealing temperature in the first amplification cycle was 66 °C, which was decreased by 1 °C per cycle over the next nine cycles. Additional 30 or 33 amplification cycles (TEF exon and β-tub, respectively) were then performed: 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The PCR amplification of ITS was initiated by denaturation at 94 °C for 1 min followed by 35 cycles of three steps: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, with a final

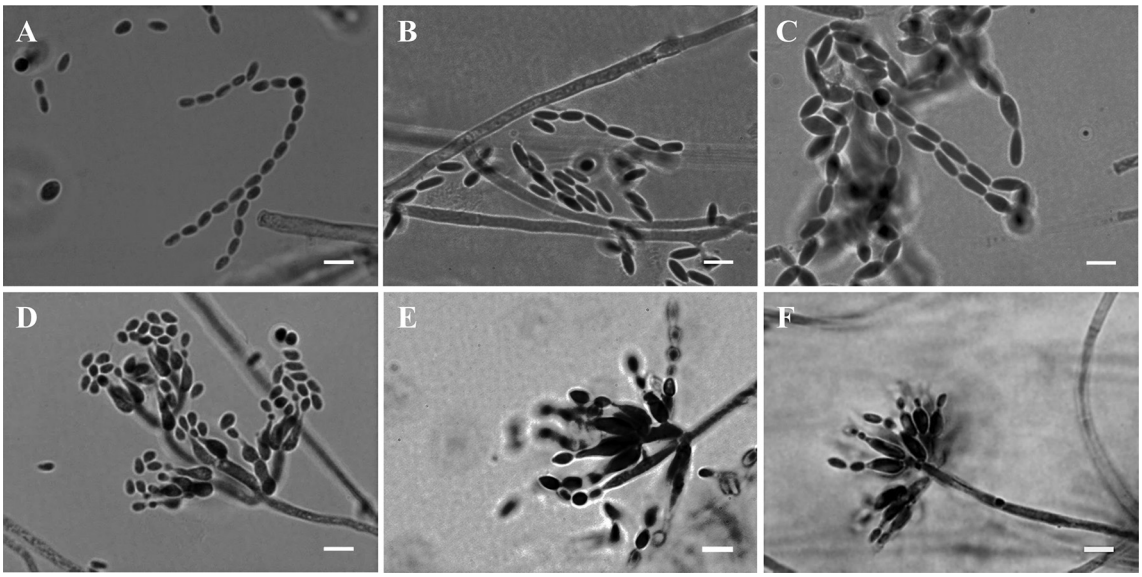

Fig 1 – Conidia and conidiogenous cells of *Isaria* species. (A). *I. fumosorosea* CHE-CNRCB 393: chains of conidia. (B). *I. javanica* CHE-CNRCB 310: chains of conidia. (C). *I. javanica* CHE-CNRCB 307: chains of conidia. (D). *Isaria fumosorosea* CHE-CNRCB 393: Phialides with developing conidia. (E). *I. javanica* CHE-CNRCB 324: Phialides with developing conidia. (F). *I. javanica* CHE-CNRCB 310: Phialides with developing conidia. White scale bars represent 5 μm.

extension step at 72 °C for 5 min. The amplicons were visualized in agarose gels (1 %), and prepared for sequencing by purification with the Wizard® SV Gel and PCR Clean-Up System Kit (Promega, Madison, USA). The samples were sequenced at the 'Laboratorio Nacional de Genómica para la Biodiversidad' (LANGEBIO – CINVESTAV – Guanajuato, Mexico).

Phylogenetic analyses

Sense and antisense sequences were edited and assembled using the BioEdit 7.2.0 software (Hall 1999), and multiple alignments were performed using MAFFT (Katoh *et al.* 2002) (http://mafft.cbrc.jp/alignment/server/). All the sequences were deposited in GenBank (Table 1), and the final alignments are available from TreeBASE under submission ID 17866. Additionally, GenBank accession numbers of all the isolates used in phylogenetic analyses are summarized in Table S1.

