

Specific Diversity of *Metarhizium* Isolates Infecting *Aeneolamia* spp. (Hemiptera: Cercopidae) in Sugarcane Plantations

C HERNÁNDEZ-DOMÍNGUEZ¹, AW GUZMÁN-FRANCO¹, MG CARRILLO-BENÍTEZ¹, R ALATORRE-ROSAS¹, E RODRÍGUEZ-LEYVA¹, JA VILLANUEVA-JIMÉNEZ²

¹Colegio de Postgraduados, Campus Montecillo, Posgrado en Fitosanidad, Entomología y Acarología, 56230 Texcoco, Estado de México, Mexico

²Colegio de Postgraduados, Campus Veracruz, 91690 Veracruz, Mexico

Keywords

In vitro growth, intraspecific variation, microbial control, sustainable pest management

Correspondence

AW Guzmán-Franco, Posgrado en Fitosanidad, Entomología y Acarología, Colegio de Postgraduados, Km, 36.5 Carretera Mexico-Texcoco, Montecillo, Texcoco, Estado de Mexico 56230, Mexico
gariel@colpos.mx

Edited by Fernando L Cônsoli – ESALQ/USP

Received 2 June 2015 and accepted 14 September 2015

Published online: 5 October 2015

© Sociedade Entomológica do Brasil 2015

Abstract

Spittlebugs from the genus *Aeneolamia* are important pests of sugarcane. Although the use of the entomopathogenic fungus *Metarhizium anisopliae* s.l. for control of this pest is becoming more common in Mexico, fundamental information regarding *M. anisopliae* in sugarcane plantations is practically non-existent. Using phylogenetic analysis, we determined the specific diversity of *Metarhizium* spp. infecting adult spittlebugs in sugarcane plantations from four Mexican states. We obtained 29 isolates of *M. anisopliae* s.str. Haplotype network analysis revealed the existence of eight haplotypes. Eight selected isolates, representing the four Mexican states, were grown at different temperatures in vitro; isolates from Oaxaca achieved the greatest growth followed by isolates from Veracruz, San Luis Potosi and Tabasco. No relationship was found between in vitro growth and haplotype diversity. Our results represent a significant contribution to the better understanding of the ecology of *Metarhizium* spp. in the sugarcane agroecosystem.

Introduction

Sugarcane is one of the most important crops in the world; during 2012, more than 1,800 million metric tonnes were produced (FAOSTAT 2012). Spittlebugs, including species in the genus *Aeneolamia*, are serious pests of pastures and sugarcane plantations in neotropical countries (Thompson 2004) such as Brazil, Venezuela, Ecuador and Mexico (Gómez 2007). Among the most important biological control agents of this pest is the entomopathogenic fungus *Metarhizium anisopliae* sensu lato (Li *et al* 2010). There is some information reporting the virulence of *M. anisopliae* isolates against different species of the spittlebug under laboratory (Arango *et al* 1994, Garcia *et al* 2012, de la Torres *et al* 2013) and field (Badilla *et al* 1996, Matabanchoy Solarte *et al* 2012) conditions. Furthermore, the use of *M. anisopliae* isolates for the control of field populations of the spittlebug in

countries such as Guatemala, Costa Rica, Panama, Venezuela, Ecuador and Brazil is common (Garcia *et al* 2012). Brazil is an example of the most extensive use of *M. anisopliae* for the control of spittlebugs in sugarcane and pastures, with more than 1.5 million ha sprayed with this fungus during 2011 (Vega *et al* 2012). In Mexico, the use of *M. anisopliae* for control of this pest is also increasing; unfortunately, information regarding the impact of the treatments on spittlebug field populations is non-existent. In addition, data regarding the origin of the isolates (host), their virulence under laboratory and field conditions, and data describing their biological attributes such as germination, conidial production, and optimal temperature for in vitro growth are seldom published, or only appear in unofficial reports that are difficult or impossible to access.

A greater understanding of the ecology and composition of indigenous populations of entomopathogenic

fungi is required and must be considered in the development of these microorganisms as biological control agents in Mexico. For instance, it has been shown that particular habitats can harbour different species of fungi and different genetic groups within those species (Meyling *et al* 2009, Ormond *et al* 2010). As a first step, accurate species identification is important based on the revised taxonomic status of *M. anisopliae*, which now includes cryptic species that cannot be separated morphologically (Bischoff *et al* 2009). At least two comprehensive studies have been published describing the existence of cryptic species within the *M. anisopliae* lineage (Bischoff *et al* 2009, Kepler *et al* 2014). Previous research reported genetic variation amongst *M. anisopliae* s.l. isolates (Becerra Velásquez *et al* 2007, Freed *et al* 2011), but these did not consider the existence of cryptic speciation. At this respect, to our knowledge, there is no information regarding the identification of *Metarhizium* species infecting spittlebugs using phylogenetic methods for Mexico or elsewhere.

Therefore, our aims were first to establish the taxonomic status of the *Metarhizium* isolates infecting spittlebug populations from the genus *Aeneolamia* spp. collected in five plantations from four of the most important Mexican states producing sugarcane. Secondly, using a reduced number of isolates, but including representatives from all geographical origins, *in vitro* growth profiles at different temperatures were studied, in order to establish the potential relationships between genetic variation, growth profiles and geographical origin.

