COLEGIO DE POSTGRADUADOS

INSTITUCIÓN DE ENSEÑANZA E INVESTIGACIÓN EN CIENCIAS AGRÍCOLAS

CAMPUS MONTECILLO

POSTGRADO DE FITOSANIDAD

ENTOMOLOGÍA Y ACAROLOGÍA

INTERACCIÓN DEL TIPO DE CULTIVO CON LA DIVERSIDAD DE ESPECIES DE HONGOS ENTOMOPATÓGENOS

JOSÉ ALFREDO CABRERA MORA

T E S I S

PRESENTADA COMO REQUISITO PARCIAL
PARA OBTENER EL GRADO DE:

MAESTRO EN CIENCIAS

MONTECILLO, TEXCOCO, ESTADO DE MÉXICO

2018

CARTA DE CONSENTIMIENTO DE USO DE LOS DERECHOS DE AUTOR Y DE LAS REGALIAS COMERCIALES DE PRODUCTOS DE INVESTIGACION

En adición al beneficio ético, moral y académico que he obtenido durante mis estudios en el Colegio de Postgraduados, el que suscribe José Alfredo Cabrera Mora, Alumno (a) de esta Institución, estoy de acuerdo en ser participe de las regalías económicas y/o académicas, de procedencia nacional e internacional, que se deriven del trabajo de investigación que realicé en esta institución, bajo la dirección del Profesor Ariel Wilbert Guzmán Franco, por lo que otorgo los derechos de autor de mi tesis Interacción del tipo de cultivo con la diversidad de especies de hongos entomopatógenos y de los productos de dicha investigación al Colegio de Postgraduados. Las patentes y secretos industriales que se puedan derivar serán registrados a nombre del colegio de Postgraduados y las regalías económicas que se deriven serán distribuidas entre la Institución, El Consejero o Director de Tesis y el que suscribe, de acuerdo a las negociaciones entre las tres partes, por ello me comprometo a no realizar ninguna acción que dañe el proceso de explotación comercial de dichos productos a favor de esta Institución.

Montecillo, Mpio. de Texcoco, Edo. de México, a 09 de Julio de 2018

Firma del
Alumno (a)

Dr. Ariel Wilbert Guzmán Franco
Vo. Bo. del Consejero o Director de Tesis

La presente tesis titulada: **Interacción del tipo de cultivo con la diversidad de especies de hongos entomopatógenos**, realizada por el alumno: **José Alfredo Cabrera Mora**, bajo la dirección del Consejo Particular indicado, ha sido aprobada por el mismo y aceptada como requisito parcial para obtener el grado de:

MAESTRO EN CIENCIAS
FITOSANIDAD
ENTOMOLOGÍA Y ACAROLOGÍA

CONSEJO PARTICULAR

CONSEJERO

DR. ARIEL W. GUZMÁN FRANCO

ASESORA

DRA. MA. TERESA SANTILLÁN GALICIA

ASESOR

DR. FERNANDO TAMAYO MEJÍA

Montecillo, Texcoco, Estado de México, Junio de 2018

INTERACCIÓN DEL TIPO DE CULTIVO CON LA DIVERSIDAD DE ESPECIES DE HONGOS ENTOMOPATÓGENOS

José Alfredo Cabrera Mora, M. en C.
Colegio de Postgraduados, 2018

RESUMEN

Beauveria y *Metarhizium* fueron aislados de suelo y larvas de gallina ciega de una variedad de cultivos en Puebla y Guanajuato, México. Los aislamientos se identificaron a nivel de especie usando la información de secuencia de Bloc y Factor de elongación 1-α. Aunque se encuentra ampliamente dispersa, *B. bassiana* solo se aisló del suelo y no de larvas de gallina ciega infectadas. Por el contrario, *B. pseudobassiana* se aisló predominantemente de larvas de gallina ciega (solo un aislamiento de suelo). El análisis de haplotipos de *B. bassiana* s.s. identificó 25 haplotipos que indican diversidad genética sustancial; ni el origen geográfico ni el tipo de cultivo explicaron esta variación genética. *Metarhizium brunneum* y *M. robertsii* también se aislaron solo de suelo, mientras que *M. anisopliae* s.s. y *M. pingshaense* solo se aislaron de larvas de gallina ciega. *Metarhizium anisopliae* s.s. solo se encontró infectando especies de *Paranomala* mientras que *M. pingshaense* solo se encontró infectando especies de *Phyllophaga*. La diversidad de especies en *Metarhizium* fue influenciada por el tipo de cultivo. Se discuten los roles ecológicos potenciales de estas especies.

Palabras clave: Especies crípticas, diversidad de especies, taxonomía molecular, rizosfera-competente, endófito

INTERACTIONS BETWEEN CROP TYPE AND SPECIES DIVERSITY OF ENTOMOPATHOGENIC FUNGI

José Alfredo Cabrera Mora, M. en C.

Colegio de Postgraduados, 2018

ABSTRACT

Beauveria and *Metarhizium* were isolated from soils and white grub larvae from a range of crops in Puebla and Guanajuato, Mexico. Isolates were identified to species level using Bloc and Elongation Factor 1-α sequence information. Although widespread, *B. bassiana* was only isolated from soil and not from infected white grubs. In contrast, *B. pseudobassiana* was predominantly isolated from white grub larvae (only one isolate from soil). Haplotype analysis of *B. bassiana* s.s. identified 25 haplotypes indicating substantial genetic diversity; neither geographical origin nor crop type explained this genetic variation. *Metarhizium brunneum* and *M. robertsii* were also only isolated from soil, while *M. anisopliae* s.s. and *M. pingshaense* were only isolated from white grub larvae. *Metarhizium anisopliae* s.s. was only found infecting *Paranomala* species while *M. pingshaense* was only found infecting *Phyllophaga* species. Species diversity in *Metarhizium* was influenced by crop type. The potential ecological roles of these species is discussed.

Key words: Cryptic species, species diversity, molecular taxonomy, rhizosphere-competent, endophyte

DEDICATORIA

A Dios por escucharme, orientarme y ayudarme a cumplir mis metas.

A mis padres Salomon y María de Lourdez por apoyarme sin importar el enorme sacrificio que han realizado y siguen haciendo por mis estudios.

A mi esposa Carolina por su apoyo, comprensión y amor incondicional.

A mi hermano Javier por ser un ejemplo de superación para mí.

A mi hermano Yosser por demostrarme una gran fortaleza para triunfar.

AGRADECIMIENTOS

A Dios por escuchar mis plegarias enfocadas a mi vida personal y académica. Gracias por guiarme a un futuro placentero en compañía de mis seres queridos.

Al Consejo Nacional de Ciencia y Tecnología (CONACYT) por el apoyo económico brindado para la realización de mis estudios de postgrado.

Al Colegio de Postgraduados y a la planta docente por contribuir con mi aprendizaje en sus diferentes instalaciones.

Al Dr. Ariel W. Guzmán Franco por su voto de confianza, dirigir esta investigación, compartir sus conocimientos y experiencia.

A la Dra. Ma. Teresa Santillán Galicia por su apoyo, disponibilidad y revisión del trabajo de investigación.

Al Dr. Fernando Tamayo Mejía por su tiempo y revisión de la investigación.

Al Mc. Jorge M. Valdez Carrasco por su apoyo en la toma de fotografías empleadas en esta investigación.

A mis padres Salomon y María de Lourdez que gracias a sus correcciones, enseñanzas, paciencia, sacrificios, amor y consejos han hecho de mí una persona íntegra y de beneficio, capaz de explorar nuevos horizontes.

A mi esposa Carolina por su amor, fortaleza y ser un pilar de vida indispensable para mí.

A mis hermanos Javier y Yosser por su apoyo incondicional en esta etapa de mi vida.

A mis compañeros del laboratorio de patología de insectos por su amistad, observaciones y consejos.

A los administrativos y directivos del Colegio de Postgraduados que de alguna manera contribuyeron para lograr mi objetivo.

CONTENIDO

LISTA DE CUADROS

LISTA DE FIGURAS

INTRODUCCIÓN GENERAL

El suelo es considerado un recurso natural extremadamente complejo que involucra factores como textura, capacidad de intercambio catiónico, materia orgánica, pH, temperatura y humedad que inciden en la diversidad y comportamiento de microorganismos como virus, bacterias, protozoos, nematodos y hongos (Inglis *et al*., 2001). Estos últimos pertenecen al reino Fungi, el cual consiste de alrededor de 1.5 millones de especies con 110 000 descritas, de este registro 90 géneros y 700 especies son patógenos de insectos (Roy *et al*., 2010). Se consideran así porque estos microorganismos establecen una simbiosis parasitaria con su hospedero, provocan su muerte y desarrollan a partir de éste (Raffel *et al*., 2008).

Los hongos entomopatógenos son filogenéticamente diversos con reproducción sexual y asexual, se consideran hongos verdaderos y pertenecen a cuatro divisiones: Chytridiomycota, Basidiomycota, Zygomycota y Ascomycota, en esta última se encuentran los géneros de hongos entomopatógenos más comunes y abundantes como *Beauveria*, *Metarhizium*, *Isaria*, *Lecanicillium* y *Tolypocladium*; en otras divisiones también tenemos a *Hirsutella* y *Nomuraea* con capacidad de controlar insectos plaga (Inglis *et al*., 2001).

Los hábitats comunes de estas especies fúngicas entomopatógenas son áreas no perturbadas, bosques y cultivos agrícolas; estos enemigos naturales se encuentran en el suelo como micelio saprófito o propágulos inactivos hasta su adhesión a un hospedero apropiado y compatible con su microambiente (Goettel *et al*., 2005). Se ha documentado que en agroecosistemas con producción anual se afectan las poblaciones de hongos mediante labores de labranza con remoción de suelo y exposición a rayos ultravioleta; estas acciones pueden

ocasionar que las comunidades fúngicas de estos agroecosistemas sean diferentes a la de hábitats menos perturbados; es probable que la presión de selección por diversas circunstancias promueva la diversidad (Meyling y Eilenberg, 2006).

