GENETIC CHARACTERISATION OF *Metarhizium anisopliae* (Metchnikoff) SOROKIN ISOLATES FROM SUGARCANE FIELDS AND THEIR PATHOGENICITY AGAINST *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae)

Caracterización genética de aislados de *Metarhizium anisopliae* (Metchnikoff) Sorokin de cañaverales y su patogenicidad contra *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae)

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ABSTRACT. A commercial strain of *Metarhizium anisopliae* (Metchnikoff) Sorokin was used by farmers in 2005 to control the sugarcane froghopper *Aeneolamia postica* (Walker) in Los Ríos, Tabasco, Mexico. The persistence and the effect of this introduced isolate on the genetic diversity and pathogenicity of the native fungus population are unknown. The purpose of this study was to characterise the genetic diversity and pathogenicity of *M. anisopliae* isolates collected from sugarcane fields in the region. The *Galleria* baiting method was used to sample the fungus from the soil, whereas the insect netting method was used to capture adults infected by *A. postica*. Samples were collected from a total of 33 sugarcane fields. Ten isolates of *M. anisopliae* were obtained, three in 2007 and seven in 2008; seven isolates were collected near the Usumacinta river. Random amplified polymorphic DNA markers and amplified ribosomal DNA restriction analysis of the 10 isolates revealed the presence of seven subgroups. Two of the isolates obtained in 2008 were genetically similar to the commercial strain used in 2005. At least two genotypes of *M. anisopliae* were found in this region. No clear relationship between genotype similarity and isolate pathogenicity against *A. postica* was recorded. Some of the native *M. anisopliae* isolates are promising candidates as agents of biological control against *A. postica*.
Key words: Sugarcane, entomopathogenic fungi, biological control, molecular characterisation, pathogenicity.

RESUMEN. En 2005, una cepa comercial de *Metarhizium anisopliae* (Metchnikoff) Sorokin fue aplicada por agricultores contra la mosca pinta *Aeneolamia postica* (Walker) en Los Ríos, Tabasco, México. Se desconoce la persistencia y el efecto de este aislado introducido sobre la diversidad genética y patogenicidad de las poblaciones nativas del hongo. El objetivo de este estudio fue caracterizar la diversidad genética y patogenicidad de aislados de *M. anisopliae* colectados en cañaverales de esta región. La técnica del insecto trampa con larvas de *Galleria* se usó para colectar el hongo del suelo, mientras que una red entomológica se usó para capturar adultos infectados de *A. postica*. Las muestras se colectaron en un total de 33 cañaverales. En total, se obtuvieron 10 aislados de *M. anisopliae*, tres en 2007 y siete en 2008; siete de los aislados se colectaron cerca del Río Usumacinta. Marcadores del ADN polimórfico amplificado al azar y análisis de restricción de ADN ribosomal amplificado de los diez aislados, revelaron siete subgrupos. Dos de los aislados obtenidos en 2008 fueron genéticamente similares a la cepa comercial usada en 2005. Al menos dos genotipos de *M. anisopliae* se encontraron distribuidos en esta región. No hubo una clara relación entre la similitud genética y la patogenicidad de los aislados contra *A. postica*. Algunos de los aislados nativos de *M. anisopliae* son candidatos promisorios como agentes de control biológico contra *A. postica*.
Palabras clave: Caña de azúcar, hongos entomopatógenos, control biológico, caracterización molecular, patogenicidad.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) production is currently the most important agroindustry in Mexico. The cultivation of sugarcane involves 812 000 ha distributed over 15 states, and 57 sugar factories. With an average yield of 69 ton ha^{-1}, Mexico is one of the 10 main producers of sugarcane in the world and, with a per capita consumption of 50 kg year^{-1}, it is the second greatest sugar consumer. Approximately three million Mexicans depend on this economic activity, which also generates an important amount of foreign currency (Salgado *et al.* 2005). Approximately 28 000 ha of sugarcane are grown in the regions of La Chontalpa and Los Ríos in the state of Tabasco. Mean sugarcane yield in Tabasco is 60 ton ha^{-1} (Salgado *et al.* 2010). Sugarcane production is limited by the froghopper *Aeneolamia postica* (Walker) (Hemiptera: Cercopidae), the most damaging insect pest present over extensive areas of the Gulf of Mexico and Pacific Ocean coastal plains. This pest reduces sugarcane yield up to 60 %. It is controlled mainly by pesticides, which increases production costs substantially (Bautista-Gálvez & Gonzalez-Cortes 2005). As the froghopper has become resistant to many insecticides, sugarcane producers in Tabasco have searched for alternative control methods. One such alternative is *Metarhizium anisopliae* (Metchnikoff) Sorokin (Moniliales: Moniliaceae), an entomopathogenic fungus that is widely used in the biological control of insect pests (Zimmermann 2007).

In 2005, some farmers applied a commercial strain of *M. anisopliae* as part of an integrated pest management strategy against *A. postica* in the sugarcane fields of Los Ríos, Tabasco (Bautista-Gálvez & Gonzalez-Cortes 2005). To this day, there is no information on whether the strain has managed to survive in the region's sugarcane fields. Some studies have indicated that *M. anisopliae* is a highly genetically diverse species (Riba *et al.* 1985; Fegan *et al.* 1993; Becerra *et al.* 2007; Zimmermann 2007), however the diversity of the native populations of this fungus in the Los Ríos region, their pathogenicity against *A. postica* and their potential as biological control agents are similarly unknown. The present study was carried out in order to increase understanding of these issues and to characterise the genetic diversity and pathogenicity of Metarhizium isolates collected from the main sugarcane growing areas in the Los Rios region of Tabasco, Mexico. Polymerase chain reaction (PCR)-based methods, such as the random amplified polymorphic DNA (RAPD) markers and the internal transcribed spacer (ITS-rDNA) sequence analysis, were used to determine the genomic variability of this fungus. These methods have been applied in studies of genomic variation of *M. anisopliae* (Cobb & Clarkson 1993; Fegan *et al.* 1993; Bridge *et al.* 1997; Driver *et al.* 2000; Castrillo *et al.* 2003; Entz *et al.* 2005; Becerra *et al.* 2007; Freed *et al.* 2011).