Material and Methods

Isolate collection

One hundred and six dead spittlebug adults were collected during August 2012 from the soil of five sugarcane plantations distributed in the states of Veracruz, San Luis Potosi (two plantations), Oaxaca and Tabasco (Table 1). Each dead insect was placed individually into 2-mL Eppendorf tubes; no visible signs of infection were observed in the collected insects. The insects were transported to laboratory and maintained at 5°C prior to processing, but for no longer than 2 weeks.

All collected insect cadavers were surface-sterilised by individually immersing them in 5% sodium hypochlorite for 2 min and then rinsing them twice in sterile distilled water. Surface-sterilised cadavers were blot dried with sterile filter paper and incubated individually in 60-mm-diameter Petri dishes on sterile damp filter paper at 25°C in complete darkness for 72 h.

Table 1 Collection sites for infected spittlebug adults in sugarcane plantations in Mexico.

Estate	Locality	Geographical position	
		Latitude	Longitude
Oaxaca	Guadalupe Reyes	96° 38' 2"	18° 33' 16"
Veracruz	Paso Nuevo	96° 32' 37"	18° 36' 43"
San Luis Potosi	El Sabinito	99° 27' 23.6"	22° 30' 36.6"
""	Protacio F. Guerra	99° 27' 10.5"	22° 49' 57.6"
Tabasco	Cardenas	93° 22' 15.61"	18° 0' 5.30"

After this time, samples of conidia were taken from sporulating cadavers using a bacterial loop in a laminar flow sterile hood. Samples were placed onto 25 mL of Sabouraud dextrose agar (SDA) in a 90-mm-diameter Petri dish and incubated at 25°C in complete darkness. If no growth was observed on the SDA plates, a second sample of conidia was taken from the same sporulating cadaver, deposited into 25 mL of Sabouraud dextrose broth (SDB) and incubated at 25°C and 250 rpm for 5 days. Any resulting mycelium was washed with sterile distilled water, inoculated into SDA plates and incubated as described above. From each isolate we obtained, a monosporic colony was produced by randomly selecting one colony-forming unit from a SDA plate inoculated with a 1×10^6 conidia mL⁻¹ suspension made from the original isolation. Both the original and monosporic isolates were stored at -80°C in 2-mL cryovials (Nalgene, Thermo Fisher Scientific, Rochester, NY, USA) containing 10% sterile glycerol. Only monosporic isolates were used in subsequent experiments.

From the 106 dead insects processed, 29 produced external sporulation and *Metarhizium* spp. were successfully isolated (Table 2). The isolates obtained were identified morphologically to genus and species. For this, an undetermined number of conidia from each isolate were collected from a 15-day-old colony grown on SDA plates. The conidia were placed onto a glass slide and covered with a coverslip without any mounting liquid. Preliminary observations showed that immersing conidia in mounting liquid, created a diffuse area surrounding each conidium. This made it very difficult to establish the exact borderline of each conidium for accurate measurements. Without the mounting liquid, the borderline of each conidium was clear and could be photographed and measured accurately. One hundred photos of conidia were taken for each isolate using a Carl Zeiss microscope attached to a digital camera PaxCam 3. From these digital photos, the length and width of approximately 300 conidia per isolate were measured using the software Image Tool for Windows 3.0 (Wilcox *et al* 2002). The mean conidial size for the isolates was $8.10-4.68 \times 4.34-2.09$ μm , placing them in

Table 2 List of *Metarhizium anisopliae* s.str. isolates obtained from infected *Aeneolamia* species adults collected in sugarcane plantations in Mexico. Haplotype assignment for each isolate is shown after the haplotype network analysis (Fig 2).