Phylogenetic hypotheses for TEF exon, β-tub, and ITS were analysed independently with Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI) methods with these parameters: (i) MP – Equally weighted parsimony analysis was performed with the MEGA 6 software (Tamura *et al.* 2013); parsimony bootstrap (MP BS) analysis was performed with 1000 replicates, each with 2000 random taxon addition sequences, and branch swapping set to subtree pruning and regrafting (SPR). (ii) ML – The analysis was run in the MEGA 6 software based on the two-parameter model of Kimura (1980) for ITS and β-tub; and generalized time-reversible (GTR) model (Tavaré 1986) for TEF exon. A discrete gamma distribution was applied for each analysis with 1000 bootstrap replicates (ML BS). (iii) BI – MrBayes 3.2.5 (Ronquist & Huelsenbeck 2003) was used to determine posterior probabilities (BI PP). The analyses were run with 1 400 000, 2 500 000 or 1 200 000 generations for TEF exon, β-tub, and ITS respectively, sampling every 200 generations under the GTR model with discrete gamma distribution. The MCMC runs were repeated twice to confirm the topology of the Bayesian majority rule tree obtained after each run. The resulting trees were visualized into FigTree 1.4.2 (Rambaut 2014), and a 50 % consensus tree was computed. Clades were considered significantly supported with 95 % BI PP, 70 % ML BS, and 70 % MP BS or greater values (Bischoff *et al.* 2009). The phylograms (Figs 2–4) are the consensus trees with the mean branch lengths from the Bayesian analyses.

ISSR DNA fingerprinting

In a first step, we screened 43 different 2-, 3-, 4-, and 5-base-pair repeat primers for their effectiveness in producing useful, repeatable and interpretable levels of variation on inter-simple sequence repeat (ISSR) lengths from *Isaria javanica* and *Isaria fumosorosea* species. Four primers (i.e., $(AG)_8$, $HVH(TG)_7$, $(GTG)_5$, and $(ACC)_6$) were selected to survey ISSR variation across the *Isaria* species. Briefly, reactions were performed in a final volume of 25 μL using the GoTaq® G2 Flexi DNA Polymerase Kit (Promega, Madison, USA) following the manufacturer's instructions, 0.5 mM of ISSR primer, and 1 ng of genomic DNA. Thermal cycling parameters were: 94 °C for 5 min followed by 35 cycles at 94 °C for 1 min, 50 °C ($(AG)_8$)/52 °C ($HVH(TG)_7$ and $(GTG)_5$)/54 °C ($(ACC)_6$) for 1 min, and 72 °C for 2 min, with a final extension step at 72 °C for 10 min. Amplification products were separated by capillary electrophoresis using the Fragment Analyzer™ Automated

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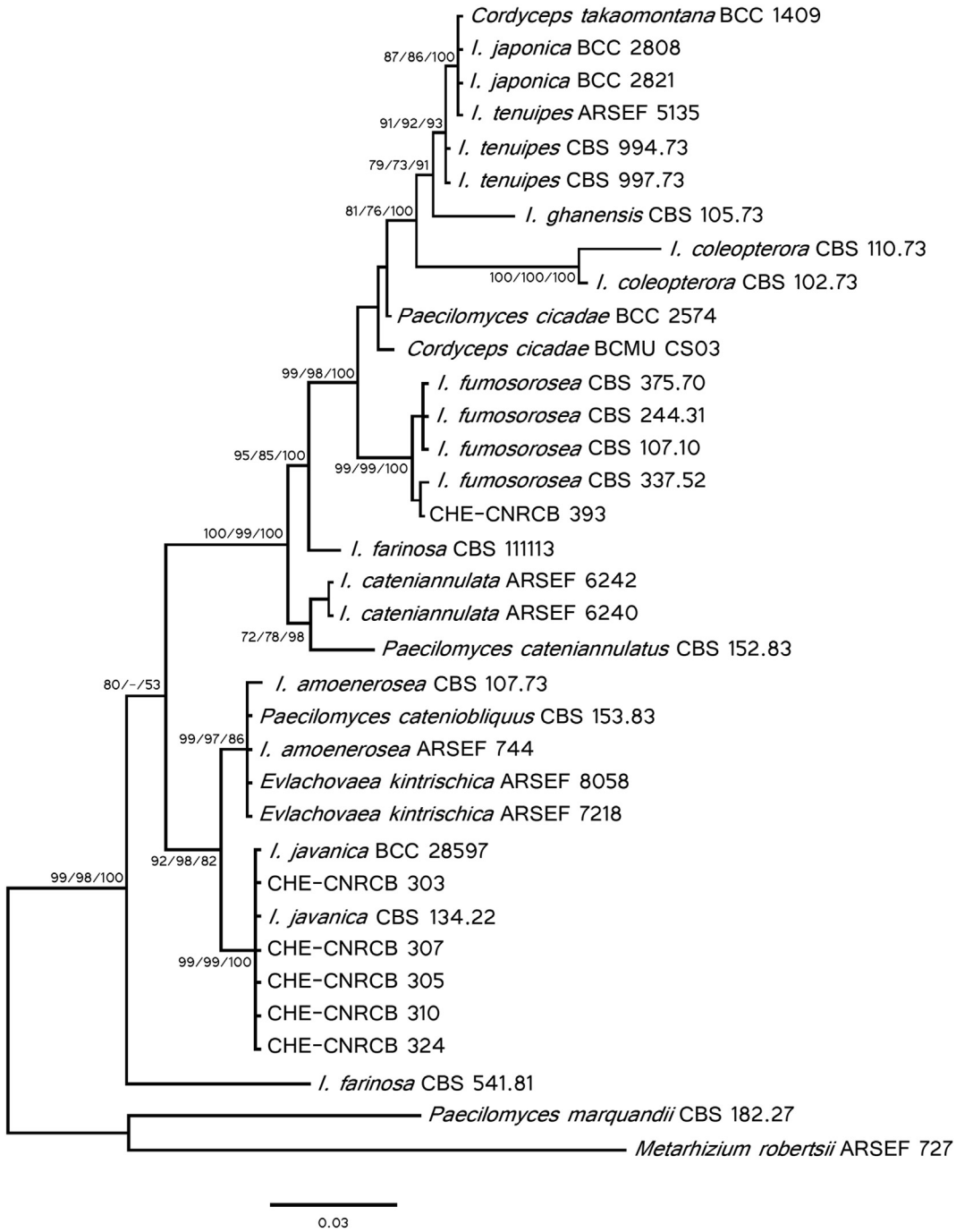

