Isolation of Metarhizium guizhouense and Metarhizium robertsii Strains from Soil-Exposed Amblyomma americanum (Acarina: Ixodidae) from Northwest Arkansas, USA

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Isolation of *Metarhizium guizhouense* and *Metarhizium robertsii* strains from soil-exposed *Amblyomma americanum* (Acarina: Ixodidae) from northwest Arkansas, USA

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Abstract

The lone star tick, *Amblyomma americanum* (L.) (Acarina: Ixodidae), is the most abundant tick species found in Arkansas and is involved in the transmission of pathogens of medical and veterinary importance. When not feeding, most non-nidicolous tick species shelter in the soil and leaf litter where they may be exposed to and potentially infected with entomopathogenic fungi that reside naturally in the soil. Entomopathogenic fungi in the genus *Metarhizium* Sorokīn (Hypocreales: Clavicipitaceae) have shown promise as biological control agents of ticks. Here, the first study to isolate and identify Arkansas-derived isolates of *Metarhizium* from *A. americanum* ticks is presented. We exposed 320 ticks artificially to native soil from Savoy. Of these soil exposed ticks, 2.5% of adults and 1.5% of nymphs displayed signs of infection with *Metarhizium*. Of the infected Savoy adults, 3.3% were females and 1.7% were males. Similarly exposed ticks from West Fork resulted in only 2.4% of nymphal ticks being infected with this fungus. Eight isolates of *Metarhizium* were cultured from infected ticks exposed to soil from these locations. Four of these *Metarhizium* isolates (3 from Savoy and 1 from West Fork) were identified to species by sequencing of the ITS locus and the EF1-*α* genes. Three Savoy strains (P10N1, P10AF1, and P2AM1) had identical sequences and were identified as *Metarhizium robertsii* (Bischoff, Rehner & Humber) (Hypocreales: Clavicipitaceae). The strain from West Fork (P9N2) was identified as *Metarhizium guizhouense* (Chen & Guo) (Hypocreales: Clavicipitaceae). The ITS and the EF1-*α* sequences of the Savoy strains showed 100% similarity to *M. robertsii* strains ARSEF 2575 and ART 500, respectively. The ITS and EF1-*α* sequences of the West Fork strain showed 99% similarity to *M. guizhouense* strains ARSEF 977 and CBS 258.90, respectively. This study demonstrates that entomopathogenic fungi *M. guizhouense* and *M. robertsii* are pathogenic to and can be isolated from *A. americanum*. Furthermore, the EF1-*α* genetic marker was shown to be a very effective tool for distinguishing different species of *Metarhizium* from ticks when used in conjunction with ITS sequence data. Standardizing the use of ticks in soil exposure methods for isolating entomopathogenic fungi could be useful for obtaining isolates that are highly virulent to *A. americanum*. The isolation and identification of *Metarhizium* spp. from *A. americanum* in Arkansas indicates that further exploration of entomopathogenic fungi as biological agents to control *A. americanum* is warranted.

Key Words: lone star tick; entomopathogenic fungi; isolates; identification; Savoy; West Fork

Resumen

La garrapata estrella solitaria, *Amblyomma americanum* (L.) (Acarina: Ixodidae), es la especie de garrapata más abundante encontrada en Arkansas y está involucrada en la transmisión de patógenos de importancia médica y veterinaria. Cuando no se alimentan, la mayoría de las especies de garrapatas no nídicas se refugian en el suelo y la hojarasca donde pueden estar expuestas y potencialmente infectadas con hongos entomopatógenos que residen naturalmente en el suelo. Los hongos entomopatógenos del género *Metarhizium* Sorokīn (Hypocreales: Clavicipitaceae) se han mostrado prometedores como agentes de control biológico de las garrapatas. Aquí, se presenta el primer estudio para identificar los aislados de *Metarhizium* derivados de garrapatas de *A. americanum* en de Arkansas. Expusimos 320 garrapatas artificialmente a suelo nativo de Saboya. De estas garrapatas expuestas al suelo, el 2,5% de los adultos y el 1,5% de las ninfas mostraron señales de infección por *Metarhizium*. De los adultos de Saboya infectados, el 3,3% fueron hembras y el 1,7% fueron machos. Las garrapatas expuestas de manera similar de West Fork dieron como resultado que solo el 2,4% de las garrapatas ninfales se infectaran con este hongo. Se cultivaron ocho cepas de *Metarhizium* de garrapatas infectadas expuestas al suelo de estos lugares. Cuatro de estos aislados de *Metarhizium* (3 de Savoy y 1 de West Fork) se identificaron en especies mediante la secuenciación del locus ITS y los genes EF1-α. Tres cepas de Savoy (P10N1, P10AF1 y P2AM1) tenían secuencias idénticas y se identificaron como *Metarhizium robertsii* (J.F. Bischoff, S.A. Rehner & Humber) (Hypocreales: Clavicipitaceae). La cepa de West Fork (P9N2) se identificó como *Metarhizium guizhouense* (Chen & Guo) (Hypocreales: Clavicipitaceae). Las secuencias ITS y EF1-α de las cepas Savoy mostraron un 100% de similitud con las cepas ARSEF 2575 y ART 500 de *M. robertsii*, respectivamente. Las secuencias ITS y EF1-α de la cepa West Fork mostraron un 99% de similitud con las cepas ARSEF 977 y CBS 258.90 de *M. guizhouense*, respectivamente. Este estudio demuestra que los hongos entomopatógenos *M. guizhouense* y *M. robertsii* son patógenos y pueden aislarse de *A. americanum*. Además, se demostró que el marcador genético EF1-α es una herramienta muy eficaz para distinguir diferentes