MATERIALS AND METHODS

Study Area
The study took place in the sugarcane fields of the Los Rios region, municipality of Tenosique, Tabasco, Mexico. Sampling was carried out in the fields that supply the AZSUREMEX S.A. de C.V. sugar refinery, that is located on the La Palma highway, Tenosique, at 17°25' N, 91°24' W, and 60 m altitude, with a cultivated area of 4,210 ha (Bautista-Gálvez & Gonzalez-Cortes 2005).

Fungal sampling
Samples were collected from the fields of 33 farmers (two fields with and 31 fields without the use of *M. anisopliae* to control *A. postica*), representing 5.1 % of the sugarcane producers and 4.7 % of the area cultivated with sugarcane in the Los Ríos region. On average, each farmer had 6 ha of sugarcane crops. The area is predominantly characterised by a Cambisol soil type. Soil samples were taken to obtain *A. postica* adults. Five sampling points were located in each field, one at each corner and another at the centre. Soil samples were collected down to a depth of 10 cm within the rhizosphere, as described by Almeida *et al.* (1997). Each soil sample consisted of five 0.5 kg soil sub-samples of the upper 10 cm of soil, close to the crop's rhizosphere, with a distance of 5 cm between each sample. The samples were placed in plastic bags and transported to the

laboratory in insulated boxes at 4 °C. Samples of *A. postica* adults were collected near the base of the sugarcane stems using an insect net. The captured insects were placed in Petri dishes (100 x 15 mm) and transported to the laboratory. All *A. postica* adults were placed in a sterile Petri dish containing a moistened filter paper and sealed with Parafilm to promote fungus sporulation (Goettel & Inglis 1997). The samples were collected between September and December of 2007 and 2008.

Fungal isolation

The *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae baiting method was used to isolate *M. anisopliae* from the soil (Vanninen 1997). The soil samples, each containing five sub-samples per field, were sieved through a 2 mm metallic mesh sieve, after which 300 g of soil were moistened and placed in a 500 mL plastic cup. Five larvae were then placed in each cup. The cups were sealed with sellotape and incubated for seven days at 25 °C, with a photoperiod of L:D of 16:8 h (Bedding & Akurst 1975; Doberski & Tribe 1980). The larvae were examined after incubation. The moribund insects were separated from the soil and disinfected in a 0.025 % sodium hypochlorite (NaOCl) solution for one minute, followed by 70 % alcohol for five seconds. They were then rinsed three times in sterile distilled water, and the excess water was removed using tissue paper (Inglis *et al.* 2008). The larvae were then placed in a 100 x 15 mm Petri dish with a double layer of damp filter paper and subjected to the aforementioned incubation conditions to favour fungus development and sporulation (Hatting et al. 1999). Mycosed insects were observed under a stereoscopic microscope to detect the body areas with the greatest sporulation. The procedures mentioned above for G. mellonella mycosed larvae were followed in order to isolate entomopathogenic fungi from the field-collected samples of *A. postica* adults. In total, 33 soil samples and 165 *A. postica* adults were processed. The isolated fungi were identified following Tulloch's (1976) taxonomic keys.

Fungal genomic DNA purification

Monosporic cultures were used as inoculi and were grown on potato dextrose agar (PDA) (Lilly & Barnett 1951). Fungal inoculi were incubated and agitated for three days, at 27 °C, in a potato dextrose broth, and the mycelium was collected on a sterile filter. The mycelium was ground with liquid nitrogen to a fine dust, and transferred to a sterile 1.5 mL plastic microcentrifuge tube. Each sample was mixed with lysis buffer (containing Tris HCL 50 mM, pH 7.0; EDTA 50 mM; SDS 3 %; and 1-2 β-mercaptoethanol 1 %), incubated at 65 °C for 1 h, extracted with phenol:chloroform and precipitated with isopropanol (Lee & Taylor 1990). The resulting DNA was analysed on 0.8 % agarose gel in 1x SB buffer (sodium borate 10 mM), stained with ethidium bromide and observed under ultraviolet light (UV). DNA samples were stored at 4°C (Gómez-Leyva *et al.* 2008).

PCR amplification of the rDNA ITS region and restriction analysis (ARDRA)

The Internal Transcribed Spacer (ITS) region of the rDNA of each sample was amplified by PCR using the following reaction mixture: 1x buffer, pH 8.5, ITS1 and ITS4 primers at 20 pmol each (White *et al.* 1990), 0.2 mM dNTPs, 2.0 mM of $MgCl_2$, 2.5 U of Taq polymerase DNA and 100 pmol DNA, and the following thermocycler conditions: 3 min at 94 °C followed by 30 cycles of: 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C and a final extension of 7 min at 72 °C. The amplified products were extracted after electrophoresis on agarose 1.2 % gel using a DNA extraction kit (Promega, Madison, WI). The PCR products were ligated in the TOPO TA vector (Invitrogen) and cloned in *Escherichia coli* Top10. The recombinant TOPO vector was sequenced in both directions and the results were compared with GenBank records using the DNASTAR Lasergene software (DNASTAR Inc. 2009). In total, 14 isolates were sequenced dideoxi sequencing, Sanger method.