Fig 2 – Majority rule consensus phylogram from the Bayesian analysis based on the sequences of the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) for 35 isolates (*Paecilomyces marquandii* CBS 182.27 and *Metarhizium robertsii* ARSEF 727 as out-group). Support values are shown for MP BS, ML BS, and BI PP respectively (- = value not supported).

CE system associated with the DNF-915 dsDNA Reagent Kit (i.e., DNA sizing range: 35 bp–5000 bp), as recommended by the manufacturer's protocol (Advanced Analytical Technologies Inc., Ames, USA). For each isolate and ISSR primer, at least three technical repetitions were performed ($n = 3$). Data analysis was performed with the program PROSize™ 2.0 (Advanced Analytical Technologies Inc., Ames, USA). Only product fragments between 200 and 2100 bp, with relative fluorescent units (RFU) above 200, and peak width above 5 s were scored. The matching fragments were analysed in a binary matrix with the Dice similarity coefficient (Bonin *et al.* 2007) and expressed as percentage.

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Results

Morphological analysis

Morphological features of the six *Isaria* isolates and their comparisons are summarized in Table 1 and illustrated in Fig 1. The isolates showed an ellipsoidal to fusiform-elliptical conidial shape with overall dimensions of 3.48–4.92 × 1.54–2.11 μm (i.e., length × width) (Table 1). However, significant differences in conidial sizes were noted between the isolates (Table 1). Isolates 303, 305, 307, 310, and 324 showed similarities in conidial sizes, whereas isolate 393 presented a conidial length significantly lower, and a conidial width significantly higher than the others isolates (i.e., long ovoid conidial shape) (Table 1 and Fig 1A–C).

The phialides of the isolates were characterized by a wide globose basal portion with a long distal neck, and overall dimensions of 4.81–5.73 × 1.86–2.39 μm (Table 1 and Fig 1D–F). Significant differences were observed in phialide sizes between the isolates, and particularly the isolate 393 showed significantly a lower phialide length compared with the others isolates (Table 1 and Fig 1D–F).

Phylogenetic analyses

Sequences of the ITS region, β-tub, and TEF exon genes were aligned and analysed separately by MP, ML, and BI analyses. The resulting trees were compared (Figs 2–4, respectively), and phylogenies from the three analyses were largely congruent to confirm the identification of the six isolates.