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especies de *Metarhizium* de las garrapatas cuando se usa junto con los datos de la secuencia ITS. La estandarización del uso de garrapatas en los métodos de exposición del suelo para aislar hongos entomopatógenos podría ser útil para obtener aislamientos altamente virulentos para *A. americanum*. El aislamiento e identificación de especies de *Metarhizium* sobre *A. americanum* en Arkansas indica que se justifica una mayor exploración de los hongos entomopatógenos como agentes biológicos para controlar *A. americanum*.

Palabras Clave: garrapata estrella solitaria; hongos entomopatógenos; aislamientos; identificación; Saboya; West Fork

In Arkansas and throughout much of the southeastern USA, one of the most abundant tick species is the lone star tick, *Amblyomma americanum* (L.) (Acarina: Ixodidae) (Childs & Paddock 2003; Goddard & Varela-Stokes 2009; Trout 2010; K. M. Loftin & E. E. Smith, University of Arkansas Cooperative Extension Service, personal communication). This tick species feeds on humans and a wide variety of domesticated and wild animals but preferentially feeds on the white-tailed deer, *Odocoileus virginianus* (Zimmermann) (Artiodactyla: Cervidae), which is a preferred host for all host-seeking life stages of *A. americanum* (Childs & Paddock 2003; Paddock & Yabsley 2007; Holderman & Kaufman 2013). Upsurges in white-tailed deer populations have led to a subsequent increase in the densities of lone star tick (Paddock & Yabsley 2007). White-tailed deer populations also are involved in the distribution of *A. americanum* to other habitats and regions throughout the US. Recent modeling of the geographic range of this species from acarological survey data in the US (Ragahaven et al. 2019) found that this tick has been expanding its range further northward and westward than previously expected.

Amblyomma americanum transmits medically important bacterial, viral, and protozoan pathogens including the causal agents of human ehrlichiosis (*Ehrlichia chaffeensis* [Anderson, Dawson, Jones, & Wilson] and *Ehrlichia ewingii* [Anderson, Greene, Jones, & Dawson] [both Rickettsiales: Anaplasmataceae]), tularemia (*Francisella tularensis* [McCoy & Chapin] [Thiotrichales: Francisellaceae]), some *Rickettsia* species, bobcat fever (*Cytauxzoon felis* [Kier] [Piroplasmida: Theileriidae]), Bourbon virus, and Heartland virus (Childs & Paddock 2003; Trout 2010; Holderman & Kaufman 2013; Loftin & Hopkins 2014; Nicholson et al. 2018). Furthermore, saliva of *A. americanum* may induce an allergic reaction to mammalian red meat known as alpha-gal syndrome (Commins et al. 2011; Nicholson et al. 2018).

Historically, most tick control measures have used synthetic chemical acaricides that provide a quick knockdown of ticks with long-term residual control (e.g., over 1 mo) (Stafford & Williams 2017; White & Gaff 2018). However, development of acaricide resistance in some species of ticks and concerns of possible negative environmental effects (Kunz & Kemp 1994; Abbas et al. 2014) have led to the search for alternative control measures.