In the case of the amplified ribosomal DNA restriction analysis, the ITS-PCR products were digested using *Hae* II according to the manufacturer's instructions (this enzyme was obtained from Promega, Madison, WI). The restriction fragments were separated on 1.4 % agarose gel in 1x SB buffer. Ethidium bromide gel staining was applied, and the

DNA fragments were observed under UV light and photographed. The molecular size of the fragments was estimated by comparison with a 100 bp DNA ladder (Entz *et al.* 2005; Inglis et al. 2008).

The ARDRA technique was applied to a group of 19 fungus samples consisting of 10 *M. anisopliae* isolates obtained from the sampled sugarcane fields and the following nine reference samples: one commercial strain of M. anisopliae from the "Tiemelonlà Nich Klum" laboratory, four *M. anisopliae* isolates (M370, M371, M372 and M374) characterised and provided by the Centro Nacional de Referencia de Control Biológico (CNRCB), Tecomán, Colima, Mexico, two *Beauveria bassiana* (Balsamo) Vuillemin isolates, and two unidentified fungus isolates obtained from the sugarcane fields sampled in this study.

RAPD-PCR analysis

Fungal isolates were characterised by RAPD analysis (Williams *et al.* 1990). The OPB07 (GGT-GACGCAG) and OPB09 (TGGGGGACTC) Series B Operon primers were used (Operon Technologies Inc., Alameda, CA) (Cobb & Clarkson 1994; Guerrero *et al.* 2000; Kendall & Rygiewicz 2005). The reaction mixture consisted of 0.2 mM dNTPs, 10 μM of primer, 2.5 U of Taq polymerase DNA (Promega, Madison, WI) and 100 ng of DNA. Polymerase Chain Reaction (PCR) amplifications were carried out in a MJ Research thermocycler programmed as follows: 2 min at 94°C followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C, 90 s at 72 °C, and then 7 min at 72 °C and 4 °C until removed. The amplified fragments were separated by horizontal gel electrophoresis in 1.2 % agarose (Ultrapure, Gibco BRL, Gaithersburg, MD). The gels were stained with ethidium bromide and photographed on a UV Cole Palmer 97500 transilluminator.
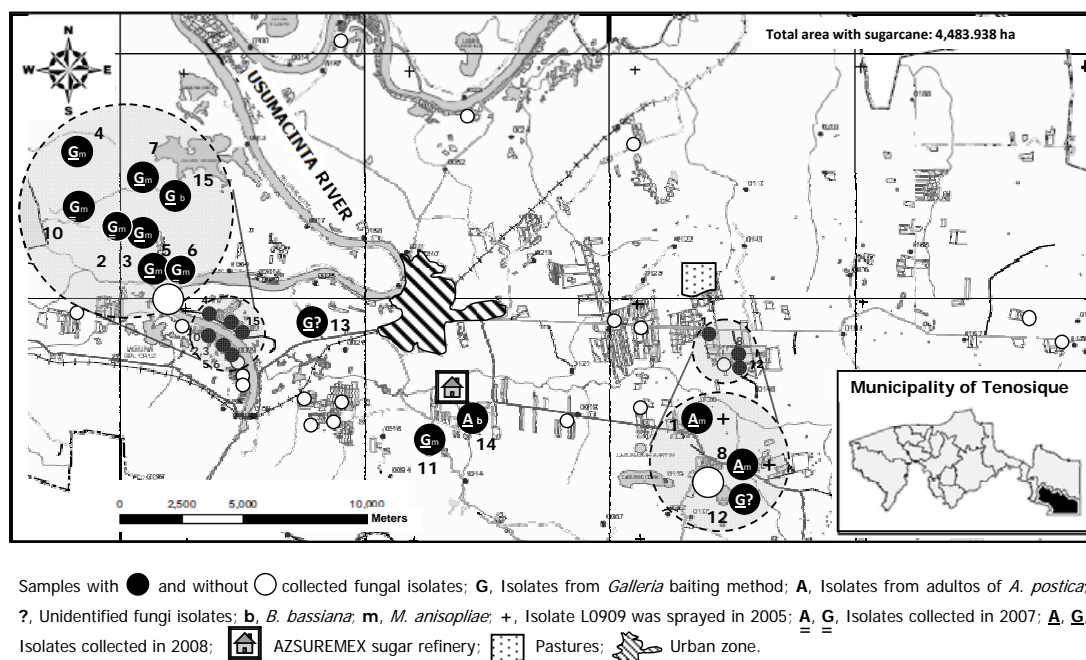
The RAPD technique was applied to a group of 10 *M. anisopliae* samples that included the first nine isolates obtained from the sugarcane fields and the L0909 commercial strain provided by the "Tiemelonlà Nich Klum" laboratory (Palenque, Chiapas, Mexico), which was used as the reference isolate.

Estimation of fungal genetic diversity

The genetic diversity of the 17 fungus samples was analysed through the polymorphism expressed by the primers. A 0,1 matrix, referring to the absence or presence of bands in each isolate, was generated from the RAPD amplifications and ARDRA results. Variations in band intensity were not considered as differences. The matrix was prepared using the Neighbour-Joining coefficient. Dendrograms were generated by a UPGMA analysis and NTSYS 2.0 (Numeral Taxonomy and Multivariate Analysis System) programme (Rohlf 1993).