The phylogenetic analyses of ITS showed that the five isolates 303, 305, 307, 310, and 324 form part of the *Isaria javanica* clade, including the ex-type isolate *I. javanica* CBS 134.22 (Fig 2). The branch was strongly and significantly supported from all three analyses (i.e., 99 % MP BS, 99 % ML BS, and 100 % BI PP), without conflict. Additionally, the analyses showed that isolate 393 forms part of the *Isaria fumosorosea* clade, including *I. fumosorosea* CBS 107.10 (ex-type isolate) (Fig 2). This branch was also significantly supported by all three analyses (i.e., 99 % MP BS, 99 % ML BS, and 100 % BI PP) without conflict.

The phylogenetic relationships of TEF exon and β-tub exon genes confirmed that the five isolates 303, 305, 307, 310, and 324 were included in the *I. javanica* clade (Figs 3 and 4). This branch was strongly supported from all the three analyses (i.e., 100/99 % MP BS, 98/98 % ML BS, and 100/100 % BI PP, respectively for TEF exon and β-tub). In the case of the isolate 393, the phylogeny of TEF exon gene confirmed its placement in the *I. fumosorosea* clade with strong support (i.e., 100 % MP BS, 100 % ML BS, and 100 % BI PP). Whereas, the β-tub region was the only gene whose phylogeny did not significantly support the monophyly of *I. fumosorosea* clade (Fig 4). However, isolate 393 showed a close relationship with *I. fumosorosea* isolates in a clade including *Isaria cicadae*, *Isaria coleopterora*, *Isaria ghanensis*, *I. fumosorosea*, *Isaria japonica*, and *Isaria tenuipes*.

ISSR DNA fingerprinting

The four selected ISSR primers produced 55 countable fragments of which 53 fragments (96.4 %) were polymorphic across the six *Isaria* isolates. Only isolates 305 and 310 presented an identical pattern with the four primers, indicating polymorphisms between isolates of the same species. An example of the ISSR electropherogram pattern of the isolates generated using the $(GTG)_5$ primer is presented in Fig S1.

The whole set of ISSR markers was considered to estimate the Dice similarity index between samples (Table 2). Isolates 303, 305, and 307 showed the highest similarities with *Isaria javanica* isolates 310 and 324. In particular, these three isolates were close to isolate 310, with values of similarity from 96.30 to 100 %. In contrast, isolates 303, 305, and 307 showed the lowest similarity with *Isaria fumosorosea* isolate 393, with very low values of similarity (from 8 to 8.33 %).

Discussion

EPF as mycoinsecticides are one of the best strategies to prevent pest insects from damaging field crops. However, complete and correct identification of these fungi is required in order to develop an efficient biological control program. Previous work demonstrated the great potential of three *Isaria* isolates (i.e., 303, 305, and 307) to control *Diaphorina citri* (Ramírez-Balboa *et al.* 2012). Here, we have provided a complete characterization of these isolates, which are being used in the ARCOS (Sánchez *et al.* 2015). In contrast to the earlier morphological identification, which determined these isolates as being *Isaria fumosorosea*, the present analysis correctly identified the isolates as *Isaria javanica*. In addition, the new morphological identification was validated by phylogenetic analysis of the TEF exon, β-tub genes, and ITS region, which placed isolates 303, 305, and 307 in the *I. javanica* clade that includes isolate CBS 134.22. Finally, we demonstrated that three other *Isaria* isolates from the CHE-CNRCB (i.e., 310 and 324: *I. javanica* formerly *I. fumosorosea*; 393: *I. fumosorosea* formerly *Isaria farinosa*) were misidentified.

Based on the work of Scorsetti *et al.* (2008) and Zimmermann (2008), the morphological analysis (i.e., conidial and phialides sizes) established that isolates 303, 305, 307, 310, and 324 were similar to *I. javanica*, and 393 was similar to *I.*

Table 2 – Dice similarity coefficients of the whole set of inter-simple sequence repeat-polymerase (ISSR) primer expressed in percentage between each fungal isolate.

Fungal isolate (CHE-CNRCB)	303	305	307	310	324	393
303	–					
305	98.11	–				
307	98.18	96.30	–			
310	98.11	100.00	96.30	–		
324	72.73	74.07	74.07	74.07	–	
393	8.16	8.00	8.00	8.33	8.00	–

For each strain and ISSR primer, three technical repetitions were at least performed ($n = 3$).