Entomopathogenic fungi are some of the most effective biological control agents for ticks (Samish & Rehacek 1999; Samish et al. 2008). These fungi may infect all tick life stages and can penetrate directly through the integument (Samish et al. 2008). This is important because pathogens that infect hosts per os would not be likely to work for blood-feeding ticks (Ostfeld et al. 2006; Samish et al. 2008). Entomopathogenic fungi occur naturally in the leaf litter and soil where ticks spend 90% of their life cycle (Samish et al. 2008; Tuininga et al. 2009; Burtis et al. 2019). Studies in Europe, Africa, and South and North America have examined natural associations of ticks with entomopathogenic fungi and have isolated several well-known fungal pathogens from ticks (Samsinakova et al. 1974; Estrada-Peña 1990; Kalsbeek et al. 1995; Samish & Rehacek 1999; Zhioua et al. 1999; da Costa et al. 2002; Benoit et al. 2005; Fernandes & Bittencourt 2008; Samish et al. 2008; Tuininga et al. 2009; Greengarten et al. 2011; Fernandes et al. 2012). Of these, strains of *Metarhizium anisopliae* sensu lato (s.l.) (Metschnikoff) Sorokīn (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Balsamo-Crivelli) Vuillemen (Hypocreales: Cordycipitaceae) are the most extensively studied and may be the most effective biocontrol agents for ticks (Fernandes & Bittencourt 2008; Samish et al. 2008; Fernandes et al. 2012). To date, few studies have reported on the isolation of *M. anisopliae* s.l. from ticks (da Costa et al. 2002; Benoit et al. 2005; Tuininga et al. 2009). Furthermore, little is known about entomopathogenic fungi that infect *A. americanum* and their potential for use as a biocontrol agent for this tick species. The objectives of this study were to isolate entomopathogenic fungi from *A. americanum* using soil exposure methods and to identify fungal pathogens using morphological and molecular techniques.

Materials and Methods

TICK COLLECTIONS

Ticks were collected from 2 locations in Washington County, Arkansas, USA: the University of Arkansas Agricultural Experiment Station, Savoy Research Complex, Beef Cattle Research Area (36.128°N, 94.331°W) on 4 Apr 2018 and 18 May 2018, and West Fork (35.96°N, 94.151°W) on 7 Jun 2018. These locations were 24.7 km apart from each other. Collection sites at both locations were within forested areas or on the edge of forested areas adjacent to open pastureland. Ticks were collected with carbon dioxide traps consisting of 1.9 L Igloo coolers (Igloo Products Corp., Katy, Texas, USA) filled with 0.5 kg of dry ice and placed on 1 m^2 white cloth (Mays et al. 2016). Carbon dioxide traps were placed 130 m apart at 10 different collection sites around each location, and were exposed to the environment for 2 h. In addition, drag samples were collected at both locations using 58 × 114 cm drag cloths (Bioquip Products Inc., Rancho Dominguez, California, USA) in 5 to 10 transects for 130 m (Mays et al. 2016). Ten soil samples each were collected from Savoy and West Fork by removing the top 5 mm of soil, 5 m from each carbon dioxide trap. Cloths containing ticks collected by carbon dioxide were placed in 7.6 L plastic bags then placed in a refrigerator at 4 °C along with bags of soil samples until sorted. Ticks remained in storage anywhere from 1 to 42 d (Savoy), or 1 to 7 d (West Fork). Soil samples were held in storage from 5 to 7 d. Ticks collected by dragging were placed in plastic vials containing a small blade of grass, and stored at room temperature (29–28 °C) for 1 to 5 d (Savoy) or 1 to 7 d (West Fork).

Ticks from all sampling locations were sorted by location, species, sex, and life stage. Ticks were identified to species using the taxonomic keys of Lancaster (1973) and an online tick identification key from Georgia Southern University (Bischof & Beati 2014). This latter key was inspired by the taxonomic pictorial key published by Keirans & Litwak (1989).

SOIL EXPOSURE ASSAYS

Soil exposure assays were used for collecting entomopathogenic fungi by the forced contact of field-collected *A. americanum* ticks to soil samples using methods modified from Tuininga et al. (2009). Only *A. americanum* ticks were exposed to soil because they comprised 99% of collected ticks. Ticks were placed in 3 sets of 10 plastic Petri dishes (100 × 15 mm) (VWR International, Radnor, Pennsylvania, USA), each