Fungal pathogenicity

Netting was used to collect *A. postica* adults in the field between the hours of 06:00 to 09:00. The captured insects were transported to the laboratory in 500 mL bottles with perforated tops to allow ventilation. They were fed cane leaves to favour survival during transportation. Once in the laboratory, the insects fed on the Chontalpo-variety grass *Brachiaria decumbens* Stapf during the experiment. Pathogenicity was compared for nine *M. anisopliae* isolates collected in the sugarcane fields, two isolates provided by the CNRCB and the standard stock provided by the "Tiemelonlà Nich Klum" laboratory. The control consisted of a sterile distilled water solution of Inex with a coadjuvant (Inex, 2 μlL^{-1}). The insects were treated with a 1x10^8 conidia mL^{-1} solution of each isolate. The conidia concentration was determined using a hemocytometer (Goettel & Inglis 1997). The adult insects were submerged in the fungal solution contained in a Petri dish for 1 minute using a soft paintbrush. Each experimental unit consisted of a 100 x 15 mm box with 10 *A. postica* insects. Four replicates were used. Insect mortality was recorded 24 h after starting the experiment. Each dead insect was placed in a 100 x 15 mm Petri dish with a double layer of damp filter paper and incubated at 25 ± 1 °C to favour fungus development and sporulation and to corroborate the inoculated species. The mortality percentage was calculated by relating the number of dead insects to the total number of insects at the start of the experiment. The bioassays were repeated when the control mortality was ≥ 20 % (Alves *et al.* 1998). An analysis of variance and a Tukey multiple Benchmark test of averages

Samples with ● and without ○ collected fungal isolates; **G**, Isolates from *Galleria* baiting method; **A**, Isolates from adultos of *A. postica*; **?**, Unidentified fungi isolates; **b**, *B. bassiana*; **m**, *M. anisopliae*; **+**, Isolate L0909 was sprayed in 2005; **A**, **G**, Isolates collected in 2007; **A**, **G**, Isolates collected in 2008; ⌂ AZSUREMEX sugar refinery; Pastures; Urban zone.

Figure 1. Map of the sampling sites and location of the fungi isolates collected in sugarcane fields in the Los Ríos region of Tabasco, Mexico. Numbers are the isolate numbers in Table 1.
Figura 1. Mapa que muestra los sitios muestreados y la localización de los aislados de los hongos colectados en cañaverales de la región de los Ríos de Tabasco, México. Los números se refieren al número del aislado de la Tabla 1.

(p < 0.01) were used to analyse the data. Before running the variance analysis, the percentage data were normalised by arcsine transformation. The analyses were carried out using the Minitab® Statistical Software (Minitab Inc. 2007).

Relationship between fungal genetic diversity, fungal pathogenicity and geographic distance

The Spearman's rank correlation test (Minitab Inc. 2007) was used to analyse the relationships among the genetic diversity of *M. anisopliae* isolates (Jaccard coefficient), their pathogenicity against *A. postica* (mortality percentage) and the geographic distance between the sites where the isolates were collected (Figure 1).

RESULTS

Fungal isolates collected in the sampled sugarcane fields

Fourteen fungal isolates were collected in the sugarcane fields in this study (Table 1; Figure 1).

Three were collected in 2007 and 11 in 2008, representing 9.1 % and 33.3 % of the sampled sites, respectively. In total, 10 isolates were morphologically identified as *M. anisopliae* (Tulloch 1976), two as *B. bassiana* (Humbert 1997), and two were not identified. In 2008, two *M. anisopliae* isolates were obtained from *A. postica* adults that were collected in sugarcane fields where the reference isolate of *M. anisopliae* (L0909) was sprayed in 2005. The other eight *M. anisopliae* isolates were obtained with the *Galleria* baiting method in sugarcane fields without *M. anisopliae* application. Most of the isolates (seven) were collected in the western area of the Los Ríos region where *M. anisopliae* has not been used against *A. postica*, due to a historically low pest population.

PCR amplification of the rDNA ITS region and restriction analysis (ARDRA)

Six hundred to 800 bp of DNA fragments were obtained from the PCR amplification of the rDNA ITS1-ITS4 region for the analysed fungal isolates

Table 1. Information on the identity, origin and morphological characteristics of the conidia and mycelium of the fungi isolates under study.
Tabla 1. Información de la identidad, origen y características morfológicas de la conidia y micelio de los aislados de los hongos estudiados.