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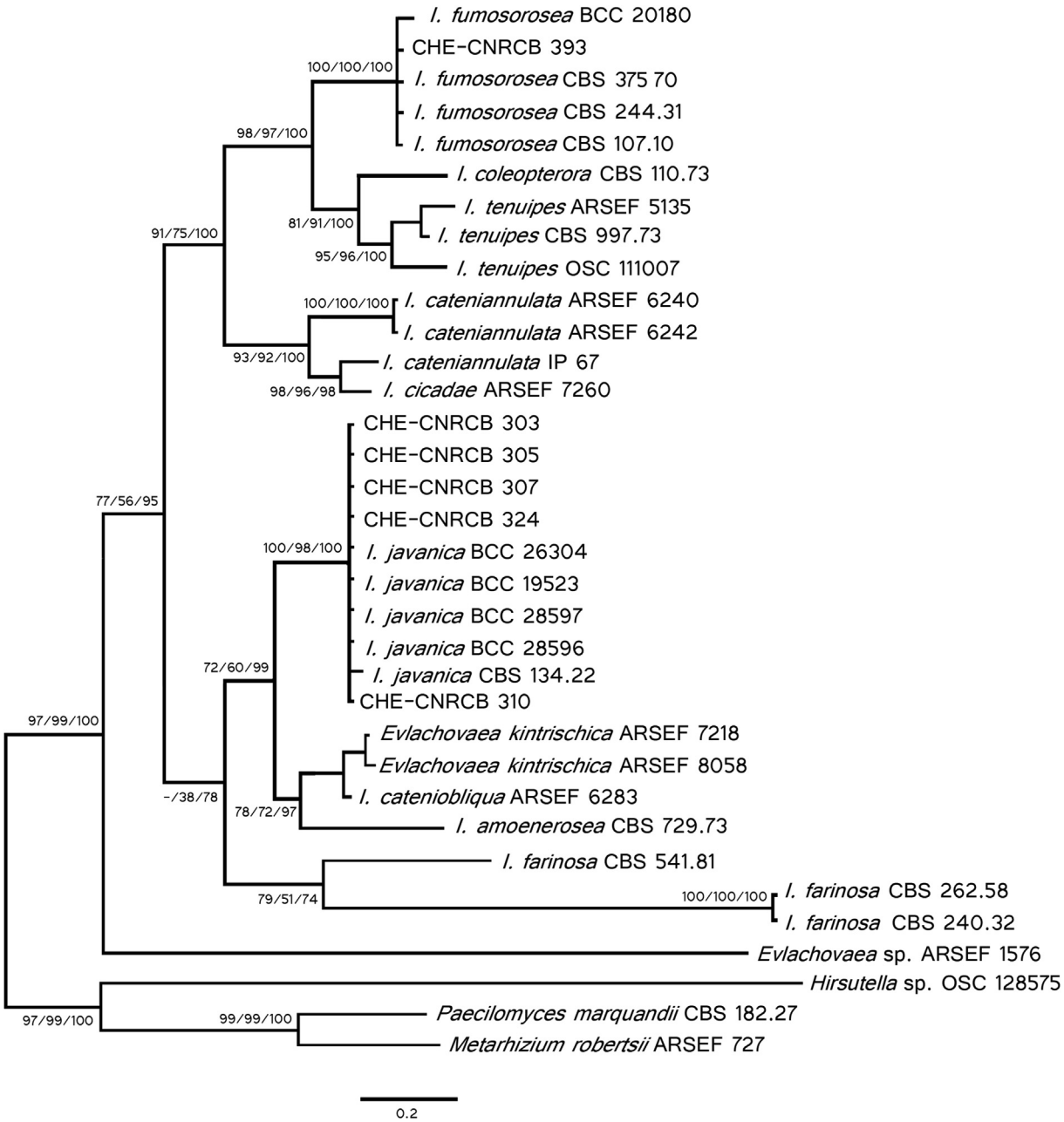

Fig 3 – Majority rule consensus phylogram from the Bayesian analysis based on the sequences of the exon region of translation elongation factor 1-α (TEF exon) for 34 isolates (*Paecilomyces marquandii* CBS 182.27, *Metarhizium robertsii* ARSEF 727 and *Hirsutella* sp. OSC 128575 as out-group). Support values are shown for MP BS, ML BS, and BI PP respectively (- = value not supported).

fumosorosea. In particular, we noted a significant difference in the conidial shape of isolate 393, which had a long ovoid shape in contrast to the ellipsoidal to fusiform of the others isolates. Additionally, the most important difference between the isolates was observed in the colour of the colonies on SDAY/4 medium. In fact, one of the most clear morphological characteristic to distinguish *I. javanica* from *I. fumosorosea* is the different colouration on medium (Cabanillas *et al.* 2013). During the early growth (data not shown), we observed that isolates 303, 305, 307, 310, and 324 showed white colonies, which turned to brownish grey when mature, whereas isolate 393 in the same culture medium showed bright pink colonies, attributed to conidial colouration.