filled with 8 g of moist soil from 1 of the 10 collection sites from Savoy. Due to the difficulty in recovering ticks from the Savoy soil samples, West Fork soil samples were sifted through an 850 µm mesh prior to exposing ticks to soil samples. This procedure removed bulky organic matter for easier recovery of ticks from soil. To compensate for the loss in soil mass and volume due to elimination of bulky organic matter from sifting, the amount of soil in the West Fork samples was doubled to 16 g. In total, 30 dishes of soil were used per location. Adult females (n = 4 for West Fork; n = 6 for Savoy), adult males (n = 1 for West Fork; n = 6 for Savoy), or nymphal ticks (n = 20 for Savoy; n = 21 for West Fork), depending on the availability of live, wild-captured ticks, were introduced into each dish of soil. Ticks were pooled together initially from all collection sites within either Savoy or West Fork before being exposed to soil. Soil samples then were moistened with 6 or 7 mL of deionized water, covered with filter paper, and the dishes sealed with Parafilm® (Bemis Company, Inc., Neenah, Wisconsin, USA) and white or pink labeling tape (VWR International, Radnor, Pennsylvania, USA) to prevent ticks from escaping. Each set of soil samples with ticks was wrapped in aluminum foil (to simulate conditions of complete darkness) and maintained in an incubator (28.7 ± 0.7 °C) and relative humidity of 61.4 ± 5.6%. Ticks were checked once per wk for mortality for 2 wk. Dead ticks were collected, washed in 2 separate, 50 mL baths of deionized water, placed in Petri dishes lined with filter paper moistened with 1 to 2 mL of deionized water, and checked for fungal growth after 2 wk. All remaining ticks were removed from soil exposure after 2 wk.

FUNGAL ISOLATIONS

Dead ticks that appeared to be infected with *Metarhizium* or other potential entomopathogenic fungi (i.e., fungal hyphae and conidiophores observed emerging from within the ticks' legs, mouthparts, or idiosoma) were used for isolations. Conidia from these ticks were inoculated on plates of Sabouraud Dextrose Agar (Hardy Diagnostics, Santa Maria, California, USA) (64 g per L) supplemented with yeast extract (2 g per L) and gentamicin sulfate (10 mg per L) (Goettel & Inglis 1997; Inglis et al. 2012). Conidia were transferred from infected ticks with a sterile, platinum inoculating loop into 1 mL of sterile 0.05% aqueous Tween 80 (Sigma-Aldrich, St. Louis, Missouri, USA) solution with penicillin/streptomycin (1.5 mg per L penicillin G and 2.5 mg per L streptomycin), then vortexed thoroughly for about 1 min. An aliquot of 0.2 mL conidial suspension was pipetted onto five 100 × 15 mm Sabouraud Dextrose Agar plates for each isolate and spread evenly using a sterile glass spreading rod. Plates were wrapped in aluminum foil and incubated at ambient room conditions (i.e., 26.8 ± 0.6 °C, 66.0 ± 5.2% RH). After 14 to 26 d, conidia from colony forming units were sampled and examined by phase contrast microscopy.

FUNGAL IDENTIFICATION

Morphological Identification

Conidia from infected ticks and fungal isolates grown on Sabouraud Dextrose Agar plates were mounted in lactophenol on microscope slides and examined using a Nikon® Eclipse E600 phase contrast microscope (Nikon Corporation, Tokyo, Japan) at 200× and 400× magnification. Fungi were identified to genus using the taxonomic guide of Humber (1997). Conidia from colony forming units identified as *Metarhizium* were examined additionally and measured at 630× magnification using a Zeiss® Axio Imager A1 with AxioCam 1C and Zen 2 Lite software (Carl Zeiss MicroImaging, Göttingen, Germany). Three to 4 measurements of the conidia length and width were taken for the following isolates: Savoy P10N1, Savoy P10AF1, Savoy P2AM1, and West Fork P9N2.

Molecular Identification

Cultures of 4 *Metarhizium* isolates from *A. americanum* ticks were analyzed at the Insect Genetics Lab, University of Arkansas, Fayetteville, Arkansas, USA, and at the USDA-ARS Collection of Entomopathogenic Fungal Cultures laboratory for identification. Fungi were grown in potato dextrose broth for 4 d at 25 °C, and 100 mg mycelia were collected for DNA extraction using the DNeasy® Plant Minikit (Qiagen Sciences, Germantown, Maryland, USA) following the manufacturer's protocol. DNA was eluted with 10 mM Tris: EDTA (pH 8.0) and stored at −20 °C until use. Isolates were identified to species by amplifying and sequencing the nuclear ribosomal rRNA internal transcribed spacer (ITS) locus and the translation elongation factor 1-alpha (EF1-α) gene. The ITS locus was amplified using primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) (White et al. 1990) following assay conditions reported in Taylor et al. (1996). The EF1-α gene was amplified using primers 983F (5'-GCYCCYGGHCAYCGTGAYTTYAT) and 2218R (5'-ATGACACCRACRGCRACRGTYTG), and conditions reported by Bischoff et al. (2006). Polymerase chain reaction (PCR) products were purified and concentrated with centrifugal devices from VWR™ (Radnor, Pennsylvania, USA) and sent to Eurofins Genomics (Huntsville, Alabama, USA), or purified by use of Qiagen PCR Purification kit and submitted to Cornell University Biotechnology Resource Center (Ithaca, New York, USA) for Sanger sequencing. Sequence primers were the same as those used for amplification, and both DNA strands were sequenced. Sequences were aligned and annotated with Geneious v6.16 (Kearse et al. 2012). A BLAST search was conducted for species-level identification. All 4 isolates were deposited to USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF), with the following accession numbers: Savoy P10N1 = ARSEF 14329, West Fork P9N2 = ARSEF 14330, Savoy P10AF1 = ARSEF 14331, and Savoy P2AM1 = ARSEF 14332.