No.	Code	Species of insect from which the fungus was isolated	Origin of isolate	Crop from which fungus was isolated	Type of soil in sugarcane field where fungus was collected[1]	Fungus species	Year of collection	Geographical location of site where fungus was collected (UTM units)		¿The isolate L0909 was sprayed in 2005?	Length (μm) and form of conidia[2]	¿Chains of laterally adhered conidia?	Mycelium colour[3]
1	MM0801	*A. postica*	Field	Sugarcane	Fluvisol	*M. anisopliae*	2008	679170	1930877	Yes	5-8,C	Yes	DG
2	AD0702	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2007	659604	1930538	No	5-8,C	Yes	DG
3	AD0803	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2008	659604	1930538	No	5-8,C	Yes	DG
4	CD0804	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2008	658843	1931550	No	5-8,C	Yes	Y
5	FC0805	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2008	660008	1930069	No	5-8,C	Yes	DG
6	FC0706	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2007	660008	1930069	No	5-8,C	Yes	DG
7	AS0807	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2008	659689	1931128	No	5-8,C	Yes	DG
8	GB0808	*A. postica*	Field	Sugarcane	Fluvisol	*M. anisopliae*	2008	680391	1930005	Yes	5-8,C	Yes	DG
9	L0909	*A. postica*	Laboratory[4]	Unknown	Unknown	*M. anisopliae*	Unknown	Unknown	Unknown	-	5-8,C	Yes	DG
10	BC0710	*G. mellonella*	Field	Sugarcane	Cambisol	*M. anisopliae*	2007	658894	1930898	No	5-8,C	Yes	DG
11	SF0811	*G. mellonella*	Field	Sugarcane	Fluvisol	*M. anisopliae*	2008	667870	1926323	No	5-8,C	Yes	LG
12	YR0812	*G. mellonella*	Field	Sugarcane	Fluvisol	Unknown	2008	680451	1929686	No	Unknown	Unknown	W
13	ES0813	*G. mellonella*	Field	Sugarcane	Fluvisol	Unknown	2008	662991	1931168	No	Unknown	Unknown	W
14	AR0814	*A. postica*	Field	Sugarcane	Fluvisol	*B. bassiana*	2008	669459	1927224	No	1.5-5.3,O	No	W
15	JC0816	*G. mellonella*	Field	Sugarcane	Fluvisol	*B. bassiana*	2008	659995	1930891	No	1.5-5.3,O	No	W
16	M370	*A. postica*	CNRCB[5]	Sugarcane	Unknown	*M. anisopliae* var. *anisopliae*	1994	Unknown	Unknown	-	5-8, C	Yes	DG
17	M371	*A. postica*	CNRCB	Sugarcane	Unknown	*M. anisopliae* var. *anisopliae*	1994	Unknown	Unknown	-	5-8,C	Yes	DG
18	M372	*A. postica*	CNRCB	Sugarcane	Unknown	*M. anisopliae* var. *anisopliae*	Unknown	Unknown	Unknown	-	5-8,C	Yes	DG
19	M374	*A. postica*	CNRCB	Sugarcane	Unknown	*M. anisopliae*	1995	Unknown	Unknown	-	3.7-8,C	Yes	DG

[1] Salgado et al. 2010. [2] Conidia form: C = cylindrical, O = ovoid. [3] Mycelium colour: DG = dark green, LG = light green, Y = Yellow, W = White. [4] *Metarhizium anisopliae* isolate provided by the "Tiemelonlà Nich Klum" laboratory located in Palenque, Chiapas, Mexico. [5] CNRCB = Centro Nacional de Referencia en Control Biológico, Tecoman, Colima, Mexico.

(Figure 2a). The size of the DNA fragments of the *M. anisopliae* isolates collected in sugarcane fields was very similar to that recorded for the *M. anisopliae* reference strain L0909. All the fungal isolates gave rise to a 650 bp product, with the exception of the isolate YR0812 for which the amplicon was 800 bp in length and the isolate JC0816 with a 700 bp amplicon.

The restriction rDNA of the products following treatment with *Hae* II (Figure 2b) resulted in the generation of 200 to 700 bp fragments. The restriction profile of the M371 and M372 isolates differed from that of other isolates, possibly as the result of an incomplete digestion. These results were not analysed in this study.

A comparison of the obtained rDNA ITS of the fungal isolates with respect to the GenBank (NCBI) database indicated that isolates MM0801, AD0702, AD0803, CD0804, FC0805, FC0706, AS0807, GB0808, L0909, BC0710, SF0811, M370 and M374 corresponded to *M. anisopliae* and isola-

tes AR0814 and JC0816 were from *B. bassiana*.

In the dendrogram generated from the restriction analysis (Figure 3), two neighbouring clades grouped together the reference strain of *M. anisopliae* (L0909) and the 10 isolates of this fungus that were collected in the sugarcane fields. One clone was observed in each clade, the first clone with isolates MM0801 and AD0702, and the second with isolate GB0808 and the L0909 reference strain. This last clone was genetically similar to isolate AS0807. The dendrogram indicated a genetic similarity between isolates CD0804 and FC0805. Four isolates of *M. anisopliae* collected in the sugarcane fields, two from each clade, formed solitary subgroups. Two isolates of *M. anisopliae* var. *anisopliae* provided by the CNRCB formed a separate subgroup. Both *B. bassiana* isolates (AR0814 and JC0816) were classified in the same subgroup. Finally, differences were observed between the unidentified isolates YR0812 and ES0813, and both also differed from the remainder of the fungal isolates.
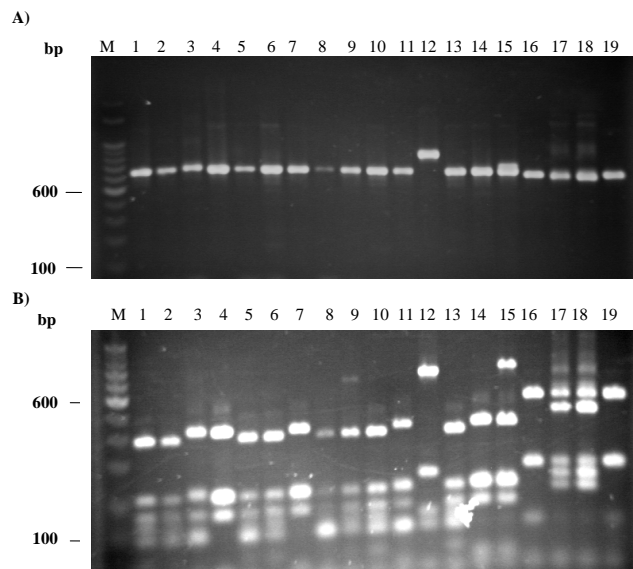
RAPD-PCR analysis

The RAPD amplification of the DNA of *M. anisopliae* isolates 1 to 10 from Table 1 with OPB07 and OPB09 and its dendrogram is presented in figures 4 and 5. Genetic variation was observed amongst the isolates, with polymorphic bands ranging between 250 and 2500 bp (Figure 4). The dendrogram formed three subgroups with two isolates each and four subgroups with only one isolate each (Figure 5). Isolate FC0805 was genetically similar to the reference isolate L0909. One of the subgroups was made up of two isolates (CD0804 and AS0807) which were collected in two sugarcane fields that were located nearby (1.8 km). However, in another subgroup, the isolates (MM0801 and AD0702) were collected in two sugarcane fields that were separated by a distance of at least 20 km (Figure 1).