En el medio ambiente, bajo condiciones ideales los hongos entomopatógenos se encuentran infectando una amplia gama de hospederos de forma natural; la especie *Metarhizium anisopliae* se ha encontrado asociada más a cultivos agrícolas (Meyling y Eilenberg, 2007) mientras que *Beauveria bassiana* es más cosmopolita prosperando en más de 700 especies hospederas (Inglis *et al*., 2001).

Para recuperar, registrar y evaluar la diversidad de hongos entomopatógenos en suelo se utiliza la técnica denominada "trampa cebo", en esta se usan inmaduros de *Tenebrio molitor* (Coleoptera: Tenebrionidae) o *Galleria mellonella* (Meyling y Eilenberg, 2007). Wakil *et al*. (2013) usando esta técnica con *G*. *mellonella* en suelos cultivados y no perturbados, identificaron las especies *B. bassiana*, *B. brongniartii*, *M. anisopliae*, *Isaria lilacinus*, *I. chlamydosporia* y *Lecanicillium attenuatum*. Por su parte, Saleh *et al*. (2016) en suelo de huertos frutales identificaron *M. anisopliae*, *L. lecanii* y *B. bassiana*, siendo esta última la más abundante. NouriAiin *et al*. (2014) y Tkaczuk *et al*. (2014) registraron *B. bassiana, I. farinosa, I. fumosorosea, Lecanicillium* sp y *M. anisopliae* en suelo de áreas no perturbadas, cultivos orgánicos y convencionales con siembra de cereales. Asimismo, Pérez-González *et al*. (2014) documentaron a *B. bassiana, B. pseudobassiana* y *M. robertsii* en suelo de maíz del estado de Guanajuato, México. Por el hábitat donde desarrollan estos microorganismos, algunos trabajos se han dirigido a entender los factores que favorecen o influencian la infección de hongos entomopatógenos sobre plagas de suelo, para considerar su posible uso como agentes potenciales

de control biológico. Tal es el caso de algunas especies de escarabajos, cuyas larvas desarrollan en el suelo y se alimentan de materia orgánica y raíces de varios cultivos, por lo que representan un serio problema como plaga en muchos agroecosistemas del mundo. En México se han identificado los géneros *Phyllophaga*, *Paranomala* (= *Anomala*) y *Cyclocephala* (Morón, 2007). Las especies de *Phyllophaga* son las más comunes, destructivas y ampliamente distribuidas (Aragón *et al*., 2005). Su importancia radica en que son plagas rizófagas y causan pérdidas económicas en maíz (*Zea mays*), papa (*Solanum tuberosum*), trigo (*Triticum aestivum*), tomate (*Lycopersicon esculentum*), frutales y pastizales (Ruiz *et al*., 2012).

En el estudio realizado por Carrillo-Benítez *et al*. (2013) se demostró la infección por *B. pseudobassiana* y *M. pingshaense* en larvas de *Phyllophaga* procedentes de parcelas de maíz. Lo que demuestra que el uso de hongos entomopatógenos en el sector agrícola es una alternativa potencial a los productos químicos. Sin embargo, debe tomarse en cuenta que la relación entre hospedante-parasito es un proceso coevolutivo, el cual implica cambios genéticos adaptativos por ambos organismos (Woolhouse *et al*., 2002). Dicho en otras palabras, la resistencia del huésped atribuye la adaptación de virulencia del patógeno y viceversa (Joop y Vilcinskas, 2016). La alta susceptibilidad del insecto en conjunto con la alta capacidad infectiva y virulencia de un hongo entomopatógeno son las causas que dan origen a epizootias, y se presentan naturalmente cuando la mayor parte de la población de insectos se infectan en la misma unidad de superficie y tiempo (Aguilera *et al*., 2017).

La apariencia física de esta infección en el ambiente es el micelio, considerado materia vegetativa y que en su entorno natural parece ser una sola unidad fisiológica y ecológica, pero en realidad es un mosaico genético (Roberts y St. Leger, 2004). En este contexto se sabe que

diferentes cepas de la misma especie muestran una variabilidad intraespecífica con respecto al tipo de hospedero, patogenicidad, morfología, producción de enzimas y toxinas; sin embargo, para tener una mejor comprensión sobre la filogenia de cepas silvestres es necesario uniformizar su genoma a través de cultivos monospóricos, además de tener un mejor control de su patogenicidad, virulencia y evitar posibles contaminantes en el aislamiento; en estudios realizados con aislamientos monospóricos y polispóricos para el control de insectos plaga se ha registrado que con estos últimos se obtiene mayor mortalidad, posiblemente por su amplia diversidad de genes virulentos (Aguilera *et al*., 2017). Con este conocimiento algunos investigadores están implementando control biológico por conservación, con la finalidad de mantener y/o mejorar las condiciones bióticas y abióticas que favorecen el desarrollo de especies fúngicas como *B. bassiana* y *M. anisopliae* para el control de plagas en agroecosistemas (Meyling y Eilenberg, 2007). La naturaleza dinámica de las poblaciones fúngicas está cambiando constantemente, es por ello que el estudio ecológico de estos microorganismos es la base para mejorar su manejo (Woolhouse *et al*., 2002).

Objetivo

Estudiar el efecto que tiene el tipo de cultivo, insecto hospedante y origen geográfico en la diversidad de especies de hongos entomopatógenos de los géneros *Beauveria* y *Metarhizium*.

Hipótesis

La diversidad de especies de hongos entomopatógenos de los géneros *Beauveria* y *Metarhizium*, será mayor al estudiar diferentes regiones geográficas, insecto hospedante y tipo de cultivo.

CAPÍTULO 1. CONTRASTING ROLES FOR GEOGRAPHICAL SEPARATION AND CROP TYPE ON SPECIES DIVERSITY IN *Metarhizium* AND *Beauveria*[1]

1.1 ABSTRACT

Beauveria and *Metarhizium* were isolated from soils and white grub larvae from a range of crops in Puebla and Guanajuato, Mexico. Isolates were identified to species level using Bloc and Elongation Factor 1-α sequence information. Although widespread, *B. bassiana* was only isolated from soil and not from infected white grubs. In contrast, *B. pseudobassiana* was predominantly isolated from white grub larvae (only one isolate from soil). Haplotype analysis of *B. bassiana* s.s. identified 25 haplotypes indicating substantial genetic diversity; neither geographical origin nor crop type explained this genetic variation. *Metarhizium brunneum* and *M. robertsii* were also only isolated from soil, while *M. anisopliae* s.s. and *M. pingshaense* were only isolated from white grub larvae. *Metarhizium anisopliae s.s.* was only found infecting *Paranomala* species while *M. pingshaense* was only found infecting *Phyllophaga* species. Species diversity in *Metarhizium* was influenced by crop type. The potential ecological roles of these species is discussed.

Key words: Cryptic species, species diversity, molecular taxonomy, rhizosphere-competent, endophyte

[1]Cabrera-Mora, J.A., Guzmán-Franco, A.W., Santillán-Galicia, M.T and Tamayo-Mejía, F. 2018. Contrasting roles for geographical separation and crop type on species diversity in *Metarhizium* and *Beauveria*. Fungal Ecology (**submitted**).

1.2 INTRODUCTION

Soil harbours a diverse community of macro and microorganisms that provide essential ecosystem services (Barrios, 2007). In particular, soil contains arthropods, plants and microorganisms such as bacteria, viruses, nematodes and fungi; the majority of microorganisms have yet to be studied (Orgiazzi *et al*., 2015). While some authors consider the soil microbial community as a 'black box' (Mommer *et al*., 2018), we do know that it is highly diverse and varies depending of soil type, climatic conditions, and how the soil is used (e.g. for agriculture) (Orgiazzi *et al*., 2015). Despite the great abundance of some microorganisms, such as fungi, knowledge about their diversity and ecology is still very scarce; from the potential 1.5 million fungal species in soil, only 10% have been studied (Hawksworth and Rossman, 1997; Hawksworth, 2001), and this includes fungi that are edible, saprophytic, endophytic and entomopathogenic (Hirsch *et al*., 2013).

It is difficult to be certain about the exact number of entomopathogenic fungi, but some authors suggest that there are approximately 90 genera with more than 700 species (Onofre *et al*., 2001). Entomopathogenic fungi have a worldwide distribution and can be found in both agricultural and forest systems including in decaying trees, leaves, insects and soil (Wakil *et al*., 2013; Sanjuan *et al*., 2015). Using molecular techniques, recent taxonomic studies have identified a number of cryptic species of *Beauveria* and *Metarhizium* that were previously all identified morphologically as *Beauveria bassiana* and *Metarhizium anisopliae* (Bischoff *et al*., 2009; Rehner *et al*., 2011). Since then there have only been a few studies done in the North America region on the occurrence of the full range of cryptic *Beauveria* and *Metarhizium* species, in soil. There are reports of *Beauveria bassiana s.s.*, *Beauveria pseudobassiana* and

Metarhizium robertsii in soil from maize crops (Pérez-González *et al*., 2014), and soil from tejocote (*Crataegus mexicana*) orchards (Muñiz-Reyes *et al*., 2014). Both of these studies used the *Galleria mellonella* L. baiting method for isolation. In the United States, Kepler *et al*. (2015) also isolated *M. anisopliae s.s.*, *Metarhizium pingshaense*, *Metarhizium lepidiotae* and *Metarhizium brunneum* from soil using selective media.

Before the identification of cryptic *Beauveria* and *Metarhizium* species, genetically different lineages in *M. anisopliae* s.l. and *B. bassiana* s.l. had been reported and were associated with specific habitats rather than different insect hosts (Bidochka *et al*., 2001, 2002). It is likely that the different genetic lineages reported by these authors, were indeed the different cryptic species subsequently reported by Bischoff *et al*. (2009) for *Metarhizium*, and by Rehner *et al*. (2011) for *Beauveria*. This could be confirmed by sequence information from mitochondrial and nuclear markers. A better understanding of the natural occurrence and ecology of entomopathogenic fungi, especially the cryptic species within the genera *Beauveria* and *Metarhizium*, will help us target these microorganisms better as biological control agents.

White grubs are a complex of species within the family Scarabaeidae, subfamilies Melolonthinae, Rutelinae and Dynastinae (Cock and Allard, 2013). White grubs are a serious problem in Mexico affecting the roots of many crops (Morón, 2010). Carrillo-Benítez *et al*. (2013) collected field-infected white grub larvae in maize crops from the central region of Mexico, and isolated *B. pseudobassiana*, *M. pingshaense*, *M. anisopliae s.s.* and *M. robertsii*.