Results

TICK COLLECTIONS

A total of 2,802 ticks were collected from Savoy and West Fork. At both locations, 99.4% of the ticks found were *A. americanum*, the majority of which were nymphs (81.2% of ticks). Adults comprised 18.3% of all ticks collected from both locations. A total of 2,301 ticks were collected from Savoy, all of which were *A. americanum*. At West Fork, the majority of individuals were lone star ticks with 2.8% *Dermacentor variabilis* (Say) (Acarina: Ixodidae) and 0.4% unidentified *Ixodes* nymphs.

SOIL EXPOSURE ASSAY

Adult and nymphal ticks exposed to soil from Savoy were observed with *Metarhizium* infection (Table 1). Ticks infected with this fungus were observed with mycelia bearing olive-green conidia bursting throughout much of the ticks' integument, particularly leg joints, mouthparts, and other orifices in the ventral abdomen (Fig. 1). The percentage of soil-exposed ticks from Savoy infected with *Metarhizium* was 1.9% with 2.5% adults and 1.5% of nymphs infected (Table 1). Of the adult ticks infected with *Metarhizium*, 1.7% of male ticks, and 3.3% of female ticks were infected with the fungus. Only nymphal ticks from West Fork were infected with *Metarhizium*. The percentage of infected *A. americanum* ticks exposed to soil from West Fork was the same as

Table 1. The number and percentage of field collected *Amblyomma americanum* ticks infected with *Metarhizium* after 2 wk of exposure to soil from northwest Arkansas. Ticks and soil samples were collected from Savoy and West Fork, Arkansas (Washington County) during the summer of 2018.

Location	Sex or life stage	Number ticks exposed[a]	Number fungi (% ticks fungi)[b]	Number infected *Metarhizium* spp. (% *Metarhizium* spp.)[c]	Number infected *Metarhizium robertsii* (% infected *M. robertsii*)[d]	Number infected *Metarhizium guizhouense* (% infected *M. guizhouense*)[e]
Savoy						
	Adult female	60	8 (13.3)	2 (3.3)	1 (1.7)	0 (0)
	Adult male	60	14 (23.3)	1 (1.7)	1 (1.7)	0 (0)
	Total adults[f]	120	22 (18.3)	3 (2.5)	2 (3.3)	0 (0)
	Nymph	200	41 (20.5)	3 (1.5)	1 (0.5)	0 (0)
	Total ticks[g]	320	63 (19.7)	6 (1.9)	3 (0.9)	0 (0)
West Fork						
	Adult female	40	15 (37.5)	0 (0)	0 (0)	0 (0)
	Adult male	10	4 (40.0)	0 (0)	0 (0)	0 (0)
	Total adult	50	19 (38.0)	0 (0)	0 (0)	0 (0)
	Nymph	210	43 (20.5)	5 (2.4)	0 (0)	1 (0.5%)
	Total ticks	260	62 (23.8)	5 (1.9)	0 (0)	1 (0.4%)

[a]Ticks were exposed to samples of moistened soil from Savoy and West Fork, Arkansas; [b]Number and percentage of ticks found with *Metarhizium* spp. and other fungi; [c]Number and percentage of ticks found with *Metarhizium* spp.; [d]Number and percentage of ticks found with *Metarhizium robertsii*; [e]Number and percentage of ticks found with *Metarhizium guizhouense*; [f]Total adults includes females plus males; [g]Total ticks includes adults plus nymphs.