Isolate	Locality	GenBank accessions numbers		Conidial size (length×width)	Haplotype
		EF1- α	ITS		
1(1) ^a	Guadalupe Reyes	KF981880	KF990284	5.03–7.99×2.06–4.83	H8
1(2)	""	KF981881	KF990285	4.24–7.99×1.86–4.54	H8
1(3)	""	KF981882	KF990286	5.12–8.22×2.24–4.29	H7
1(4) ^a	""	KF981883	KF990287	5.16–8.00×2.17–4.21	H7
1(6)	""	KF981884	KF990288	5.06–7.93×2.17–4.35	H7
1(8)	""	KF981909	KF990289	4.29–7.98×2.03–4.49	H8
3(1)	Paso Nuevo	KF981885	KF990290	4.43–8.59×2.10–4.38	H7
3(2) ^a	""	KF981886	KF990291	4.08–8.15×2.00–4.29	H7
3(3)	""	KF981887	KF990292	3.64–8.18×2.10–4.62	H8
3(4)	""	KF981888	KF990293	5.29–8.36×2.00–4.25	H7
3(5)	""	KF981889	KF990294	4.64–8.00×2.31–4.30	H7
3(9)	""	KF981890	KF990295	4.78–7.98×2.03–4.20	H6
3(10)	""	KF981891	KF990296	4.42–8.37×2.01–4.19	H2
3(11)	""	KF981892	KF990297	4.13–8.25×2.12–4.30	H4
3(12)	""	KF981893	KF990298	4.52–8.02×2.05–4.20	H7
3(13)	""	KF981894	KF990299	5.00–8.30×2.03–4.30	H1
3(14)	""	KF981895	KF990300	5.12–8.03×2.00–4.08	H7
3(15) ^a	""	KF981896	KF990301	5.22–8.28×2.05–4.49	H8
3(16)	""	KF981897	KF990302	4.59–7.98×2.09–4.50	H6
SP07 ^a	El Sabinito	KF981899	KF990304	4.30–7.99×2.03–4.09	H7
SP10	""	KF981900	KF990305	4.94–7.98×2.22–4.94	H5
SP12	""	KF981901	KF990306	3.73–8.00×2.11–4.80	H3
SP16 ^a	""	KF981902	KF990307	5.21–7.95×2.02–4.26	H8
SP17	""	KF981903	KF990308	4.55–7.79×2.09–3.97	H2
SP21 ^a	Protacio F. Guerra	KF981905	KF990310	4.60–8.09×2.03–4.09	H8
SP20	""	KF981904	KF990309	4.85–8.16×2.22–4.19	H7
SP22	""	KF981906	KF990311	5.19–8.08×2.24–4.09	H8
SP23	""	KF981907	KF990312	5.04–8.36×2.13–4.09	H8
Zona_2 ^a	Cardenas	KF981908	KF990313	4.26–8.09×2.09–4.09	H1

^a Isolates selected for the temperature experiment.

M. anisopliae s.l. Metsch. Sorokin (Table 2). Taxonomic placement of all species was made according to Humber (2012).

Phylogenetic placement of isolates

Mycelium production and DNA extraction. Conidia from each isolate were harvested from 15-day-old colonies and inoculated on to SDA plates with a sterile cellophane sheet on top of the medium. Plates were incubated at 25°C for 14 days in complete darkness. Mycelium was separated from the cellophane sheet using a sterile scalpel, placed into a sterile 20-mL glass container and lyophilised for 24 h. For DNA extraction, 0.2 g of lyophilised mycelium was placed into a 1.5-mL Eppendorf tube and plunged into liquid nitrogen for 5 min. Frozen mycelium was then ground using a pellet pestle rod (Daigger and Company Inc., Vernon Hills, IL, USA), and

DNA was extracted using the DNeasy® Plant Minikit following the manufacturer's instructions with the modifications suggested by Fargues *et al* (2002). The concentration of DNA was estimated using a NanoDrop (Thermo Fisher Scientific, Inc. Waltham, MA, USA) and stored at 4°C until required.

PCR and sequencing. Partial sequences of the nuclear gene elongation factor 1- α (EF1- α) (Bischoff *et al* 2006, 2009) and the ITS region of the rDNA were obtained. A region of the elongation factor 1- α was amplified using primers 983F and 2218R (Rehner & Buckley 2005). PCR amplifications were made in a total volume of 30 μ L, which included 6 μ L of 5X PCR buffer provided by the manufacturer, 1.2 mM of each dNTP, 1.4 μ M each of opposing amplification primers, 3.5 mM of MgCl₂ and 0.3 μ L of Taq polymerase (Promega, Madison, WI, USA). PCR was performed using a touchdown procedure (Don *et al*

1991) and the thermal conditions reported by Rehner & Buckley (2005). Reactions were made in a MyCycler thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR products were visualised on 1.5% agarose gels in 1X TAE. GelPilot® 100 bp Plus and 1 kb Plus (QIAGEN®, GmbH, Hilden, Germany) size markers were used. The gels were stained with ethidium bromide ($0.1 \mu\text{g mL}^{-1}$) and photographed. All PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN®, GmbH, Hilden, Germany) following the manufacturer's instructions, and samples were sent to Macrogen Inc. (Geumchen-gu, Seoul, Korea) for direct sequencing.

The ITS region of isolates was amplified using the universal primer set ITS5 and ITS4 (White *et al* 1990). PCR amplifications were made in 0.2-mL Eppendorf tubes in a total reaction volume of 25 μL . Each reaction contained 1.4 μM of each primer, 0.12 mM of each dNTP (QIAGEN), 1 μL (5 units) of Taq polymerase (New England Biolabs Inc., Ipswich, MA, USA) in 10 \times PCR reaction buffer and 30 ng of DNA. Thermal cycling conditions were one cycle of denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 45°C for 1 min, extension at 72°C for 1.5 min, and with a final extension at 72°C for 5 min. PCR products were visualised and sequenced as described previously.

Data analysis. All sequence traces were edited and assembled using BioEdit (Hall 1999). Multiple sequence alignments were made using the Clustal W programme (Thompson *et al* 1994). Maximum parsimony (MP) analyses were done using the Molecular Evolutionary Genetic Analysis (MEGA) software ver. 5.0 for Windows (Tamura *et al* 2011) using the close-neighbour-interchange algorithm. The robustness of branches was estimated by bootstrap analysis with 1000 repeated samplings of the data (Felsenstein 1985). Sequences from related species within the genus *Metarhizium* were retrieved from GenBank and used for comparison.