Fungal pathogenicity

Mortality of *M. anisopliae* isolates on *A. postica* adults ranged from 5 % to 55 % (Figure 6). There were significant differences regarding *A. postica* mortality of isolates AD0702 (55.0 ± 8.66 %), AD0803 (52.5 ± 6.29 %), MM0801 (50.0 ± 18.26 %), CD0804 (45.0 ± 2.89 %), BC0710 (42.5 ± 8.54 %) and SF0811 (27.5 ± 6.29 %), versus the control (0 %) (F = 5.74; df = 12, 39; p < 0.001). In this group, only the isolate MM0801 was obtained from *A. postica* adults. The others were collected using the *Galleria* baiting method.

Relationship between fungal genetic diversity, fungal pathogenicity and geographic distance

No significant association was detected between the genetic diversity of *M. anisopliae* isolates (Jaccard coefficient) and the pathogenicity against *A. postica* (mortality percentage) (S = 18318.84; P = 0.1729). Similarly, no significant associations were detected between genetic diversity and geographic distance between sites where isolates were collected (S = 14700.15; P = 0.8367), or between pathogenicity and geographic distance (S = 17366.10; P = 0.3453).
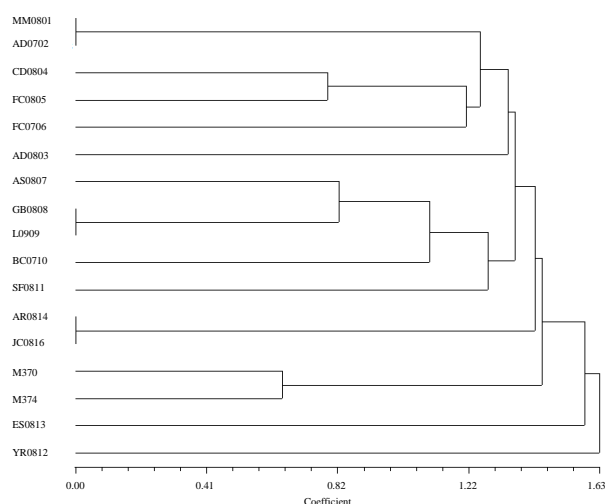

Figure 2. A) Products of amplification of the rDNA with the primers ITS1/ITS4 in different fungal isolates from Table 1. B) Amplified products of restriction (ARDRA) with the Hae II enzyme. M, 100 bp molecular marker. Lane:isolate.- 1: MM0801, 2: AD20802, 3: AD30803, 4: CD0804, 5: FC0805, 6: FC0706, 7: AS0807, 8: GB0808, 9: L0909, 10: BC0710, 11: SF0811, 12: YR0812, 13: ES0813, 14: AR0814, 15: JC0816, 16: M370, 17: M371, 18: M372, 19: M374.

Figura 2. A) Productos de amplificación de rADN con primer ITS1/ITS4 de los diferentes aislados de hongos de la Tabla 1. B) Productos amplificados de restricción (ARDRA) con la enzima Hae II. M, marcador molecular de 100 bp. Línea: aislado: 1: MM0801, 2: AD20802, 3: AD30803, 4: CD0804, 5: FC0805, 6: FC0706, 7: AS0807, 8: GB0808, 9: L0909, 10: BC0710, 11: SF0811, 12: YR0812, 13: ES0813, 14: AR0814, 15: JC0816, 16: M370, 17: M371, 18: M372, 19: M374.

DISCUSSION

Fourteen fungus isolates were collected from sugarcane fields in the Los Rios region of Tabasco, Mexico (Table 1), distributed from east to west across the study area (Figure 1). As stated by Salgado *et al.* (2010), the sampled east-west strip is wetter than the northern and southern areas of the region. Ten of the isolates were identified as *M. anisopliae* considering their morphological characteristics and comparing their genetic sequences with the GenBank database. The number of positive collections of *M. anisopliae* was lower in this study in comparison with those reported by other authors. For example, compared with the study by Inglis *et al.* (2008) that was carried out in British Colum-

bia, Canada, our 10 isolates represent a lower percentage of positive cases of this fungus from the total number of analysed samples (33 % vs 57 %). This percentage is even lower considering that three isolates were collected in 2007 and seven in 2008. These differences may be explained by the environmental conditions that predominate in both studies (temperate region vs tropical region), and also because Inglis *et al*. (2008) sampled a greater number of habitats, and sampling was restricted to rural sugarcane growing areas in our study.

Figure 3. Dendrogram based on the amplified ribosomal DNA restriction analysis (ARDRA), on different fungal isolates from Table 1, using the Jaccard coefficient.
Figure 3. Dendrograma basado en la amplificación ribosomal ADN de análisis de restricción (ARDRA) en los diferentes aislados de hongos de la Tabla 1 usando el coeficiente de Jaccard.