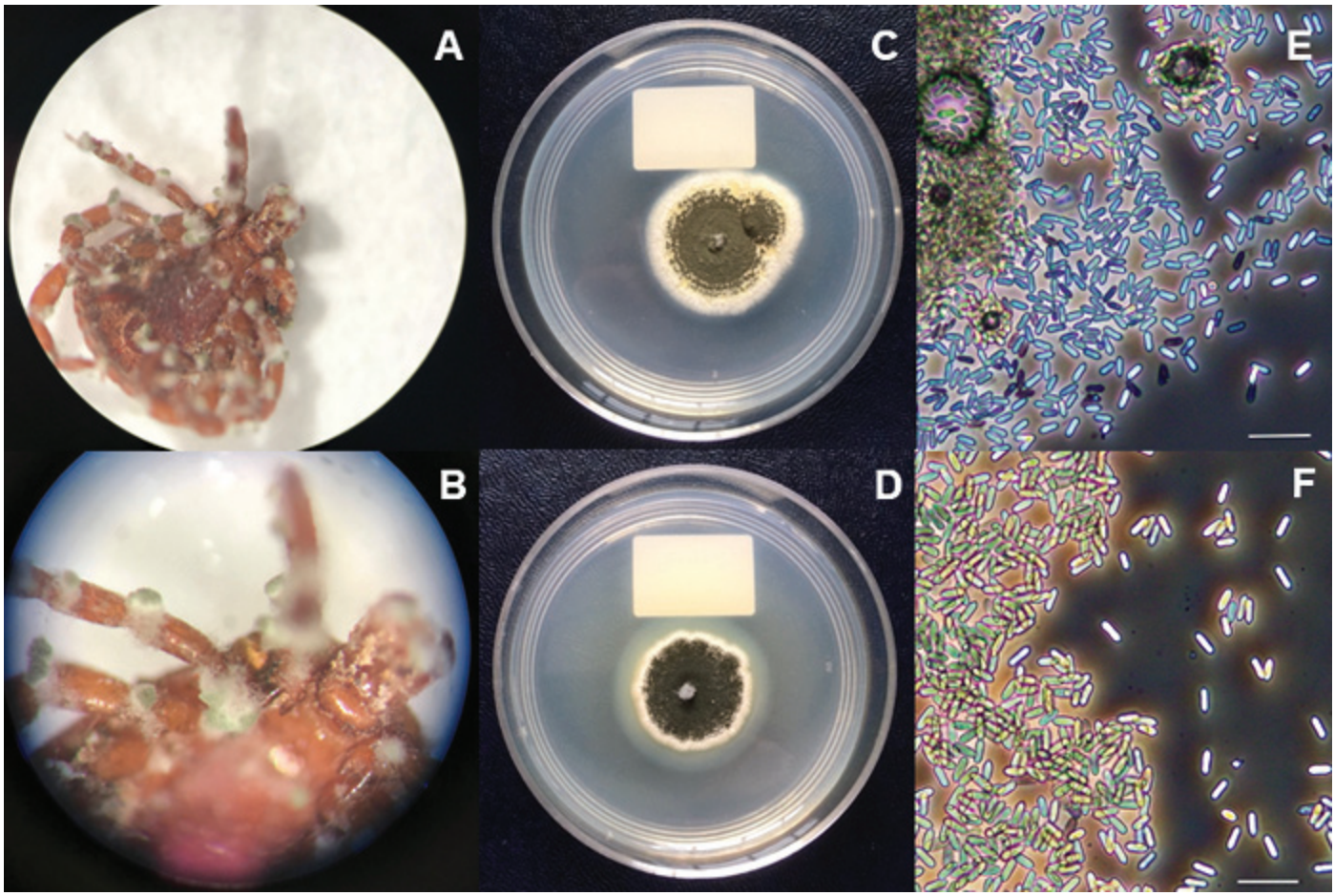

Fig. 1. *Metarhizium robertsii* (A-C, E) and *Metarhizium guizhouense* (D, F) from *Amblyomma americanum* ticks collected from northwest Arkansas (Washington County). (A) Sporulating *M. robertsii* (Savoy P2AM1/ARSEF 14332) growing on an infected adult male tick. (B) Inset of infected tick showing sporulating conidia in addition to mouthparts and coxal spurs diagnostic of *A. americanum*. (C, D) *Metarhizium robertsii* (Savoy P2AM1/ARSEF 14432) and *M. guizhouense* (West Fork P9N2/ARSEF 14330), respectively, 10 d old colony on Sabouraud Dextrose Agar (plate diam = 60 mm). (E, F) Conidia of *M. robertsii* (Savoy P2AM1/ARSEF 14332) and *M. guizhouense* (West Fork P9N2/ARSEF 14330), respectively, viewed at 200× magnification (scale = 20 μm). Photos: Austin Goldsmith (A, B) and Louela Castrillo (C-F).

Savoy, with 1.9% of soil-exposed ticks infected. The infection percentage of nymphs with *Metarhizium* was 2.4%. In addition to these infected ticks, some adult and nymphal ticks from both locations were observed to be covered with saprophytic fungi that were not identified.