As no significant topological conflicts between consensus trees generated for each loci data were observed (data not shown), sequence information data sets from two nuclear loci (EF1- α and ITS) were combined and analysed together as described previously. Genetic differences among haplotypes were detected in a maximum parsimony network (Templeton *et al* 1992) using TCS v. 1.21 (Clement *et al* 2000).

Effect of temperature on the in vitro growth of fungal isolates

For this experiment, eight *M. anisopliae* sensu stricto isolates were randomly selected but represented all sample locations. The effect of temperature on the growth of each isolate was assessed by measuring the area of

growth achieved by colonies when incubated at 20, 25, 30 and 35°C. The procedure for each isolate was the same. Sterile SDA plates were inoculated with 100 μL of a 1×10^7 conidia mL^{-1} conidial suspension. After 48 h, a 10-mm-diameter plug was taken from the growing edge, inverted and placed in the centre of a Petri dish containing 15 mL of solidified SDA. Each dish was sealed with Parafilm "M" (Pechiney Plastic Packaging Inc., Menasha, WI, USA) and incubated at 20, 25, 30 or 35°C. After 48 h, and thereafter every 24 h, the area of each colony was measured for 11 days (13 days in total), unless the colony had reached the edge of the plate within this time. For the measurements, a digitised image was created for each colony, which was then processed using the software GIMP ver. 2.8 and the area of growth quantified using Image Tool version 3.0 software (Wilcox *et al* 2002). Five replicates of each isolate were measured, and the whole experiment done on two occasions (ten replicates per isolate \times temperature combination overall).

Data analysis. The statistical package GenStat ver. 8 was used for analysis (Payne *et al* 2005). Successive colony measurements over time were analysed using an ante-dependence analysis (Kenward 1987) via the GenStat procedures ANTORDER and ANTTEST. The order of ante-dependence structure was estimated, and then, the treatment effects were estimated adjusting for the observed order of ante-dependence. The results from the ante-dependence analysis indicated those times at which treatment effects occurred as well as the overall treatment effect. A factorial treatment structure (isolates \times temperature) was incorporated into the analysis to investigate main effects of both treatments and whether an interaction existed between them. In a separate analysis, isolate comparisons were partitioned to compare geographical origins.

Results

Twenty-nine *Metarhizium* isolates were obtained from 106 dead adults of *Aeneolamia* spp. collected from five locations (sugar plantations) distributed in four Mexican states (Tables 1 and 2).

Phylogenetic placement of the Metarhizium isolates

GenBank accession numbers for sequence data generated are listed in Table 2 for all the *Metarhizium* isolates collected. After alignment and trimming, the EF1- α and ITS sequence data set consisted of 886-889 and 519-525 aligned positions,

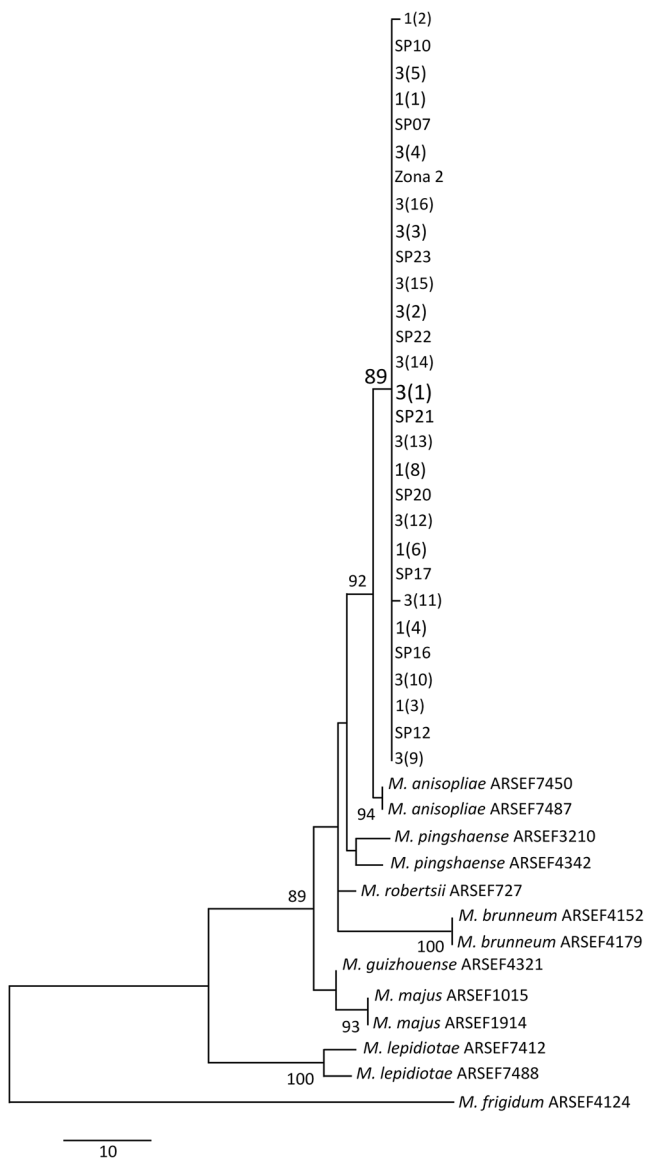


Fig 1 Phylogeny of *Metarhizium anisopliae* isolates inferred from maximum parsimony analysis of the EF1- α and ITS data. Isolates used as reference are labelled according to the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) accession numbers. Only bootstrap values above 80% are shown.

respectively. Phylogenetic analysis combining both data sets grouped all isolates as *M. anisopliae* s.str. (Fig 1).