Based on the research of Carrillo-Benítez *et al*. (2013) and Pérez-González *et al*. (2014) in the central region of Mexico on maize crops, we hypothesized that, if soil from regions that

were geographically separated were compared (including soil from different crops within each region), that the species diversity of *Beauveria* and *Metarhizium* within them would be greater than when soil from only one region or crop was studied. To test this hypothesis, we systematically sampled soil in two states in the central region of Mexico: Puebla and Guanajuato. In each state, we sampled soil from different crops, and compared the fungal diversity isolated from soil with the fungal diversity from infected white grub larvae from nearby sites.

1.3 MATERIAL AND METHODS

1.3.1 Collection of soil samples

Soil samples were collected from three crops in Puebla and four crops in Guanajuato (i.e. at a total of seven sampling sites; Table 1). Puebla and Guanajuato are 329 km apart, and the distance between crops within each state was circa 2 km. Samples from each location were taken as described by Pérez-González *et al*. (2014). Briefly, 15 soil samples, each weighing 1 kg, were taken over an area of approximately 1 ha at each site. Samples were taken from soil surrounding the roots of plants using a hand shovel that was disinfected with 70% ethanol between each sample. In the laboratory, soil samples were sieved through a 2-mm pore sieve to remove clumps and stones and maintained at 4 °C until required but never longer than six months.

1.3.2 Isolation of entomopathogenic fungi

Entomopathogenic fungi were isolated from soil using *G. mellonella* larvae as bait, and following the methods of Pérez-González *et al*. (2014). Briefly, the soil in each sample was moistened with sterile distilled water and three subsamples (80 g each) taken. Sub-samples were

each placed inside 100 mL capacity plastic containers that had previously been surface-sterilized using 70% ethanol. Three third instar *G. mellonella* larvae (PETMMAL S.A. de C.V., Cuatitlan Izcalli, Mexico) were placed onto the soil in each container. Prior to soil exposure, all larvae had been immersed in warm distilled water (56 °C) for 12 seconds, to prevent cocoon formation. All containers were sealed with a lid and incubated at 25 °C in complete darkness. During the first three days, all containers were inverted every 24 h to induce larval movement and maximise their exposure to any fungi that may have been present in the soil. A total of 945 *G. mellonella* larvae were used for the complete experiment. Larval mortality was recorded every 48 hours over three weeks.

Dead larvae were removed and surface sterilized by submerging them in 70% ethanol for ten seconds, followed by 1% sodium hypochlorite for three minutes and rinsing twice with sterile distilled water. Surface-sterilized larvae were placed in Petri dishes containing moistened filter paper and incubated at 25 °C until sporulation was evident. Under sterile conditions, a sample of conidia was taken from each sporulating larva using a bacteriological loop and transferred to Sabouraud dextrose agar (SDA) in a 90-mm Petri dish (BIOXON®, Becton Dickinson de Mexico S.A. de C.V. Cuautitlan Izcalli, Mexico) and incubated at 25 °C. A monosporic isolate was produced from each clean isolation, as described by Pérez-González *et al*. (2014). Mycelium and conidia from all the monosporic isolates were viewed under a stereomicroscope and identified to genus level based on morphological characteristics described in the taxonomic keys of Humber, (2012). Original and monosporic isolates were stored in 10% sterile glycerol at -80 °C in 2 mL cryovials (Nalgen, Thermo Fisher Scientific, Rochester, NY, USA) prior to molecular evaluation.

1.3.3 Collection of white grub larvae and fungal isolation

White grub larvae were collected in Puebla and Guanajuato. Despite previous reports from farmers that white grubs were present at the sites where we had sampled soil, we were unable to find any white grub larvae. For this reason, we identified sites with abundant white grub larvae at the time of the collection and sampled white grubs there; all were maize crops (Table 2). Methods for sampling third-instar larvae were as described by Guzmán-Franco *et al*. (2012). In the field, larvae were collected manually and placed in damp peat moss (Growing Mix®, Canada) contained within 70×40×20 cm lidded plastic trays prior to transportation to the laboratory. In the laboratory each larva was identified to genus level based on the presence of palidia in the last abdominal segment (raster) and anal aperture morphology (Morón, 1986; Ritcher, 1966), and placed individually in 100-mL plastic containers with 80 g of damp peat moss. Larval mortality was recorded every three days for 30 days. Dead larvae were handled as described previously for *G. mellonella* larvae (section 2.2). Fungal isolation and handling of isolates was also the same as described previously (section 2.2).

1.3.4 Mycelium production and DNA extraction

Conidia from monosporic isolates were sub-cultured onto SDA in 90 mm Petri dishes and incubated at 25 °C in complete darkness for five days. Using a sterile metallic spatula, mycelium from each isolate was removed and deposited into a sterile 20 mL glass vial. Sterile cellophane sheets had been placed on top of the agar prior to subculture making it easier to harvest the mycelium without collecting any of the agar. All vials were frozen at -20 °C overnight and lyophilised for 24 h.

For DNA extraction, 0.025 g of lyophilised mycelium was placed into a 1.5 mL Eppendorf tube and plunged into liquid nitrogen for five minutes. The frozen mycelium was then ground using a pellet pestle rod (Daigger and Company Inc., Vernon Hills. Il, USA) and DNA extracted using a DNeasy® Plant Minikit following the manufacturer's instructions (with the modifications suggested by Fargues *et al*. [2002]). Specifically, 20 µL of proteinase K (supplied with the kit) was added to each sample and the tubes were incubated at 65 °C for one and six hours for *Metarhizium* and *Beauveria,* respectively. The concentration of DNA was estimated using a NanoDrop (Thermo Fisher Scientific, Inc. Waltham, MA, USA) and stored at -20 °C until required.

1.3.5 Phylogenetic placement of isolates

For phylogenetic placement of the *Metarhizium* isolates, a fragment of the 5' end of the Elongation Factor 1-α (EF1-α) was amplified using the primers EF1T and EF2T (Bischoff *et al*., 2009). PCR amplifications were done as described by Hernández-Domínguez and Guzmán-Franco (2017). For the *Beauveria* isolates, partial sequences of the nuclear intergenic region Bloc (Rehner *et al*., 2011) were amplified using the primers B22U and B822L (Rehner *et al*., 2006, 2011). PCR amplifications were made as described by Pérez-González *et al*. (2014). All PCR products were visualized on a 2% agarose gel in 1X TAE buffer. The gels were stained with ethidium bromide (0.1 µg mL^{-1}) and photographed. PCR products from isolates were sent to Macrogen Inc. (Geumchen-gu, Seoul, Korea) for sequencing.

Sequence traces were edited using BioEdit v 7.1.9. (Hall, 1999). Multiple sequence alignments were made using the Clustal W. program (Thompson *et al*., 1994) implemented in

BioEdit. After alignment and trimming, the final length of the TEF sequences were 582 bp for the *Metarhizium* isolates (31 sequences), 746 bp for the *B. bassiana* isolates (41 sequences) and 743 bp for the *B. pseudobassiana* (five sequences). GenBank accession numbers are listed in Table 2. Maximum Likelihood (ML) analysis, based on the Tamura-Nei model, was done using Mega v. 5 (Tamura *et al*., 2011). The robustness of branches was estimated by bootstrap analysis with 1000 repeated samples from the data (Felsenstein, 1985). In addition, sequences from related species within the genus *Metarhizium* and *Beauveria* were retrieved from GenBank and used for comparison (Table 2).

1.3.6 Analysis of genetic variation amongst *B. bassiana* isolates based on Bloc sequences

Two analyses were done using only the sequence information for the *B. bassiana* isolates as they were the most abundant. A Haplotype Network Analysis was done using TCS v. 1.21 (Clement *et al*., 2000), with a connection limit amongst haplotypes set to a value of 95%. Using analysis of molecular variance (AMOVA) we obtained partition of genetic variation: between isolates from the two states (Puebla and Guanajuato); amongst isolates obtained from soil around different host plants; and amongst all isolates regardless of state or crop soil type., was determined by analysis of molecular variance (AMOVA), estimated by computing F-statistics using Arlequin v. 3.5 (Excoffier and Lischer 2015) with 10,000 permutations.

1.4 RESULTS

1.4.1 Occurrence of entomopathogenic fungi

A total of 63 isolates were recovered from soil; 42 were identified morphologically as belonging to the genus *Beauveria* and 21 to the genus *Metarhizium*. No *Metarhizium* isolates were recovered from soil sampled in Guanajuato, and no isolates were recovered from soil sampled from chilli crops in Puebla. In total we collected 539 larvae in Guanajuato and 779 larvae in Puebla. The number of larvae collected from the different genera at each sampling site is shown in Table 2. From these, 15 isolates were recovered from infected white grub larvae; five were identified morphologically as belonging to the genus *Beauveria* and ten to the genus *Metarhizium* (Table 2).

1.4.2 Phylogenetic placement of isolates

For the *Beauveria* isolates, GenBank accession numbers for all sequence data generated are listed in Table 3. Phylogenetic analysis of all isolates showed that 41 isolates were *Beauveria bassiana s.s.*, (Fig. 1). All *B. bassiana* isolates were obtained from soil and the majority (32) were from Puebla (only nine from Guanajuato) (Table 3). Six isolates of *B. pseudobassiana* were recovered (Table 3, Fig. 1); five of these isolates were obtained from infected white grubs (four from Puebla and one from Guanajuato) (Table 3).

For the *Metarhizium* isolates, GenBank accession numbers for all sequences are listed in Table 4. Phylogenetic analysis of all isolates showed that the most abundant species was *M. robertsii*, with 17 isolates (Fig. 2), all from soils collected in Puebla and all from maize (Table

4). Four isolates were *M. pingshaense* (Table 2; Fig. 2), all of which were obtained from infected white grubs (three from Guanajuato and one from Puebla) (Table 4). Three isolates were *M. brunneum* (Fig. 2), all of which were from soils collected in Puebla (all from bean) (Table 4). Seven isolates were *M. anisopliae s.s.* (Fig 2), six of which were from infected white grubs (five from Puebla and one from Guanajuato), and only one obtained from soil collected in Puebla (Table 4).