FUNGAL ISOLATIONS AND MORPHOLOGICAL CHARACTERIZATIONS

Out of 12 fungal isolates from ticks established on Sabouraud Dextrose Agar plates, 11 contained pure cultures while all cultures from 1 isolate were contaminated by a black, fast-growing, saprophytic mold. Specimens of conidia and conidiophores from all 11 fungal isolates were examined by phase contrast microscopy, and 8 of the fungal isolates (5 from Savoy and 3 from West Fork) were identified as *Metarhizium* based on morphological characters (Table 2). Mycelia and conidia of isolates were typical of the genus as described in Humber (1997). Fungal colonies isolated from ticks and soil from Savoy had mycelial growth that was light orange to pale yellow in appearance, exhibited a wrinkled look, and contained dark green spores borne on white mycelia (Fig. 1). The measurements reported for the Savoy *Metarhizium* isolates were 6.7 ± 0.2 × 3.1 ± 0.2 μm for Savoy P10N1, 6.6 ± 0.1 × 2.5 ± 0.4 μm for Savoy P10AF1, and 6.5 ± 0.4 × 2.7 ± 0.1 μm for Savoy P2AM1. The *Metarhizium* colonies from the West Fork isolate were found to be light yellow and somewhat smoother in appearance than the other *Metarhizium* isolates (Fig. 1). The phialides borne on white mycelia in these colonies bore conidia that were a mixture of dark green and olive-green and that measured 8.6 ± 0.5 × 3.6 ± 0.2 μm. The *Metarhizium* conidia from all isolates were rod-shaped (Fig. 1).

Table 2. *Metarhizium* spp. isolated from infected *Amblyomma americanum* ticks.

	Savoy			West Fork			
Fungi	Adult female	Adult male	Nymph	Adult female	Adult male	Nymph	Total
Metarhizium robertsii	1	1	1	0	0	0	3
Metarhizium guizhouense	0	0	0	0	0	1	1
Unidentified *Metarhizium* isolates	0	0	2	0	0	2	4
Total *Metarhizium*	1	1	3	0	0	3	8

MOLECULAR IDENTIFICATION

The 3 Savoy strains had identical ITS and EF1-*α* sequences and were identified as *M. robertsii* (Bischoff, Rehner, & Humber) (Hypocreales: Clavicipitaceae). In contrast, the West Fork strain differed in its ITS and EF1-*α* sequences, by 2 (out of 570) and 8 (out of 921) base pairs, respectively, to the the 3 Savoy strains, and was placed in the species *M. guizhouense* (Chen & Guo). The ITS and EF1-*α* sequences of the Savoy strains showed similarity to *M. robertsii* strains ARSEF 2575 (100%) and ART 500 (100%), respectively. The ITS and EF1-*α* sequences of the West Fork strain showed similarity to *M. guizhouense* strains ARSEF 977 (99%) and CBS 258.90 (99%), respectively. Species placement was based primarily on the EF1-*α* sequence data, and in comparison to reference strains of *Metarhizium* spp. reported by Bischoff et al. (2009). Sequence data from the 4 fungal strains were deposited into GenBank. Sequence accession numbers are reported in Table 3.

Discussion

Despite being some of the most pathogenic fungi on ticks, as well as some of the most extensively studied pathogens for their control, *Metarhizium* spp. have been isolated rarely from naturally infected ticks (da Costa 2002; Benoit et al. 2005; Samish et al. 2008; Tuininga et al. 2009). A study conducted in Brazil (da Costa et al. 2002) previously documented the isolation of *M. anisopliae* var. *anisopliae* (Metschnikoff) Sorokīn from engorged female *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini) (Acarina: Ixodidae) in addition to *B. bassiana*, which has been identified and isolated from ticks in other studies (Samsinakova et al. 1974; Kalsbeek et al. 1995; Tuininga et al. 2009; Greengarten et al. 2011). In addition, Benoit et al. (2005) isolated *M. anisopliae* internally and externally from *Ixodes scapularis* (Say) (Acarina: Ixodidae) and *Rhipicephalus sanguineus* (Latreille) (Acarina: Ixodidae). In a study conducted by Tuininga et al. (2009) at the Fordham University, Louis Calder Center biological field station in Armonk, Westchester County, New York, USA, entomopathogenic fungi were cultured from wild-caught *I. scapularis* nymphs. Out of the 64 plates prepared from nymphal ticks, 16 (25%) positively identified as the genera *Beauveria*, *Metarhizium*, *Paecilomyces*, and *Lecanicillium* using "microscopic examination."