Genetic variation amongst *M. anisopliae* isolates

Haplotype network analysis showed the existence of eight haplotypes amongst the 29 isolates (Table 2 and Fig 2). The most common haplotype was H7 with 11 isolates and included representatives from all locations except Santa Rosalia, Tabasco (Table 2). The second most common haplotype was H8 with nine isolates, again with representatives from all locations except Santa Rosalia, Tabasco, followed by

haplotypes H1, H2 and H6, each with two isolates, and by haplotypes H3, H4 and H5 with one isolate each (Fig 2 and Table 2).

Effect of temperature on the *in vitro* growth of fungal isolates

In the first comparison (isolate \times temperature) of *M. anisopliae* isolates, the order of ante-dependence was estimated to be seven; tests of treatment terms were therefore based on this order of ante-dependence structure. Significant differences were found in the overall growth profiles of the eight isolates ($\chi^2_{77}=404.77$, $p<0.001$). Also, significant differences were found in growth at all observation times (days) for the eight isolates ($p<0.001$). There was a significant interaction between temperature and isolate on the overall growth of the isolates ($\chi^2_{231}=738.30$, $p<0.001$). This interaction was present at all observation times ($p<0.001$). When the isolate comparison was partitioned to compare geographical origins, the order of ante-dependence structure was seven, and further comparison was based on this structure. Overall, the growth profiles differed for the *M. anisopliae* isolates from different geographical origins ($\chi^2_{33}=195.12$, $p<0.001$), and this difference was consistent at all observation times ($p<0.001$). *M. anisopliae* isolates from Oaxaca achieved the greatest colony size at 20, 25 and 30°C, followed by the isolates from Veracruz, San Luis Potosi and Tabasco (Fig 3). The overall difference between the four geographic origins of *M. anisopliae* isolates varied with temperature ($\chi^2_{99}=319.40$, $p<0.001$), and an interaction was present at all observation times ($p=0.048$ to $p<0.001$). Isolates from Oaxaca achieved their greatest colony size at 25°C (Fig 3b), whereas isolates from Tabasco obtained the smallest colony size overall (Fig 3b). Although isolates from Tabasco achieved the greatest colony size at 30°C (Fig 3c), this size was still small compared to the growth achieved by other isolates at the other temperatures (Fig 3c). Differences amongst growth profiles were less evident at 20°C (Fig 3a) and practically non-existent at 35°C (Fig 3d), where the colony size of all isolates was never greater than 1 cm².

Discussion

There are some reports indicating the pathogenicity of *M. anisopliae* s.l. isolates against spittlebug (de la Torres *et al* 2013); however, despite the importance of this pest in sugarcane plantations (Peck *et al* 2001) and the necessity to assess indigenous isolates before introducing biological control agents (Meyling & Eilenberg 2007), there are no studies reporting the occurrence and diversity of fungal species in sugarcane. Phylogenetically, all isolates collected were *M. anisopliae* s.str. We cannot be sure that this fungal species was the only one present in