1.4.3 Genetic variation amongst *B. bassiana* isolates

The haplotype network analysis of *B. bassiana* isolates showed the existence of 25 haplotypes. Three haplotypes were independent and had only one sequence each. The remaining haplotypes were distributed in seven networks. Five networks had two haplotypes, one network had three haplotypes, and one network had nine haplotypes (Fig. 3). The most common haplotype was H4, comprising 11 isolates from soil collected in maize and bean crops in Puebla. This was followed by haplotype H7 (four isolates) and haplotype H8 (three isolates), which were also from soil collected from maize and bean crops in Puebla (Table 3). There was no clear pattern in the distribution of haplotypes, except that all isolates from Guanajuato fell into haplotypes that were independent from the other networks.

The AMOVA analysis showed that 19.6% of the genetic variation could be explained by the state of origin (Puebla or Guanajuato) (Table 5). The host plant associated with the soil explained only 3.69% of the total genetic variation. However, 76.70% of the total variation was explained by comparisons amongst isolate sequences regardless of the host plant soil or state of origin (Table 5).

1.5 DISCUSSION

We studied the diversity of entomopathogenic fungi in soil from two different regions and from different crops. Despite the fact that the genera *Beauveria* and *Metarhizium* have a worldwide distribution (Meyling and Eilenberg, 2007), there are gaps in our knowledge about their distribution and ecology which requires more research. For example, many reports describing the distribution of *Beauveria* and *Metarhizium* species were done before the cryptic species within the complex of *B. bassiana* s.l. and *M. anisopliae* s.l. were described (Bischoff *et al*., 2009; Rehner *et al*., 2011).

From the genus *Beauveria* we found both *B. bassiana s.s.* and *B. pseudobassiana*. These species had already been reported from Guanajuato (Carrillo-Benitez *et al*., 2013; Pérez-González *et al*., 2014). In the current study we found that *B. bassiana* s.s. was only found in soil (using *G. mellonella* larvae as bait) and not infecting white grub larvae in the field, regardless of the region or the crop (Table 3). In contrast, *B. pseudobassiana* was predominantly found infecting white grub larvae (only one isolate was from soil), regardless of region. This represents clear niche separation between these two species. However, the role that each species plays in regulating insect populations is unclear. Preliminary experiments (data not shown) showed that selected isolates from *B. bassiana* (AP1, AP2, AG4, AG2 and AG6) and *B. psuedobassiana* (AG10) (Table 3) collected in this study, were not pathogenic to *Phyllophaga* spp. larvae, which confirms previous reports for other isolates from the same species when evaluated against *Phyllophaga* and *Paranomala* (=*Anomala*) (Guzmán-Franco *et al*., 2012; Carrillo-Benítez *et al*., 2013).

Many plant species overcome nutrient deficiencies by associating themselves symbiotically with bacteria, mycorrhiza and/or endophytic fungi (Clark and Zeto, 2000). Entomopathogenic fungi have also developed endophytic relationships with plants to acquire carbohydrates necessary for their survival (Bonfonte and Genre, 2010; Barelli *et al*., 2016). It is possible that insect pathogenicity is an adaptation of endophytic fungi to provide the plant with complex nitrogen sources from herbivores (Behie and Bidochka, 2014; Barelli *et al*., 2016). Although we did not look for *B. pseudobassiana* within plant tissues, we believe that this species has potential to be endophytic, spending part of its life cycle within the plant and, under certain (as yet unclear) circumstances, also infect white grub larvae, providing nitrogen sources to maize plants. Our findings, and others reported previously (Carrillo-Benitez *et al*., 2013; Pérez-González *et al*., 2014), suggest that a specific tritrophic relationship between maize roots, white grub larvae and *B. pseudobassiana* could exist because this fungal species has only been found infecting white grub larvae and not *G. mellonella* larvae. This tritrophic relationship suggested for *B. pseudobassiana* is not shared with isolates of *B. bassiana s.s.*, which were never found infecting white grub larvae. However, *B. bassiana s.s.* is a much more widely distributed species than *B. pseudobassiana* being found in both regions and in the soils from all crops studied except chilli (Table 3). It is likely that *B. bassiana* s.s may survive in soil because it is rizhosphere-competent, as previously reported for some isolates of *M. anisopliae s.l.* (Hu and St. Leger, 2002; St. Leger, 2008). Rhizospere competence means that these fungi can survive around the roots using the nutrients available to them in the root exudates (de Weert and Bloemberg, 2006).

Whether the *B. bassiana s.s.* isolates we obtained in this study can infect above-ground insects is unknown and currently under evaluation. The fact that the *B. bassiana s.s.* isolates we obtained in this study could infect *G. mellonella* larvae suggests that this might be possible. We

found substantial genetic variation amongst *B. bassiana s.s.* isolates which could not be related to geographical origin or crop type. We suggest this genetic variation may allow *B. bassiana* to survive in different environments, adapting to changes in biotic or abiotic conditions through a series of local extinctions and resurgences of particular genotypes. These phenomena have recently been reported for *M. anisopliae s.s.* (Hernández-Domínguez and Guzmán-Franco, 2017).

Although a combination of *B. bassiana* and *B. pseudobassiana* could, together, favour the growth of maize plants, it does not appear to contribute to reducing white grub populations in the field. Overall, few infected field-collected larvae were found and only six isolates were obtained. Most importantly, no infection was achieved by artificially inoculating white grub larvae in the genera *Phyllophaga* or *Paranomala* (data not shown; Guzmán-Franco *et al*., 2012), which are important insect pests in Mexico (Marin-Jarillo and Bujanos-Muñis, 2003).

Greater species diversity was found in the genus *Metarhizium*; there were four *Metarhizium* species compared with only two *Beauveria* species. No *Metarhizium* isolates were found in soil from Guanajuato, but 21 isolates were found in the soil from Puebla. Amongst the isolates from Puebla there was a very clear separation between crops. *Metarhizium brunneum* was isolated from bean soil and *M. robertsii* from maize soil. Wyrebek *et al*. (2011) reported an effect of plant species on *Metarhizium* diversity; *M. robertsii*, *M. brunneum* and *M. guizhouense* were associated with the soil around grasses, shrubs and trees, respectively, which was in line with our findings.

In our study a different set of species were found infecting white grub larvae, and the genus of the white grub larvae affected the diversity (Table 3). We only isolated *M. anisopliae s.s.* from white grubs in the genus *Paranomala*, and *M. pingshaense* only from white grubs in the genus *Phyllophaga*. This suggests that *M. robertsii* and *M. brunneum* may have a partially endophytic life cycle as previously suggested by Sasan and Bidochka, (2012). Furthermore, given that the soil samples were taken from the area surrounding the roots, it is possible that *M. brunneum* and *M. robertsii* could also be rhizosphere-competent. We believe *M. anisopliae* and *M. pingshaense* are predominantly insect regulators and use white grub larvae as their primary nitrogen source (Barelli *et al*., 2016). Despite low levels of infection in the field, *M. pingshaense* is known to be highly virulent against larvae of *Paranomala* (= *Anomala*) *cincta* in the laboratory (Guzmán-Franco *et al*., 2012). It is unclear why *P.* (= *A.*) *cincta* is so susceptible to *M. pingshaense* in the laboratory while *Ph. polyphylla* is not (Guzmán-Franco *et al*., 2012). We suggest that species within the genus *Phyllophaga* are co-evolved to survive in the soil environment and have developed mechanisms to avoid infection in the field. However, as mentioned earlier, under specific, but currently unknown circumstances, some larvae might become infected, possibly to fulfil a nutriment requirement of the plant. While *M. pingshaense* and *M. anisopliae* seem to have an insect-regulation role, the reason for differential susceptibility between *Paranomala* and *Phyllophaga* species is unclear, despite white grubs from both genera co-existing in the same areas (Marin-Jarillo and Bujanos-Muñis, 2003) and presumably under the same selection pressure from the microbial community.

We believe that, in the white grub ecosystem, entomopathogenic fungi may have evolved into a more endophytic or rhizosphere-competent relationship with plants rather than relying exclusively on infecting insects for their survival. This change in behaviour is most likely to be

because it requires a lower energy investment compared with infecting white grubs, which may have evolved a more complex defence system against infection as a result of continuous exposure to the soil microbiota. This needs experimental confirmation.

In conclusion, *Metarhizium* had a higher species diversity than *Beauveria* in the regions studied. We found no effect of geographical separation, but species diversity in *Metarhizium* was influenced by crop type. The host used for isolation (i.e. *G. mellonella* as a soil bait or direct from white grubs) strongly influenced which species would be isolated. We believe all species found could have some sort of endophytic relationship with plants, and that niche separation suggests that each species has a different role and relationship with particular plants. Our results contribute to testing the hypothesis that these fungal species have more complex roles in the ecosystem than just as insect regulations.

1.6 ACKNOWLEDGEMENTS

JACM received a scholarship from CONACYT-Mexico for his MSc studies. The Colegio de Postgraduados funded this research through resources allocated to all students.

1.7 REFERENCES

Barelli L, Moonjely S, Behiel SW, Bidochka MJ, 2016. Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. *Plant Molecular Biology* **90**: 657-664.

Barrios E, 2007. Soil biota, ecosystem services and land productivity. *Ecological Economics* **64**: 269-285.

Behie SW, Bidochka MJ, 2014. Ubiquity of insect-derived nitrogen transfer to plants by endophytic insect-pathogenic fungi: an additional branch of the soil nitrogen cycle. *Applied and Environmental Microbiology* **80**: 1553-1560.

Bidochka MJ, Kamp AM, Lavender TM, Dekoning J, Amritha de Croos JN, 2001. Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: Uncovering cryptic species? *Applied and Environmental Microbiology* **67**: 1335-1342.

Bidochka MJ, Menzies FV, Kamp AM, 2002. Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences. *Archives of Microbiology* **178**: 531-537.

Bischoff JF, Rehner SA, Humber RA, 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia* **101**: 512-530.

Bonfante P, Genre A, 2010. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nature Communications* **1**: 48.