Eight of the *M. anisopliae* isolates were obtained with the *Galleria* baiting method and two by netting *A. postica* adults, suggesting that this fungus is generally easier to collect from the soil, possibly because it is more abundant there. The ability of *M. anisopliae* to survive in soil is well recognised (Vanninen *et al*. 2000; Bidochka *et al*. 2001; Sallam *et al*. 2007; St. Leger 2008).
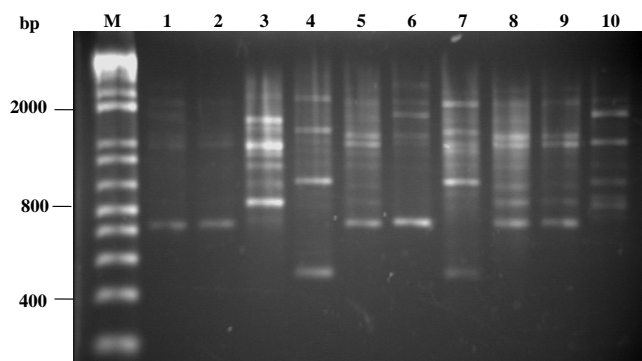
Regarding isolate geography, the two isolates obtained from *A. postica* were collected in the eastern area of the Los Rios region (Figure 1). Historically, sugarcane fields in this area have suffered more damage from this pest given the close proximity to pastures, and some sugarcane farmers have used *M.*

anisopliae to control *A. postica* (Bautista-Gálvez & Gonzalez-Cortes 2005). It is worth mentioning that the soil in the sugarcane fields is even wetter in the western area of the region, where seven *M. anisopliae* isolates were collected (Figure 1), as this area tends to flood occasionally when the Usumacinta river overflows. The higher frequency of *M. anisopliae* in this area supports reports of increased fungus survival in wet soils (Zimmermann 2007). The presence of B. bassiana in the sugarcane fields was lower than that of *M. anisopliae*, with only two isolates, one obtained with the *Galleria* method and the other by capturing *A. postica* adults. The lower presence of B. bassiana was possibly because this fungus has not been applied to control other pests like the sugarcane borer *Diatraea saccharalis* (F.) in the Los Ríos region.

From the genetic point of view, the isolates were separated into two large groups or clades, one grouping six isolates in two subgroups of two and two subgroups of one each, and the other grouping four isolates in one subgroup of two and two subgroups of one each. These groups demonstrate the existence of genetic heterogeneity among the *M. anisopliae* isolates collected in the study area, in agreement with the observations of other authors that have reported a high level of genetic diversity (Riba *et al*. 1985; Becerra *et al*. 2007). The genetic analysis suggests that two of the *M. anisopliae* isolates obtained in 2008, AS0807 isolated from *Galleria* larvae and GB0808 isolated from *A. postica* adults, collected in the western and eastern areas of the Los Rios region respectively, and separated by a distance of approximately 20 km (Figure 1), are similar to the L0909 reference isolate that was used by some producers in 2005 to control *A. postica*. This suggests two points. First, that commercial *M. anisopliae* products can persist in sugarcane fields for at least three years after application, in agreement with experiments carried out by Milner *et al*. (2003) who reported a persistence of 3.5 years for *M. anisopliae* in a sugarcane crop in Australia. Second, from its origin in commercial applications, a fungus can disperse great distances from the original treated site, possibly aided by farmers' practices like the shared use of tractors to plough the soil and

of lorries to transport sugarcane from fields to sugar refineries. Indeed, Bidochka *et al.* (2001) suggested that certain agricultural practices may favour the dispersion of *M. anisopliae*. Taking into consideration our results, it may be stated that the L0909 isolate has spread throughout the Los Rios region from a few sprayings, presenting the possibility of recombination between introduced and native strains of the species, as well as the eventual impact that this phenomenon could have on the genetic diversity of *M. anisopliae* in the region and its pathogenicity towards *A. postica*. Although the methods used in this study did not allow detection of recombination between fungal isolates, recombination events have been detected using isoenzyme techniques (Bidochka et al. 2001).
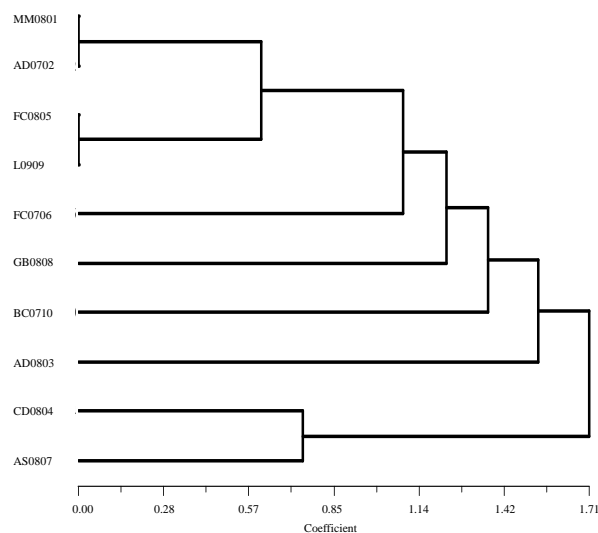

Figure 4. Products of RAPD-PCR amplification using the OPB07 and OPB09 primers. M, 100 bp molecular marker. The DNA template was (lane:isolate).- 1: MM0801, 2: AD0702, 3: AD0803, 4: CD0804, 5: FC0805, 6: FC0706, 7: AS0807, 8: GB0808, 9: L0909, 10: BC0710.