To confirm the morphological identification of the isolates, we performed a molecular phylogenetic study. To date, no unambiguous MLST procedure is available for the genus *Isaria*. Consequently, this work was based on the most informative molecular markers (i.e., ITS, β-tub, and TEF exon) currently

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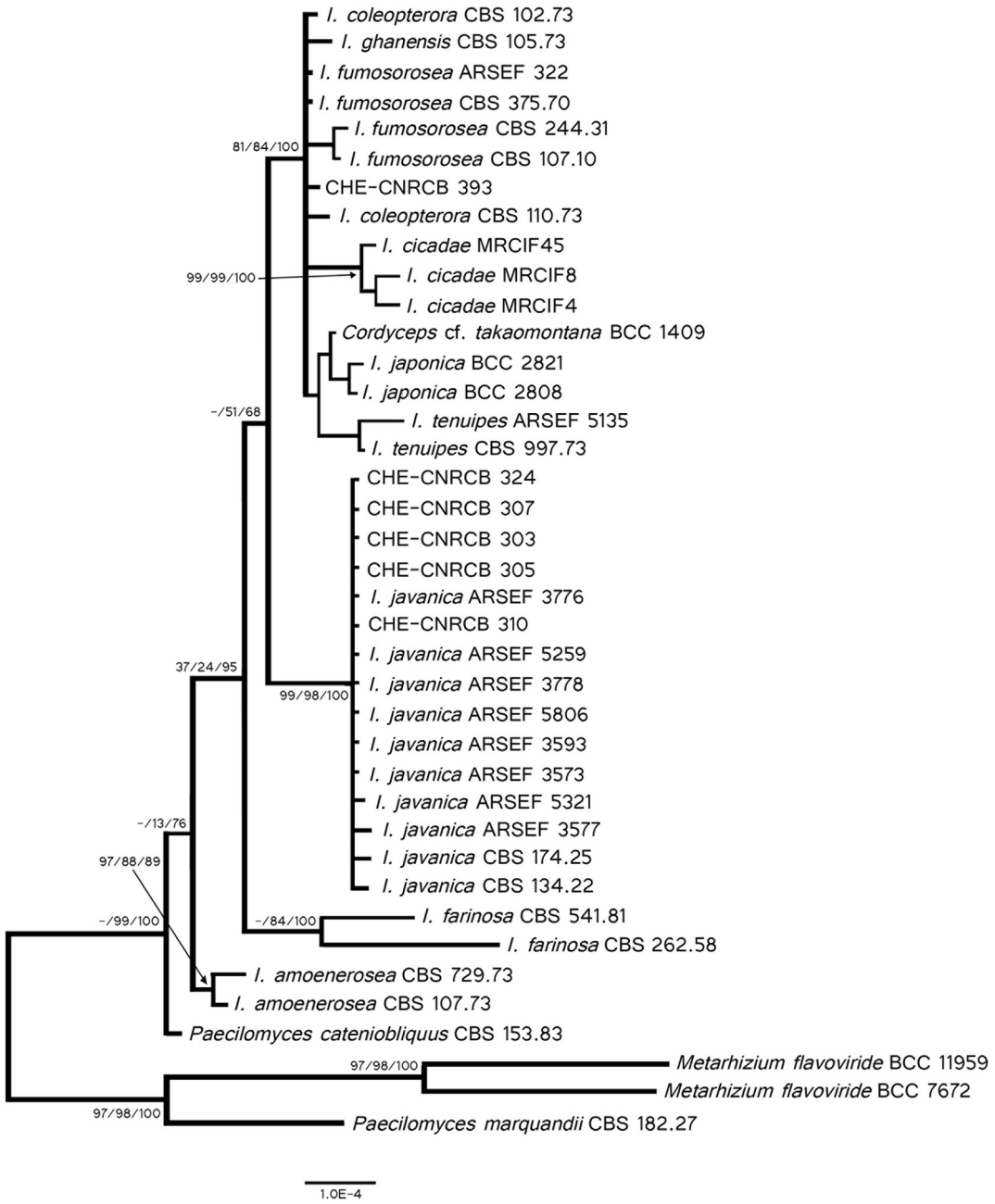