Carrillo-Benítez MG, Guzmán-Franco AW, Alatorre-Rosas R, Enríquez-Vara JN, 2013. Diversity and genetic population structure of fungal pathogens infecting white grub larvae in agricultural soils. *Microbial Ecology* **65**: 437-449.

Clark RB, Zeto SK, 2000 Mineral acquisition by arbuscular mycorrhizal plants. *Journal of Plant Nutrition* **23**: 867-902.

Clement M, Posada D, Crandall K, 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657-1660.

Cock MJW, Allard GB, 2013. Observations on white grubs affecting sugar cane at the Juba Sugar Project, South-Western Somalia, in the 1980s, and implications for their management. *Insects* **4**: 241-272.

De Weert S, Bloemberg GV, 2007. *Rhizosphere competence and the role of root colonization in biocontrol*. In: Gnanamanickam S.S. (ed.) Plant-Associated Bacteria. Springer, Netherlands, pp 317-333.

Fargues J, Bon MC, Manguin S, Couteaudier Y, 2002. Genetic variability among *Paecilomyces fumosoroseus* isolates from various geographical and host insect origins based on the rDNA-ITS regions. *Mycological Research* **106**: 1066-1074.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.

Guzmán-Franco AW, Hernández-López J, Enríquez-Vara JN, Alatorre-Rosas R, Tamayo-Mejía F, Ortega-Arenas LD, 2012. Susceptibility of *Phyllophaga polyphylla* and *Anomala cincta* larvae to *Beauveria bassiana* and *Metarhizium anisopliae* isolates, and the interaction with soil properties. *BioControl* **57**: 553-563.

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Hawksworth DL, 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycological Research* **105**: 1422-1432.

Hawksworth DL, Rossman AY, 1997. Where are all the undescribed fungi? *Phytopathology* **87**: 888-891.

Hernández-Domínguez C, Guzmán-Franco AW, 2017. Species diversity and population dynamics of entomopathogenic fungal species in the genus *Metarhizium*: a spatiotemporal study. *Microbial Ecology* **74**: 194-206.

Hirsch J, Galidevara S, Strohmeier S, Uma DK, Reineke A, 2013. Effects on diversity of soil fungal community and fate of an artificially applied *Beauveria bassiana* strain assessed through 454 pyrosequencing. *Microbial Ecology* **66**: 608-620.

Hu G, St. Leger RJ, 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Applied and Environmental Microbiology* **68**: 6383-6387.

Humber RA, 2012. *Identification of entomopathogenic fungi*. In: Lacey LA (ed.) Manual of Techniques in Invertebrate Pathology, 2nd edition. Academic Press, London, pp 151-187.

Kepler RM, Ugine TA, Maul JE, Cavigelli MA, Rehner SA, 2015. Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. *Environmental Microbiology* **17**: 2791-2804.

Marin-Jarillo A, Bujanos-Muñiz R, 2003. Especies del complejo "Gallina Ciega" del género Phyllophaga en Guanajuato, México. *Agricultura Técnica en México* **34**: 349-355.

Meyling NV, Eilenberg J, 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biological Control* **43**: 145-155.

Mommer L, Cotton TEA, Raaijmakers JM, Termorshuizen AJ, van Ruijven J, Hendriks M, van Rijssel SQ, van de Mortel JE, van der Paauw JW, Schijlen EGWM, Smit-Tiekstra AE, Berendse F, De Kroon H, Dumbrell AJ, 2018. Lost in diversity: the interactions between soil-borne fungi, biodiversity and plant productivity. *New Phytologist* **218**: 542-553.

Morón MA, 1986. El género Phyllophaga en México: morfología, distribución y sistemática supraespecifica (Insecta: Coleoptera) Publ. 20, Instituto de Ecología, México.

Morón MA, 2010. Diversidad y Distribución del Complejo "Gallina Ciega" (Coleoptera: Scarabediae). In: Plagas del Suelo. Rodríguez-del-Bosque LA y Morón MA (eds). Mundi Prensa. México, pp 41-63.

Muñiz-Reyes E. Guzman-Franco AW, Sánchez-Escuder J., Nieto-Angel R, 2014. Occurrence of entomopathogenic fungi in tejocote (*Crataegus mexicana*) orchard soils and their pathogenicity against *Rhagoletis pomonella*. *Journal of Applied Microbiology* **117**: 1450-1462.

Onofre SB, Miniuk CM, De Barros NM, Azevedo JL, 2001. Pathogenicity of four strains of entomopathogenic fungi against the bovine tick *Boophilus microplus*. *American Journal of Veterinary Research* **62**: 1478-1480.

Orgiazzi A, Bonnet DM, Panagos P, Arjen GG, Lemanceau P, 2015. Soil biodiversity and DNA barcodes: opportunities and challenges. *Soil Biology and Biochemistry* **80**: 244-250.

Pérez-González VH, Guzmán-Franco AW, Alatorre-Rosas R, Hernández-López J, Hernández-López A, Carrillo-Benítez MG, Baverstock J, 2014. Specific diversity of the entomopathogenic fungi *Beauveria* and *Metarhizium* in Mexican agricultural soils. *Journal of Invertebrate Pathology* **119**: 54-61.

Rehner SA, Buckley E, Infante F, Castillo A, Vega FE, 2006. Phylogenetic origins of African and Neotropical *Beauveria bassiana* s.l. pathogens of the coffee berry borer. *Journal of Invertbrate Pathology* **93**: 11-23.

Rehner SA, Minnis AM, Sung G-H, Luangsa-ard JJ, Devetto L, Humber RA, 2011. Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. *Mycologia* **103**: 1055-1073.

Ritcher PO, 1966. *White grubs and their allies. A study of North American Scarabaeid larvae*. Studies in Entomology No. 4. Oregon State University Press, Corvallis 219 pp.

Sanjuan TI, Franco-Molano AE, Kepler RM, Spatafora JW, Tabima J, Vasco-Palacios AM, Restrepo S, 2015. Five new species of entomopathogenic fungi from the Amazon and evolution of neotropical *Ophiocordyceps*. *Fungal Biology* **119**: 901-916.

Sasan RK, Bidochka MJ, 2012. The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *American Journal of Botany* **99**: 101-107.

St. Leger RJ 2008. Studies on adaptations of *Metarhizium anisopliae* to life in the soil. *Journal of Invertebrate Pathology* **98**: 271-276.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731-2739.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Wakil W, Ghazanfar MU, Riasat T, Kwon YJ, Qayyum MA, Yasin M, 2013. Occurrence and diversity of entomopathogenic fungi in cultivated and uncultivated soils in Pakistan. *Entomolological Research* **43**: 70-78.

Wyrebek M, Huber C, Sasan RK, Bidochka MJ, 2011. Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. *Microbiology* **157**: 2904-2911.

Table Legends

Table 1. Sites in Guanajuato and Puebla states, Mexico, where soil was collected for isolation of entomopathogenic fungi. ** = zucchini (*Cucurbita pepo* L.), cucumber (*Cucumis sativus* L.) and beans (*Phaseoulus vulgaris* L.).

State	Municipality	Locality	Crop
Puebla	Tetela de Ocampo	San Nicolas	Maize (*Zea mays* L.)
	""	Tonalapa	Bean (*Phaseoulus vulgaris* L.)
	""	Cuapancingo	Chilli (*Capsicum annuum* L.)
Guanajuato	Salvatierra	Urireo	mixed **
	Comonfort	5 de Febrero	Maize
	Comonfort	5 de Febrero	Sorghum (*Sorghum bicolor* (L.) Moench)
	Comonfort	Morales	Jicama (*Pachyrhizus erosus* (L.) Urb.)

Table 2. Sites (all maize crops) in Guanajuato and Puebla states, Mexico, where white grub larvae were collected for subsequent isolation of entomopathogenic fungi. The number of white grubs collected (No), and the number of isolates from the genera *Beauveria* and *Metarhizium*, are shown. Ma = *M. anisopliae s.s.*, Mp = *M. pingshaense*, Bp = *B. pseudobassiana*.

State	Municipality	Locality	Genus	No	No. isolates	
					Beauveria	*Metarhizium*
Puebla	Tetela de Ocampo	San Nicolas	*Paranomala*	230	0	4 (Ma)
			Phyllophaga	122	1 (Bp)	1 (Mp)
			Cyclocephala	9	2 (Bp)	0
	""	Acatlán	*Paranomala*	13	0	1 (Ma)
			Phyllophaga	350	1 (Bp)	0
			Cyclocephala	4	0	0

Guanajuato	Jerecuaro	San Lorenzo	*Paranomala*	69	0	1 (Ma)
			Phyllophaga	282	1 (Bp)	3 (Mp)
			Cyclocephala	44	0	0

Table 3. Isolates of *Beauveria* species obtained and sequenced for phylogenetic analysis. GenBank accession numbers and haplotype assignment (only for *B. bassiana*) following Haplotype Network Analysis are shown.