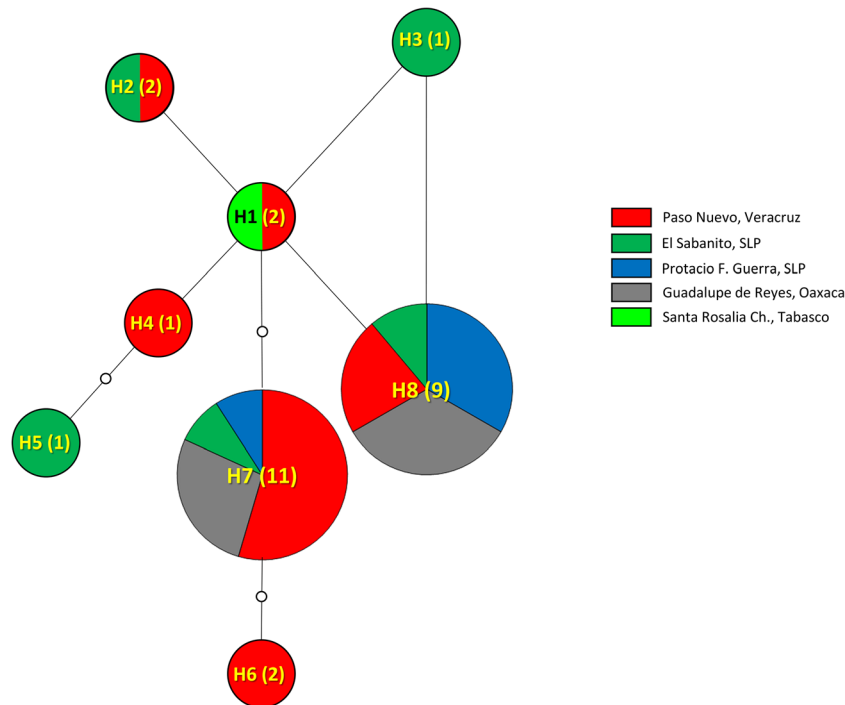


Fig 2 The most parsimonious haplotype network for *Metarhizium anisopliae* isolates. Haplotypes are connected with a 95% confidence limit. Each line in the network represents a single mutational change. Small circles indicate missing haplotypes. Numbers of samples per haplotype are shown in parentheses. Colours indicate geographical origin within each haplotype.

sugarcane plantations or whether other *Metarhizium* species might be present in this agroecosystem but not infecting the spittlebug. It would be important to

investigate the presence of other potential *Metarhizium* species in soil, roots and leaves in sugarcane plantations. We also found two isolates for each of the species *Isaria*

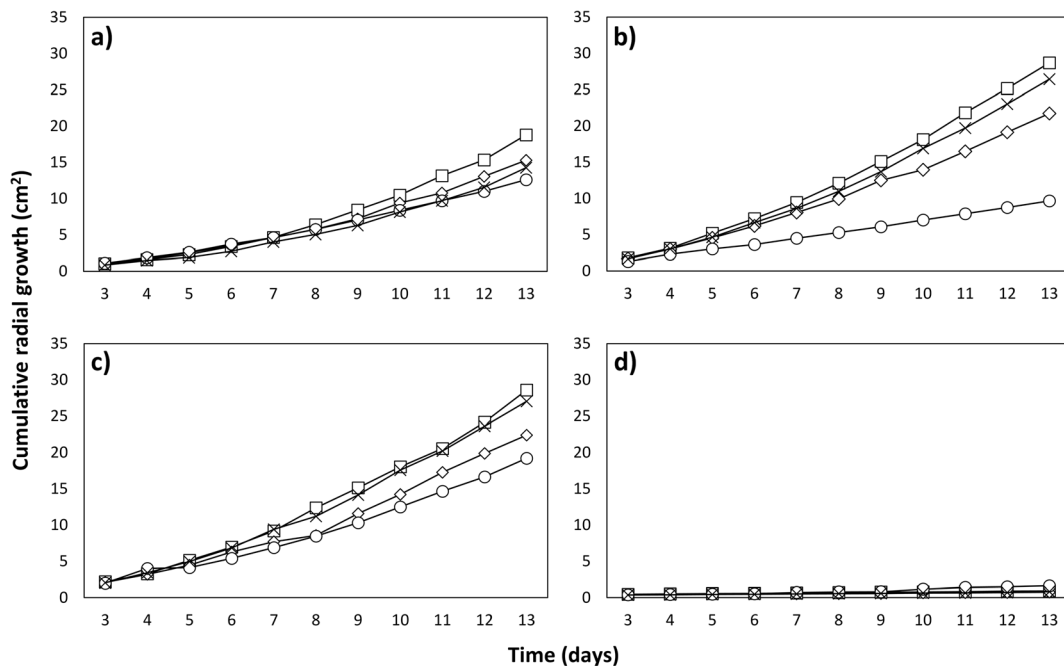


Fig 3 Cumulative growth of eight *Metarhizium anisopliae* isolates grouped into four geographical origins are expressed in square centimetres. Three isolates were from San Luis Potosi, two isolates each were from Oaxaca and Veracruz and one isolate was from Tabasco (Table 2). Oaxaca (□), San Luis Potosi (◇), Veracruz (×) and Tabasco (○). Growth profiles represent mean values of all isolates at a 20°C, b 25°C, c 30°C and d 35°C.

farinosa and *Isaria fumosorosea* (data not shown). These were not included in the molecular study, but warrant further investigation, particularly in relation to their interactions with the spittlebug.

Genetic variation was found amongst the *M. anisopliae* s.str. isolates as confirmed by the haplotype network (Fig 2); however, geographical distance between locations seems not to be an important factor driving genetic variation. It is always desirable to analyse as many samples as possible; however, not all the dead insects collected showed sporulation, which suggested that they may have died from other causes than fungal infection, thus reducing the number of potential isolates. However, we believe that our results are still valid and represent an important step forward in the study of the ecology of *Metarhizium* in this important agroecosystem. The genetic variation found amongst isolates collected in Paso Nuevo (Veracruz) could be related to the fact that they have their own small factories that produce *M. anisopliae*; however, there are no consistent and/or strict procedures in place to maintain and to produce large quantities of *M. anisopliae* conidia. For instance, field applications are made using multispore isolates and sometimes, when isolates fail to grow while being mass-produced in vitro, it is common to obtain a different isolate from field-infected insects to substitute the failing isolate. In addition, fungi with asexual reproduction such as *M. anisopliae* are haploid, which means they express all their genes and, therefore, are exposed to mutations leading to the presence of genetically different nuclei in the cytoplasm of the hyphae. Interestingly, this may result in the ratio of nuclear types within each isolate varying according to environmental conditions, eventually allowing the isolate to alter the nuclear ratio in response to prevailing environmental conditions (Deacon 1997), and as a consequence, genetically diverse isolates may be obtained from the same location. In addition, a further source of genetic diversity in fungal populations is recombination events, such as the exchange of nuclei during hyphal fusion events (anastomoses), which can be followed by nuclear recombination (parasexual cycle) (Burdon & Silk 1997). However, this event has been demonstrated only in the laboratory, as reported for *Beauveria bassiana* (Castrillo *et al* 2004), and its impact in field fungal populations remains unclear (Burdon & Silk 1997, Read *et al* 2010).