Fig 4 – Majority rule consensus phylogram from the Bayesian analysis based on the sequences of the β-tubulin (β-tub) for 39 isolates (*Paecilomyces marquandii* CBS 182.27 and *Metarhizium flavoviride* BCC 7672 and BCC 11959 as out-group). Support values are shown for MP BS, ML BS, and BI PP respectively (- = value not supported).

available (Luangsa-ard *et al.* 2005; Cabanillas *et al.* 2013; D'Alessandro *et al.* 2013; Humber *et al.* 2013). The phylogenetic analyses by MP, ML, and BI of each of the three markers were clearly consistent in identifying the six isolates. The results demonstrated that isolates 303, 305, 307, 310, and 324 were *I. javanica* and 393 was *I. fumosorosea*, in agreement to the phylogenetic relationships of the genus *Isaria* (Luangsa-ard *et al.* 2005; Cabanillas *et al.* 2013).

It is interesting to note that few conflicts were observed in the *Isaria* species relationship established by the individual gene analyses. For example, the *I. farinosa* isolate CBS 111113 was placed at the basal level of the *I. fumosorosea* clade by ITS phylogeny (i.e., Danish isolate that equates to the type locality (Holm 1781)), whereas *I. farinosa* isolates were generally grouped in a same clade at the basal level of the *I. javanica* clade. The same observation was also made by Luangsa-ard *et al.* (2005) through ITS phylogeny (i.e., *I. farinosa* isolated CBS 262.58 and CBS 541.81 were grouped, and CBS 111113 was unsupported). As another example, the low informative level of the β-tub phylogeny in order to support the

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phylogenetic relationships of the *Isaria cicadae*, *Isaria coleopterora*, *Isaria ghanensis*, *I. fumosorosea*, *Isaria japonica*, and *Isaria tenuipes* species (i.e., only a good relationship and support were observed by Neighbor Joining method; data not show) in contrast to the well supported ITS or TEF exon phylogenies for the relationships between these species. As pointed out by Humber *et al.* (2013), it is not yet possible to ensure that these genes alone provide enough information to allow reliable identifications at the species level. In addition to the low number of *Isaria* sequences available in databases, our work demonstrated the need to refine the taxonomy of the monophyletic group '*Isaria* clade' by studying additional taxa and increasing the sequence data available to strengthen phylogenetic hypotheses.

The ISSR method, based on surveying variation in length of spacers between short simple repeats (SSRs) (Reddy *et al.* 2002), assessed the genetic diversity of the six isolates. The first interesting result was the low Dice similarity values obtained for the five isolates identified as *I. javanica* (i.e., 303, 305, 307, 310, and 324) compared with isolate 393, identified as *I. fumosorosea*. These low values indicated a higher genetic distance between the isolate 393 and the five other isolates, confirming that 393 belongs to a different species. Secondly, this method provides sufficient resolution for identification of genotypes within species. In fact, among the five *I. javanica* isolates studied, only isolates 305 and 310 showed the same genotype using the ISSR method. Finally, with the ability to distinguish the isolates, this method could be developed as an efficient tool to monitor field application of isolates 303, 305, and 307.

In conclusion, the full morphological and molecular characterization performed on *Isaria* isolates 303, 305, and 307 has facilitated their correct identification as *I. javanica*. Additionally, phylogenic analysis using the molecular markers ITS, β-tub, and TEF exon, clearly supported placement of the isolates in the *I. javanica* clade, along with the ex-type isolate CBS 134.22. Moreover, this work contributed to the correct identification of three other isolates from the CHE-CNRCB: 310 and 324 as *I. javanica* and 393 as *I. fumosorosea*. In addition to these results, Cabanillas *et al.* (2013) demonstrated the misidentifications of 12/16 *Isaria* isolates from the ARSEF or CBS collections, clearly underlining the need to develop a MLST strategy for the genus *Isaria* that would allow the correct identification of isolates. This strategy could facilitate and improve the further development of biological control programs using isolates from the genus *Isaria*. Finally, the ability of the ISSR markers to discriminate isolates 303, 305, and 307 may underline the potential for these markers to be used as a powerful tool to monitor the persistence of the isolates in field conditions. However, further studies are required to validate the specificity and sensitivity of the ISSR markers.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.11.009.

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