State	Isolate	Crop	Species	GenBank	Haplotype
Isolates obtained from soil					
Puebla	AP1	bean	*B. bassiana*	**MH374233**	H19
	AP9	bean	*B. bassiana*	**MH374216**	H4
	AP20	bean	*B. bassiana*	**MH374219**	H8
	AP26	bean	*B. bassiana*	**MH374231**	H3
	AP27	bean	*B. bassiana*	**MH374223**	H4
	AP39	bean	*B. bassiana*	**MH374226**	H8
	AP40	bean	*B. bassiana*	**MH374227**	H7
	AP2	maize	*B. bassiana*	**MH374211**	H7
	AP3	maize	*B. bassiana*	**MH374213**	H4
	AP4	maize	*B. bassiana*	**MH374251**	H18
	AP5	maize	*B. bassiana*	**MH374214**	H4
	AP6	maize	*B. bassiana*	**MH374246**	H17
	AP7	maize	*B. bassiana*	**MH374215**	H7
	AP8	maize	*B. bassiana*	**MH374232**	H16
	AP10	maize	*B. bassiana*	**MH374239**	H15
	AP11	maize	*B. bassiana*	**MH374217**	H4
	AP12	maize	*B. bassiana*	**MH374248**	H14

	AP18	maize	*B. bassiana*	**MH374218**	H4
	AP19	maize	*B. bassiana*	**MH374244**	H13
	AP21	maize	*B. bassiana*	**MH374220**	H12
	AP22	maize	*B. bassiana*	**MH374238**	H11
	AP23	maize	*B. bassiana*	**MH374221**	H7
	AP25	maize	*B. bassiana*	**MH374222**	H8
	AP28	maize	*B. bassiana*	**MH374250**	H10
	AP29	maize	*B. bassiana*	**MH374249**	H9
	AP30	maize	*B. bassiana*	**MH374224**	H4
	AP38	maize	*B. bassiana*	**MH374225**	H4
	AP41	maize	*B. bassiana*	**MH374228**	H4
	AP42	maize	*B. bassiana*	**MH374229**	H6
	AP43	maize	*B. bassiana*	**MH374247**	H5
	AP44	maize	*B. bassiana*	**MH374230**	H4
	AP45	maize	*B. bassiana*	**MH374234**	H1
Guanajuato	AG1	maize	*B. bassiana*	**MH374212**	H4
	AG3	maize	*B. bassiana*	**MH374243**	H24
	AG5	maize	*B. bassiana*	**MH374242**	H22
	AG6	maize	*B. bassiana*	**MH374236**	H20
	AG8	maize	*B. bassiana*	**MH374245**	H2
	AG9	maize	*B. bassiana*	**MH374235**	H20
	AG2	sorghum	*B. bassiana*	**MH374241**	H25
	AG4	sorghum	*B. bassiana*	**MH374237**	H23
	AG7	jicama	*B. bassiana*	**MH374240**	H21
	AG10	bean	*B. pseudobassiana*	**MH374254**	-----
Isolates obtained from infected white grubs					

Puebla	GP3	maize	*B. pseudobassiana*	**MH374255**	-----
	GP9	maize	*B. pseudobassiana*	**MH374252**	-----
	GP10	maize	*B. pseudobassiana*	**MH374256**	-----
	GP11	maize	*B. pseudobassiana*	**MH374253**	-----
Guanajuato	GG4	maize	*B. pseudobassiana*	**MH422574**	-----

Table 4. Isolates of *Metarhizium* species obtained and sequenced for phylogenetic analysis. GenBank accession numbers are shown.

State	Isolate	Crop	Species	GenBank
Isolates obtained from soil				
Puebla	AP13	bean	*M. brunneum*	**MH374191**
	AP14	bean	*M. brunneum*	**MH374192**
	AP34	bean	*M. brunneum*	**MH374193**
	AP47	bean	*M. anisopliae*	**MH374190**
	AP15	maize	*M. robertsii*	**MH374208**
	AP17	maize	*M. robertsii*	**MH374209**
	AP24	maize	*M. robertsii*	**MH374205**
	AP31	maize	*M. robertsii*	**MH374199**
	AP32	maize	*M. robertsii*	**MH374198**
	AP33	maize	*M. robertsii*	**MH374197**
	AP35	maize	*M. robertsii*	**MH374203**
	AP36	maize	*M. robertsii*	**MH374196**
	AP37	maize	*M. robertsii*	**MH374207**
	AP46	maize	*M. robertsii*	**MH374194**
	AP48	maize	*M. robertsii*	**MH374204**
	AP49	maize	*M. robertsii*	**MH374210**

	AP50	maize	*M. robertsii*	**MH374195**
	AP51	maize	*M. robertsii*	**MH374200**
	AP52	maize	*M. robertsii*	**MH374201**
	AP53	maize	*M. robertsii*	**MH374206**
	AP54	maize	*M. robertsii*	**MH374202**
Isolates obtained from infected white grubs				
Puebla	GP1	maize	*M. anisopliae*	**MH374186**
	GP2	maize	*M. anisopliae*	**MH374187**
	GP4	maize	*M. anisopliae*	**MH374188**
	GP5	maize	*M. anisopliae*	**MH374189**
	GP8	maize	*M. anisopliae*	**MH374185**
	GP7	maize	*M. pingshaense*	**MH374183**
Guanajuato	GG1	maize	*M. pingshaense*	**MH374180**
	GG2	maize	*M. pingshaense*	**MH374181**
	GG3	maize	*M. pingshaense*	**MH374182**
	GG5	maize	*M. anisopliae*	**MH374184**

Table 5. Results of AMOVA analysis of Bloc sequences from *B. bassiana* isolates.

Source of variation	d.f.	Sum of squares	Variance components	Variation (%)	*F* statistic	*P* value
Amongst groups (states)	1	58.429	2.9887	19.61	0.1960	0.09
Amongst populations (host plant soil) within groups (states)	4	43.063	0.5626	3.69	0.0459	0.40
Within populations	36	420.92	11.692	76.70	0.2329	0.01
Total	40	522.41	15.243			

Figure Legends

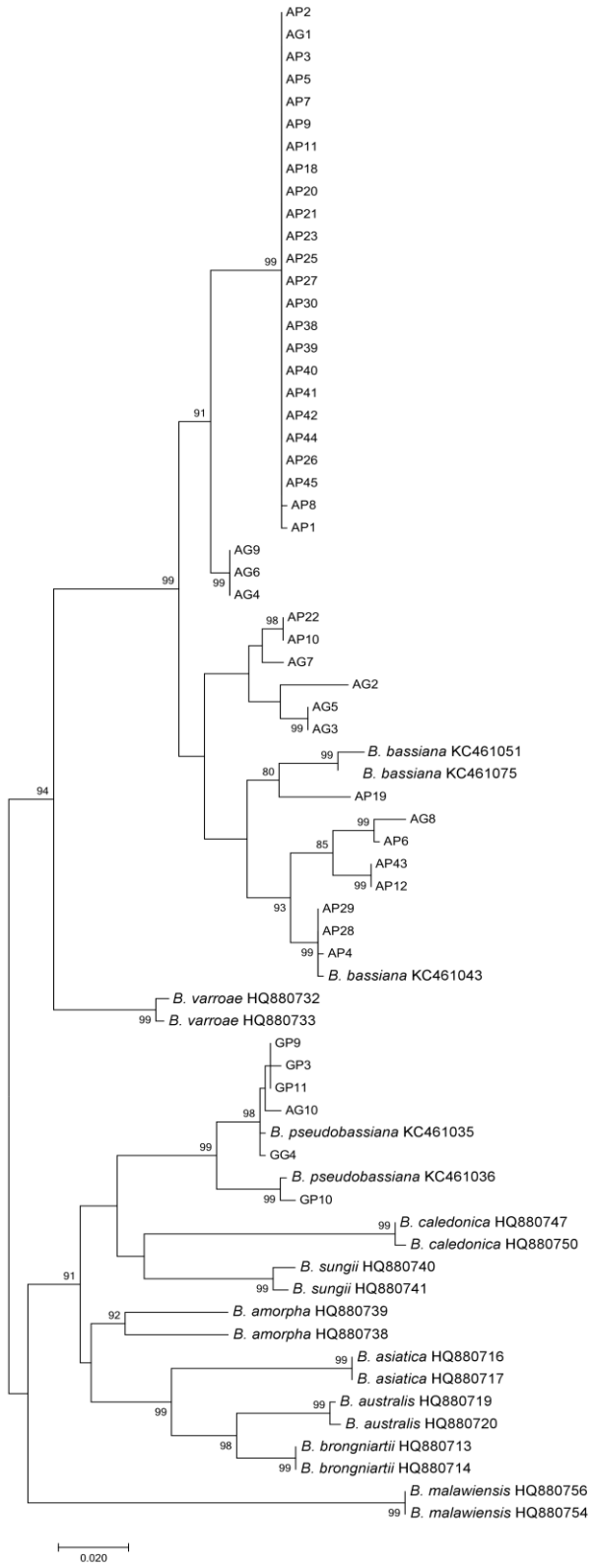

Figure 1. Phylogeny of *Beauveria* species and isolates inferred from Maximum Likelihood (ML) analysis of the Bloc sequence data. Isolates used as references are labelled according to their GenBank accession numbers. Only bootstrap values above 80% are shown.