Temperature is one of the most important abiotic factors affecting the biology and ecology of entomopathogenic fungi (Tanada & Kaya 1993, Inglis *et al* 2001). Therefore, it is important to evaluate in vitro growth profiles of entomopathogenic fungi as a clue to the field behaviour of the fungi for a better understanding of the influence of abiotic factors on the diversity and distribution of fungal species. Overall, our results were similar to those previously reported, with optimal growth occurring between 20 and 25°C for *M. anisopliae* s.l. (Ekesi *et al* 1999, Dimbi *et al* 2004). When isolates of

M. anisopliae were grouped by geographical origin and compared, isolates from Oaxaca normally achieved the greatest growth at all temperatures (Fig 3), and isolates from Tabasco consistently achieved the smallest colony size at all temperatures. We consider that it is now very important to assess the ability of the isolates collected to infect spittlebug adults at different temperatures and to determine whether there is any potential relationship between in vitro growth and virulence.

We were not able to find a relationship between in vitro growth of the isolates and genetic variation. We accept that if we had used a larger number of isolates from each location, some significant trends could have been observed. However, our samplings were done during 1 month and focused only in isolates infecting the spittlebug; therefore, we consider the number of isolates obtained to be significant and important in underpinning our understanding of the organisation and ecology of *Metarhizium* in this agroecosystem. It is also likely that different results regarding genetic variation could be obtained using a multigene approach (Kepler *et al* 2014) or a different method such as mass spectrometry (Kepler *et al* 2014).

In summary, we only found *M. anisopliae* s.str. infecting adults of *Aeneolamia* in Mexican sugarcane plantations. The *M. anisopliae* isolates were genetically diverse but without any obvious geographic structure. More detailed studies of the ecology of the genus *Metarhizium* within this agroecosystem are needed to elucidate the possible role of this fungal genus in insect population dynamics, particularly the presence of other *Metarhizium* species in soil, adjunct vegetation or infecting other insects at the same niches.

Acknowledgments CHD received a scholarship from CONACYT, Mexico. This research was funded by the project ‘Diseño de un programa contemporáneo de manejo integrado de mosca pinta en caña de azúcar’, granted by Fundación Produce Veracruz-COFUPRO-SAGARPA, Mexico. Grant No. 2009-056.

References

- Arango GL, Torres C, Lapointe SL (1994) Pathogenicity of three strains of *Metarhizium anisopliae* to eggs and nymphs of *Aeneolamia varia* (Fabricius) (Homoptera: Cercopidae). *Rev Colomb Entomol* 20:43–46
- Badilla F, Toledo JC, Barreno C (1996) Pathogenicity of *Metarhizium anisopliae* on *Aeneolamia albofasciata* and *Prosapia* spp. (Homoptera: Cercopidae) adult insects in sugarcane in Escuintla, Guatemala. *Manejo Integrado de Plagas* 42:39–44
- Becerra Velásquez V, Paredes Cárcamo M, Rojo Meriño C, France Iglesias A, Franco Durán J (2007) Intraspecific differentiation of Chilean isolates of the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* as revealed by RAPD, SSR and ITS markers. *Genet Mol Biol* 30:89–99

