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-ROMES, 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

**ARTICLE INFO**

Article history:
Received 12 August 2015
Received in revised form 26 October 2015
Accepted 29 November 2015
Available online 17 December 2015

Corresponding Editor: Kevin D. Hyde

Keywords:
Biological control
Entomopathogenic fungi
*Isaria javanica*
ISSR markers
Morphology
Phylogeny

**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.

© 2015 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.

**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).
http://dx.doi.org/10.1016/j.funbio.2015.11.009
1878-6146/© 2015 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
genus Paecilomyces sensu Samson (1974), who divided this genus into two sections: section Paecilomyces (thermophilic) and section Isarioidae (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 (Cams et al. 2005).

Molecular phylogenetic studies have resurrected the genus Isaria (Luangsaa-ard et al. 2005; Sung et al. 2007). The polyphyletic nature of the genus Paecilomyces (i.e., including the sect. Isarioidae) has been demonstrated several times previously by analyses of the large and small subunit rRNA genes (Obornik et al. 2001; Luangsaa-ard et al. 2004). However, using the β-tubulin gene and the nuclear ribosomal internal transcribed spacer (ITS) region, Luangsaa-ard et al. (2005) investigated the phylogenetic relationships of Paecilomyces sect. Isarioidae species, and established the existence of a monophyletic group named ‘Isaria clade’, which includes Isaria amoecerosa Henne., Isaria catenamultula (Z.Q. Liang) Samson & Hywel-Jones, Isaria catenioliqua (Z.Q. Liang) Samson & Hywel-Jones, Isaria cicadae Miq., Isaria coleopetora (Samson & H.C. Evans) Samson & Hywel-Jones, Isaria farinosa (Holmsk.) Fr., Isaria fumosorosea Wize, Isaria ghannasi (Samson & H.C. Evans) Samson & Hywel-Jones, Isaria javonica (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, Luangsaa-ard et al. (2011) showed that Paecilomyces lilacinus, placed in the Ophiocordycipitaceae, was not related to Paecilomyces and Isaria, or still await transfer into appropriate genera.

‘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: ARGOS) (Sánchez et al. 2015). Ramirez-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 (Luangsaa-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 ARSEP or ‘Centraalbureau voor Schimmecultures’ (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 ARGOS.

### 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...