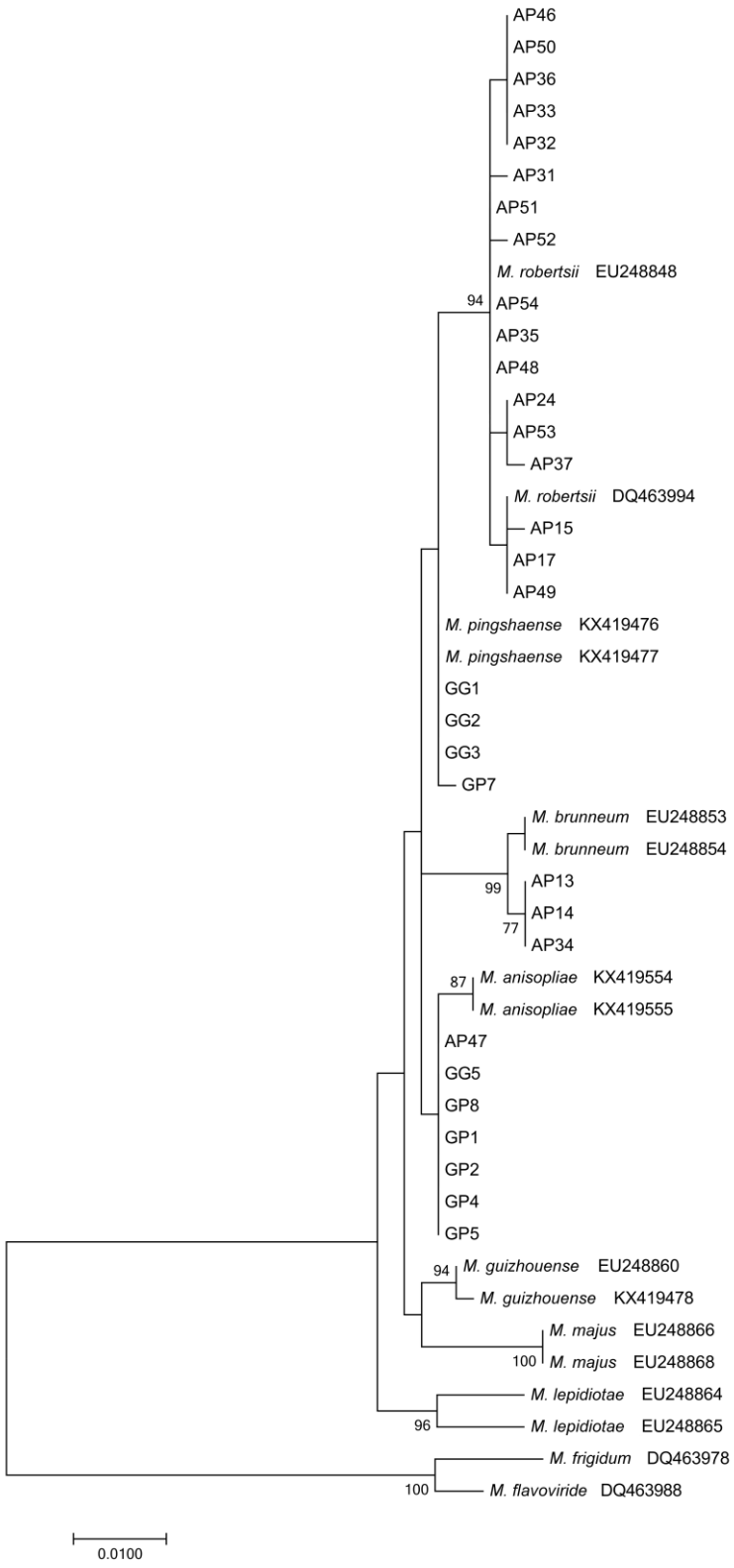

Figure 2. Phylogeny of *Metarhizium* species and isolates inferred from Maximum Likelihood (ML) analysis of the EF1-α sequence data. Isolates used as references are labelled according to their ARSEF accession numbers. Only bootstrap values above 80% are shown.

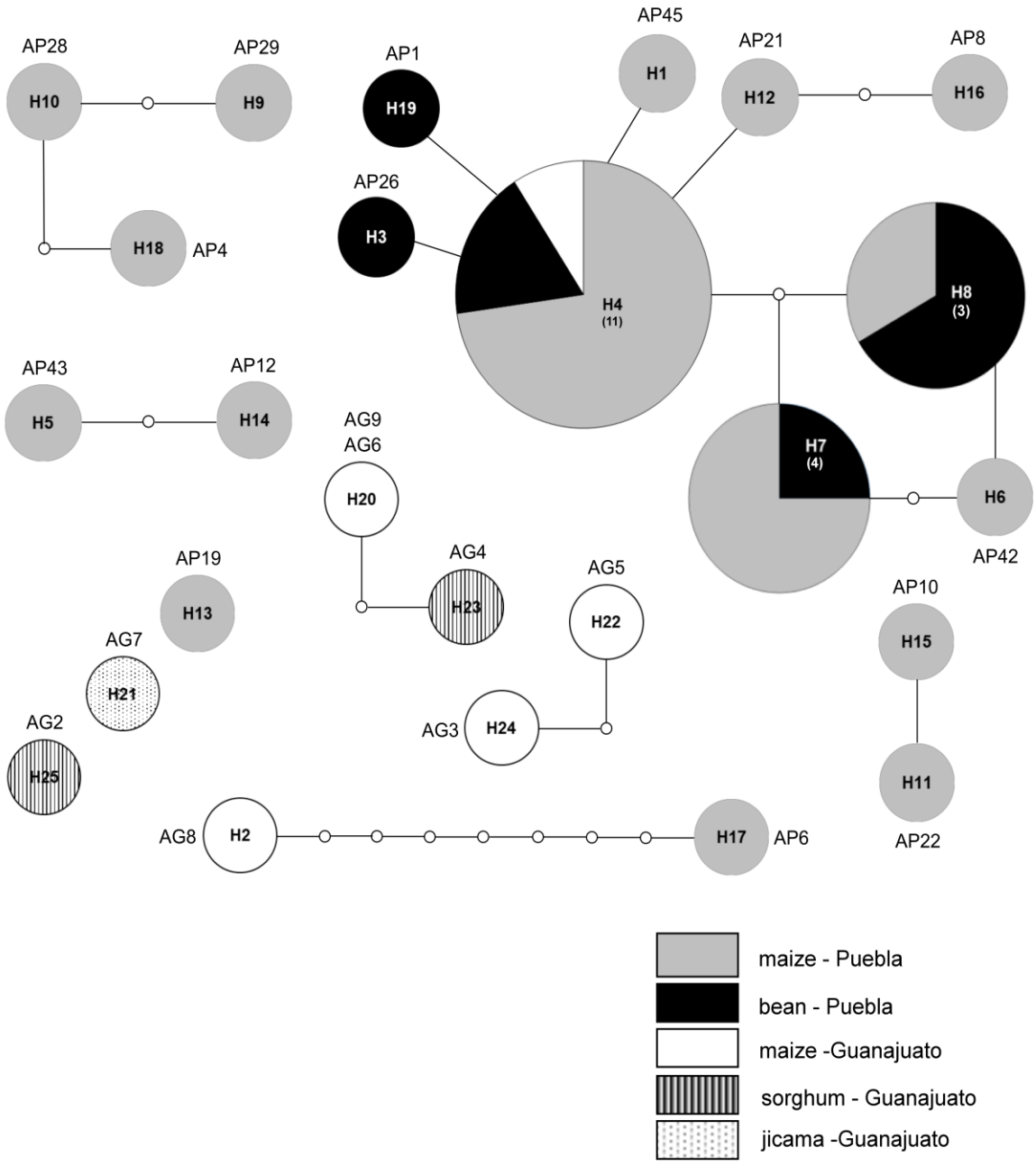

Figure 3. Most parsimonious haplotype network for the 25 haplotypes found in *B. bassiana* isolates from Mexico. Haplotypes are connected with a 95% confidence limit. Each line in the network represents a single mutational change. Small white circles indicate missing haplotypes. Numbers of samples per haplotype are shown in parentheses.

DISCUSIÓN GENERAL

En esta investigación se utilizaron larvas de *G. mellonella* para realizar el trampeo de hongos entomopatógenos en suelo de diferentes cultivos de las entidades federativas Puebla y Guanajuato. En total se obtuvieron 63 aislamientos de *Beauveria* y *Metarhizium*. *B. bassiana* fue la especie más abundante en ambos estados, seguido de *M. robertsii*, *M. brunneum*, *B. pseudobassiana,* y *M. anisopliae*. El mayor registro de *Beauveria* y *Metarhizium* se obtuvo en el cultivo de maíz en la localidad de San Nicolás, en el estado de Puebla y Ejido 5 de Febrero, en el estado de Guanajuato. Lo cual coincide con lo reportado por Pérez-González *et al*. (2014) quienes colectaron muestras de suelo en parcelas de maíz de 11 localidades de Guanajuato e identificaron *B. pseudobassiana, M. robertsii* y *B. bassiana,* este último con el mayor registro de aislamientos. Igualmente, Meyling y Eilenberg (2006) reportaron la especie *B. bassiana* en suelo de un campo cultivado orgánicamente e *I. fumosoroseus* en suelo de setos vivos que rodeaban el campo agrícola. En un estudio realizado en Isparta, Turquía se tomaron 196 muestras de suelo y se utilizaron 1080 larvas de *G. mellonella*; los géneros registrados fueron *Beauveria* spp (164 aislamientos) y *Metarhizium* spp (40 aislamientos) (Baydar *et al*., 2016). Por otra parte, Saleh *et al*. (2016) con una metodología similar a la nuestra, al remover y obtener suelo lo más próximo al tallo de la planta de huertas frutales y utilizar larvas de *G. mellonella* identificaron *B. bassiana*, *M. anisopliae* y *L. lecanii*. Medo y Cagán (2011) tomaron 430 muestras de suelo y mediante el trampeo de hongos obtuvieron cuatro especies siendo *B. bassiana* la más abundante en hábitats diversos, pero principalmente en bosques, *M. anisopliae* se presentó en campos cultivados y praderas, *I. farinosa* en bosques e *I. fumosorosea* en setos vivos. Con los resultados de estos trabajos que desarrollaron metodologías similares, incluyendo las nuestras se pudo

indicar que los géneros de hongos con mayor abundancia y distribución geográfica fueron *Beauveria* y *Metarhizium*.

El análisis de variación genética de los aislamientos de *B. bassiana* en este estudio reveló la existencia de 25 haplotipos distribuidos en siete redes y tres haplotipos independientes, hubo más variabilidad genética en aislamientos obtenidos del cultivo de maíz seguido de frijol, ambos de Puebla, posiblemente por contar con un mayor registro de hongos. El análisis de varianza molecular (AMOVA) reveló que ni origen geográfico (Puebla y Guanajuato) o cultivos fueron factores importantes en la diversidad genética. Medo *et al*. (2016) obtuvieron 15 haplotipos de aislamientos de *B. bassiana*, mientras que Coates *et al*. (2002) obtuvieron 24 haplotipos de 96 aislamientos de *B. bassiana* procedentes de diferentes órdenes de insectos; el análisis AMOVA indicó que no existe correlación significativa entre los haplotipos y el orden taxonómico al que pertenece el hospedante u origen geográfico de donde fueron aislados los hongos. Carrillo-Benítez *et al*. (2013) reportaron 17 aislamientos infectando inmaduros de escarabajos (= gallina ciega), todos identificados como *B. pseudobassiana* y pertenecientes a un solo haplotipo. Goble *et al*. (2015) al hacer bioensayos con el hongo entomopatógeno *B. brongniartii* infectando *Schizonycha affinis* y *T. molitor* señalaron que los aislamientos que comparten el mismo haplotipo no varían en virulencia, pero si entre haplotipos a pesar de obtenerse en la misma epizootia o área de estudio.