Our study is the first to report on the isolation of *M. guizhouense* and *M. robertsii* from the lone star tick, *A. americanum*. Because other studies reporting "*M. anisopliae*" from ticks were based on morphological characters (da Costa et al. 2002, Benoit et al. 2005, Tuininga et al. 2009), these can be classified only as *M. anisopliae* s.l., and thus the diversity of *Metarhizium* species and strains pathogenic to ticks cannot be assessed definitively from earlier reports. Although morphological characters such as colony description, conidiophore shape or size, and conidial dimensions are useful for determining genera in entomopathogenic fungi, these factors are of limited value for delimiting different species within a genus, particularly for *Metarhizium* (Bischoff et al. 2006, 2009). Colony, conidiophore, and conidia morphology among different *Metarhizium* species can be quite similar if not identical to one another. Conversely, morphological characteristics also can vary markedly in one species or among cultures of the same isolate. Therefore, it is extremely difficult to distinguish most species of *Metarhizium* from each other by morphological characters alone. Moreover, several authors have reported that ITS markers by themselves have proven to be insufficient for resolving many of the terminal lineages (e.g., phylogenetic species) within the *Metarhizium* genus (Driver et al. 2000, Huang et al. 2005a, b) despite its usefulness in deliminating the genus as a single clade (Driver et al. 2000). As a result, this molecular marker might be considered unreliable for species level-idenitification within *Metarhizium* (Bischoff et al. 2006, 2009). However the the EF-α gene, used alone or in conjunction with other genetic markers, has resolved phylogenetic species linages and relationships within the genus (Bischoff et al. 2006, 2009), and has been demonstrated as a useful tool for species identification (Mesquita et al. 2020). A recent study by Mesquita et al. (2020) reported the isolation of strains of *M. anisopliae* sensu strictu (s.s.) and *Metarhizium pingshaense* (Chen & Guo) (Hypocreales: Clavicipitaceae) from soil that were pathogenic to the cattle tick *R.* (*B.*) *microplus*. These strains also were identified by sequencing of the EF1-α gene, and along with our results indicate that a number of diverse species of soil-borne *Metarhizium* exist that are pathogenic to ticks.

A standard method used for isolating entomopathogenic fungi from soil is by the "insect baiting method" (Zimmermann 1986; Meyling 2007; Tuininga et al. 2009; Bharadwaj & Stafford 2011). This method usually involves exposing highly susceptible arthropod hosts (e.g., larvae of *Galleria mellonella* L. [Lepidoptera: Pyralidae] or *Tenebrio molitor* L. [Coleoptera: Tenebrionidae]) to soil samples that might contain entomopathogenic fungi. Our study used a slightly novel approach of this method by substituting *G. mellonella* or *T. molitor* larvae with field-collected *A. americanum* ticks. Because ticks naturally associate with soil and leaf litter (Samish 2008; Burtis et al. 2019), their use in the arthropod soil baiting method might be advantageous for isolating entomopathogenic fungi that have a specific virulence to a particular tick species. However, it must be mentioned that a strain of entomopathogenic fungus isolated from a given arthropod host does not necessarily mean that such a fungal strain is more virulent to this particular host than fungi isolated from other hosts (Fernandes et al. 2006; Samish et al. 2008). Further research should explore standardization and application of the soil baiting method with ticks as a means of surveying for and isolating potential entomopathogenic fungi that can be developed for tick management. Before these soil-baited tick isolates of entomopathogenic fungi can start to undergo development as biopesticides against *A. americanum* and other tick species in the USA, studies evaluating the pathogenicity of these fungal isolates to these tick species need to be conducted in the laboratory and field.

Currently, few studies have focused on the biological control of *A. americanum* with *Metarhizium* species. Also, no study to date has

Table 3. *Metarhizium* spp. from *Amblyomma americanum* analyzed in this study.*Localities from which the isolates came were in Washington County, Arkansas.

Fungus/strain	ARSEF number	Collection site (plot number)*	Host life stage (*n*)	GenBank accession number	
				ITS	EF1-α
Metarhizium roberstsii					
Savoy P10N1	14329	Plot 10	Nymph (1)	MN255810	MW042079
Savoy P10AF1	14331	Plot 10	Adult female (1)	MW013831	MW042080
Savoy P2AM1	14332	Plot 2	Adult male (1)	MW013832	MW042081
Metarhizium guizhouense					
West Fork P9N2	14330	Plot 9	Nymph (2)	MN255811	MW042082

focused on the control of this tick species with either *M. robertsii* or *M. guizhouense* including fungal isolates from Arkansas. Therefore, additional work with these fungal species as biocontrol agents to control *A. americanum* is warranted. Further discovery of local strains of entomopathogenic fungi for the development of tick mycoinsecticides would be useful for the management of ticks in Arkansas and across the USA.

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