Figure 4. Amplificación de los Productos de RAPD-PCR usando el primer OPB07 y POB09. M, marcador molecular de 100 bp. El templete de ADN usado fue (línea: aislado): 1: MM0801, 2: AD0702, 3: AD0803, 4: CD0804, 5: FC0805, 6: FC0706, 7: AS0807, 8: GB0808, 9: L0909, 10: BC0710.

Genetic analyses also confirmed the existence of a native genotype of *M. anisopliae* that may be distributed throughout the whole region, represented by the isolates MM0801 (to the east) and AD0702 (to the west), collected in sugarcane fields a fair distance apart (ca 20 km), that were genetically identical. In contrast, a genotype that was only found in the western area of the region was represented by isolates AD0804 and FC0805. These findings suggest the existence of different adaptations to en-

vironmental conditions among the native isolates. In this respect, Bidochka *et al.* (2001) pointed out that the habitat can affect the genetic population of *M. anisopliae* as, in the absence of host insects, the fungus possesses the ability to survive in the soil where it is exposed to factors such as organic matter, pesticides, desiccation, solar radiation and fluctuations in temperature.
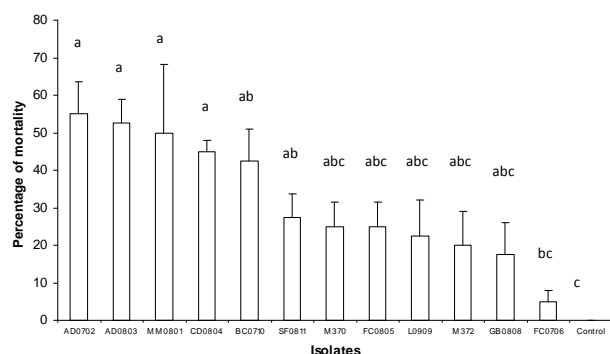

Figure 5. Dendrogram of fungal isolates obtained with the RAPD-PCR data using the Jaccard coefficient.

Figure 5. Dendrograma de los aislados de los hongos obtenidos con RAPD-PCR usando el coeficiente de Jaccard.

The genetic analyses of two isolates typified as *M. anisopliae* var. *anisopliae*, collected in sugarcane fields, one in Oaxaca, Mexico (M370) and the other in Veracruz, Mexico (M374), both provided by the CNRCB, indicated that the isolates collected in the Los Ríos region of Tabasco do not belong to this variety of Metarhizium, as the CNRCB isolates formed a genetically similar group that was different from our isolates (Figure 3).

The pathogenicity towards *A. postica* of the *M. anisopliae* isolates collected in the Los Rios region, expressed as a percentage of adult mortality, varied from very low (5 %) to intermediate (55 %). Six of the isolates caused significantly greater *A. postica* mortality than the control. These may therefore be promising candidates for the second stage of the selection process consisting of virulence tests

involving lethal concentrations and mean lethal time assays. It is clear that pathogenicity studies for the selection of *M. anisopliae* isolates are a prerequisite in a biological control programme for *A. postica*. In contrast with other similar studies on the genetic diversity of *M. anisopliae*, our study explored the relationship between pathogenicity and the degree of genetic similarity and geographical distance between isolates. According to our results, these variables are not related. In Chile, Becerra et al. (2007) found no association between the genetic diversity of *M. anisopliae* and the geographical origin. At least in the Los Rios region, there are no factors or environmental conditions that favour the spatial clustering of isolates with similar genetic and/or pathogenic characteristics. Furthermore, it is not possible to forecast isolate pathogenicity with respect to their degree of genetic similarity. Nevertheless, this study made it possible to identify several native *M. anisopliae* isolates that merit more detailed research due to their pathogenicity against *A. postica* and their widespread distribution throughout the Los Ríos region, particularly the MM0801 and AD0702 clonal isolates.

Figure 6. Average mortality rate (%) of *Aeneolamia postica* adults with several *Metarhizium anisopliae* isolates at a concentration of 1 x 108 conidia/ml. Means (± SE) with the same letter are not significantly different, using Tukey's test at 5%.

Figure 6. Tasa promedio de mortalidad (%) de adultos de *Aeneolamia postica* con varios aislados de *Metarhizium anisopliae* a una concentración de 1 x 108 conidia/ml. Los promedios (± EE) con la misma letra no son significativamente diferentes, usando la prueba de Tukey al 5%.

RAPD markers and ITS-rDNA sequence analyses were applied to determine the genetic diversity of *M. anisopliae* isolates, as has been previously done in studies of this fungus (Cobb & Clarkson 1993; Fegan *et al.* 1993; Bridge *et al.* 1997; Castrillo *et al.* 2003; Driver *et al.* 2000; Entz *et al.* 2005; Becerra et al. 2007; Freed *et al.* 2011). In order to determine the phylogenetic relationships within the *M. anisopliae* isolates from the Los Ríos region, studies that employ a multigene phylogenetic approach using near-complete sequences from nuclear encoded EF-1α, RPB1, RPB2 and β-tubulin gene regions will be necessary, as Bischoff *et al.* (2009) recently proposed.

In conclusion, a high diversity of *M. anisopliae* genotypes exists, with a wide range of pathogenicity characteristics against *A. postica*, in the sugarcane fields of Los Ríos, Tabasco, Mexico, particularly in the wetter areas of the region. It was demonstrated that the reference isolate L0909, commercially used for the control of this pest, may persist three years after its application in the sugarcane fields of the region, and may be found at sites 20 km from where it was originally applied. Finally, some of the native *M. anisopliae* isolates collected in the Los Rios region presented characteristics that make them promising candidates as agents of biological control against *A. postica*.

ACKNOWLEDGEMENTS

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