Los insectos son los hospedantes naturales de muchas especies de estos hongos, los cuales pueden ser dispersados de forma forética en algunas de sus estructuras morfológicas, se encuentran dentro y sobre estos ya sea en una asociación mutualista, de comensalismo o parásita (Blackwell, 2010). En este estudio se realizaron colectas de larvas de gallina ciega en parcelas de

maíz ubicadas en localidades del estado de Puebla y Guanajuato; tras un mes de monitoreo se obtuvo un total de 10 aislamientos de *Metarhizium* y seis de *Beauveria*; todos los aislamientos del género *Beauveria* fueron identificados molecularmente como *B. pseudobassiana*, curiosamente no se registró *B. bassiana*, por lo cual se considera que existe especificidad de esta especie de hongo con larvas de gallina ciega. En el género *Metarhizium* se identificó la especie *M. pingshaense* en la mayoría de los aislamientos obtenidos de larvas de escarabajos en Guanajuato, mientras que en Puebla fue *M. anisopliae*; el mayor registro de hongos se aisló de *Phyllophaga*, seguido de *Paranomala* y *Cyclocephala*. Al muestrear suelo y colectar larvas de gallina ciega en maíz, Solís *et al*. (2016) reportaron siete aislamientos como *M. anisopliae* y 20 como *B. bassiana* obtenidos de *Phyllophaga* sp. y *G. mellonella*, lo cual coincide con este trabajo. Los mismos autores, al realizar bioensayos con 27 aislamientos aplicados a *Phyllophaga vetula* determinaron que existía mayor patogenicidad por parte de los aislamientos de *M. anisopliae* aislados de *Phyllophaga* sp. (46.6 a 73.3%) que los de *B. bassiana*, y mayor aún que los hongos obtenidos de suelo con el insecto cebo *G. mellonella* (0 a 20%). En caña de azúcar se ha identificado *B. brongniartii* y *B. bassiana* causando epizootias en larvas, pupas y adultos del escarabeido *Hypopholis sommeri* (Goble *et al*., 2012) y *S. affinis* (Goble *et al*., 2015).

Los resultados de experimentos preliminares que se realizaron en el transcurso de este trabajo, así como los reportados por Carrillo-Benítez *et al*. (2013) revelaron que algunos de los aislamientos obtenidos no fueron patogénicos a larvas del género *Phyllophaga*, ya que las mortalidades nunca excedieron el 20%. Es probable que estos aislamientos estén jugando otro papel ecológico en los sistemas estudiados, y no precisamente el de regulador de poblaciones de insectos. Existen reportes de que estas especies de hongos podrían estar jugando un papel de asociación simbiótica con las plantas, donde la planta le provee carbohidratos esenciales al

hongo para su desarrollo, y el hongo le da acceso a fuentes complejas de nitrógeno (p.e. insectos) (Barelli *et al*., 2016).

En conclusión, las especies *B. bassiana*, *B. pseudobassiana*, *M. robertsii*, *M. brunneum* y *M. anisopliae* se aislaron de suelo procedente de diferentes cultivos agrícolas; por otro lado, *B. pseudobassiana*, *M. pingshaense* y *M. anisopliae* se encontraron infectando larvas de gallina ciega en parcelas de maíz de las entidades federativas Puebla y Guanajuato. Los resultados de este trabajo sugieren que el área geográfica y el tipo de cultivo no influyeron en la diversidad poblacional de *B. bassiana*. Esta investigación refuerza la necesidad de realizar más estudios que permitan comprender la ecología de estos microorganismos y sus hospederos, y determinar que otros papeles podrían estar jugando en el ecosistema, además de posibles reguladores de poblaciones de insectos.

LITERATURA CITADA

Aguilera S, J.A., Leclerque, A., Deymié, M.C., Torrente, K.A., Vázquez, F., Cuthbertson, A.G.S., López L, C.C and Lechner, B. 2017. Entomopathogenic fungi: are polisporic isolates more pathogenic than monosporic strains?. Revista de la Sociedad Entomológica Argentina 76: 39-43.

Aragón G, A., Morón, M.A., López-Olguín, J.F y Cervantes-Peredo, L.M. 2005. Ciclo de vida y conducta de adultos de cinco especies de *Phyllophaga* Harris, 1827 (Coleoptera: Melolonthidae; Melolonthinae). Acta Zoológica Mexicana 21: 87-99.

Barelli, L., Moonjely, S., Behiel, S.W and Bidochka, M.J. 2016. Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. Plant Molecular Biology 90: 657-664.

Baydar, R., Güven, Ö and Karaca, I. 2016. Occurrence of entomopathogenic fungi in agricultural soils from Isparta province in Turkey and their pathogenicity to *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae. Egyptian Journal of Biological Pest Control 26: 323-327.

Blackwell, M. 2010. Fungal evolution and taxonomy. In: Roy, H. E., Vega, F. E., Chandler, D., Goettel, M. S., Pell, J. K and Wajnberg, E. (Eds.). The ecology of fungal entomopathogens. Springer. pp 7-16.

Carrillo-Benítez, M.G., Guzmán-Franco, A.W., Alatorre-Rosas, R and Enríquez-Vara, J.N. 2013. Diversity and genetic population structure of fungal pathogens infecting white grub larvae in agricultural soils. Microbial Ecology 65: 437-449.

Coates, B.S., Hellmich, R.L and Lewis, L.C. 2002. *Beauveria bassiana* haplotype determination based on nuclear rDNA internal transcribed spacer PCR±RFLP. Mycological Research 106: 40-50.

Goble, T.A., Conlong, D.E and Hill, M.P. 2015. Virulence of *Beauveria brongniarti*i and *B. bassiana* against *Schizonycha affinis* white grubs and adults (Coleoptera: Scarabaeidae). Journal of Applied Entomology 139: 134-145.

Goble, T.A., Costet, L., Robene, I., Nibouche, S., Rutherford, R.S., Conlong, D.E and Hill, M.P. 2012. *Beauveria brongniartii* on white grubs attacking sugarcane in South Africa. Journal of Invertebrate Pathology 111: 225-236.

Goettel, M.S., Eilenberg, J and Glare, T.R. 2005. Entomopathogenic fungi and their role in regulation of insect populations. In: Gilbert, L. I., Latrou, K., Gill, S. (Eds.). Comprehensive molecular insect science. Elsevier, Amsterdam. pp. 361-405.

Inglis, G.D., Goettel, M.S., Butt, T.M and Strasser, H. 2001. Use of hyphomycetous fungi for managing insect pests. In: Butt, T.M., Jackson, C., Magan, N. (Eds.). Fungi as biocontrol agents. Progress, problems and potential. CABI Publishing, pp. 23-69.

Joop, G and Vilcinskas, A. 2016. Coevolution of parasitic fungi and insect hosts. Zoology 119: 350-358.

Medo, J and Cagán, L. 2011. Factors affecting the occurrence of entomopathogenic fungi in soils of Slovakia as revealed using two methods. Biological Control 59: 200-208.

Medo, J., Michalko, J., Medová, J and Cagán, L. 2016. Phylogenetic structure and habitat associations of *Beauveria* species isolated from soils in Slovakia. Journal of Invertebrate Pathology 140: 46-50.

Meyling, N.V and Eilenberg, J. 2006. Occurrence and distribution of soil borne entomopathogenic fungi within a single organic agroecosystem. Agriculture, Ecosystems and Environment 113: 336-341.

Meyling, N.V and Eilenberg, J. 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. Biological Control 43: 145-155.

Morón, M.A. 2007. Larvas de escarabajos del suelo en México (Coleoptera: Melolonthidae). Departamento de Entomología, Instituto de Ecología, A.C. Xalapa, Veracruz.

NouriAiin, M., Askary, H., Imani, S and Zare, R. 2014. Isolation and characterization of entomopathogenic fungi from hibernating sites of Sunn Pest (*Eurygaster integriceps*) on Ilam Mountains, Iran. International Journal of Current Microbiology and Applied Sciences 3: 314-325.

Pérez-González, V.H., Guzmán-Franco, A.W., Alatorre-Rosas, R., Hernández-López, J., Hernández-López, A., Carrillo-Benítez, M.G and Baverstock, J. 2014. Specific diversity of the entomopathogenic fungi *Beauveria* and *Metarhizium* in Mexican agricultural soils. Journal of Invertebrate Pathology 119: 54-61.

Raffel, T.R., Martin, L.B and Rohr, J.R. 2008. Parasites as predators: unifying natural enemy ecology. Trends in Ecology and Evolution 23: 610-618.

Roberts, D.W and St. Leger, R.J. 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. Advances in Applied Microbiology 54: 1-70.

Roy, H.E., Brodie, E.L., Chandler, D., Goettel, M.S., Pell, J.K., Wajnberg, E and Vega, F.E. 2010. Deep space and hidden depths: understanding the evolution and ecology of fungal entomopathogens. In: Roy, H.E., Vega, F.E., Chandler, D., Goettel, M.S., Pell, J.K y Wajnberg, E. (Eds.). The ecology of fungal entomopathogens. Springer. pp 1-6.

Ruiz V, J., Aquino B, T., Silva R, M.E y Girón P, S. 2012. Control integrado de la gallina ciega *Phyllophaga vetula* Horn (Coleoptera: Melolonthidae) con agentes entomopatógenos en Oaxaca, México. Revista Científica UDO Agrícola 12: 609-616.

Saleh, M.M.E., Abdel-Raheem, M.A., Ebadah, I.M and Elbehery, H. 2016. Natural abundance of entomopathogenic fungi in fruit orchards and their virulence against *Galleria mellonella* larvae. Egyptian Journal of Biological Pest Control 26: 203-207.

Solís P, O., Castillo G, A., Peña C, G., Alvear G, A., Serrano M, M.M., Suárez R, R and Hernández V, V.M. 2016. Pathogenicity, virulence and the interaction of *Metarhizium anisopliae* and *Beauveria bassiana* against *Phyllophaga vetula* (Coleoptera: Melolonthidae). Journal of Pure and Applied Microbiology 10: 2607-2612.

Tkaczuk, C., Król, A., Majchrowska-Safaryan, A and Nicewicz, L. 2014. The occurrence of entomopathogenic fungi in soils from fields cultivated in a conventional and organic system. Journal of Ecological Engineering 15: 137-144.

Wakil, W., Ghazanfar, M.U., Riasat, T., Kwon, Y.J., Qayyum, M.A and Yasin, M. 2013. Occurrence and diversity of entomopathogenic fungi in cultivated and uncultivated soils in Pakistan. Entomological Research 43: 70-78.

Woolhouse, M.E.J., Webster, J.P., Domingo, E., Charlesworth, B and Levin, B.R. 2002. Biological and biomedical implications of the co-evolution of pathogens and their hosts. Nature Genetics 32: 569-577.