- Bischoff JF, Rehner SA, Humber RA (2006) *Metarhizium frigidum* sp. nov.: a cryptic species of *M. anisopliae* and a member of the *M. flavoviridae* complex. *Mycologia* 98:737–745
- Bischoff JF, Rehner SA, Humber RA (2009) A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia* 101:512–530
- Burdon JJ, Silk J (1997) Sources and patterns of diversity in plant-pathogenic fungi. *Phytopathology* 87:664–669
- Castrillo LA, Griggs MH, Vandenberg JD (2004) Vegetative compatibility groups in indigenous and mass-released strains of the entomopathogenic fungus *Beauveria bassiana*: likelihood of recombination in the field. *J Invertebr Pathol* 86:26–37
- Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. *Mol Ecol* 9:1657–1660
- de la Torres CM, Cortez MH, Ortiz GCF, Capello GS, de la Cruz PA (2013) Caracterización de aislamientos nativos de *Metarhizium anisopliae* y su patogenicidad hacia *Aeneolamia postica*, en Tabasco, México. *Rev Colomb Entomol* 39:40–46
- Deacon JW (1997) *Modern Mycology*, 3rd. ed. Blackwell Science Ltd. Cambridge, p 303
- Dimbi S, Maniana NK, Lux SA, Mueke JM (2004) Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *BioControl* 49:83–94
- Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acid Res* 19:4008
- Ekesi S, Maniana NK, Ampong-Nyarko K (1999) Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Sci Tech* 9:177–185
- FAOSTAT (2012) Final 2012 data and preliminary 2013 data for 5 major commodity aggregates. <http://faostat.fao.org/site/339/default.aspx/> Accessed 31 Jul 2014.
- 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. *Mycol Res* 106:1066–1074
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Freed S, Jin FL, Ren SX (2011) Determination of genetic variability among the isolates of *Metarhizium anisopliae* var. *anisopliae* from different geographical origins. *World J Microbiol Biotechnol* 27:359–370
- García DA, Bustillo AE, Castro V, Arenas JB (2012) Selection of entomopathogenic fungi to control spittlebugs (Hemiptera: Cercopidae) of sugar cane in Colombia. *Rev Colomb Entomol* 38:252–259
- Gómez LA (2007) Manejo del salivazo *Aeneolamia varia* en cultivos de caña de azúcar en el valle del río Cauca. *Cenicaña (Colombia). Carta Trimestral* 29:10–17
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98
- Humber RA (2012) Identification of entomopathogenic fungi. In: Lacey LA (ed) *Manual of techniques in invertebrate pathology*, 2nd edn. Academic Press, Inc, California, pp 151–187
- Inglis GD, Goettel MS, Strasser H, Butt TM (2001) Use of hyphomycetous fungi for managing insect pests. In: Butt TM, Jackson CW, Magan N (eds) *Fungi as biocontrol agents: progress problems and potential*. CAB International, Wallingford, pp 23–70
- Kenward MG (1987) A method for comparing profiles of repeated measurements. *Appl Stat* 36:296–308
- Kepler RM, Humber RA, Bischoff JF (2014) Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. *Mycologia* 106:811–829
- Li Z, Alves SB, Roberts DW, Fan M, Jr Delalibera I, Tang J, Lopes RB, Faria M, Rangel DEN (2010) Biological control of insects in Brazil and China: history, current programs and reasons for their successes using entomopathogenic fungi. *Biocontrol Sci Tech* 20:117–136
- Matabanchoy Solarte JA, Bustillo Pardey AE, Castro Valderrama U, Mesa Cobo NC, Moreno Gil CA (2012) Eficacia de *Metarhizium anisopliae* para controlar *Aeneolamia varia* (Hemiptera: Cercopidae), en caña de azúcar. *Rev Colomb Entomol* 38:177–181
- Meyling NV, Eilenberg J (2007) Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biol Control* 43:145–155
- Meyling NV, Lübeck M, Buckley EP, Eilenberg J, Rehner SA (2009) Community composition, host range and genetic structure of the fungal entomopathogen *Beauveria* in adjoining agricultural and semi-natural habitats. *Mol Ecol* 18:1282–1293
- Ormond EL, Thomas APM, Pugh PJA, Pell JK, Roy HE (2010) A fungal pathogen in time and space: the population dynamics of *Beauveria bassiana* in a conifer forest. *FEMS Microbiol Ecol* 74:146–154
- Payne RW, Murray DA, Harding SA, Baird DB, Soutar DM (2005) *GenStat for Windows (8th Edition) Introduction*. VSN International, Hemel Hempstead
- Peck D, Castro U, Lopez F, Morales AR, Rodriguez J (2001) First record of the sugar cane and forage grass pest, *Prosapia simulans* (Homoptera; Cercopidae) from South America. *Fla Entomol* 84:402–409
- Read ND, Fleißner A, Roca MG, Glass NL (2010) Hyphal fusion. In: Borkovich KA, Ebbole DJ (eds) *Cellular and molecular biology of filamentous fungi*. American Society for Microbiology Press, Washington DC, pp 260–273
- Rehner SA, Buckley E (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1- α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97:84–98
- 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. *Mol Biol Evol* 28:2731–2739
- Tanada Y, Kaya H (1993) *Insect pathology*. Academic, San Diego, p 666
- Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* 132:619–633
- Thompson V (2004) Associative nitrogen fixation, C₄ photosynthesis, and the evolution of spittlebugs (Hemiptera: Cercopidae) as major pests of neotropical sugarcane and forage grasses. *Bull Entomol Res* 94:189–200
- 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 Res* 22:4673–4680
- Vega FE, Meyling NV, Luangsa-ard JJ, Blackwell M (2012) Fungal entomopathogens. In: Vega FE, Kaya HK (eds) *Insect pathology*, 2nd edn. Elsevier Inc, London, pp 171–220
- White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR protocols: a guide to methods and applications*. Academic, San Diego, pp 315–322
- Wilcox D, Dove B, McDavid D, Grenn D (2002) UTHSCSA Image Tool for Windows v. 3.0. The University of Texas Health Science Center, San Antonio. <http://maxrad6.uthscsa.edu>