N O T E

Quantification and Molecular Identification of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) in Soils Associated with *Megacopta cribraria* (Hemiptera: Plastaspidae)[1]

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The kudzu bug, *Megacopta cribraria* (F.) (Hemiptera: Plataspidae), is native to several areas in the Eastern Hemisphere. It was first reported in North America and the New World in 2009 aggregating on homes in northeastern Georgia. It was initially thought to be a nuisance pest and a potential pest of legumes (Eger et al. 2010, Insecta Mundi 0121: 1–11; Suiter et al. 2010, J. Integ. Pest Mgt. 1(1): F1–F4[4]). Since this initial discovery, the insect has been reported throughout the southeastern United States as far west as Louisiana and as far north as Delaware (Gardner et al. 2013, J. Entomol. Sci. 48: 118–127; www.kudzubug.org). Adults and immatures feed upon a variety of host plants, but the reproductive hosts in its expanded North American range are kudzu, *Pueraria montana* var. *lobata* (Willdenow) Maesen and Almeida, and soybean, *Glycine max* (L.) Merrill (Zhang et al. 2012, Environ. Entomol. 41: 40–50). Yield losses in soybeans are reported when populations are left unchecked (Seiter et al. 2013a, J. Econ. Entomol. 106: 1676–1683).

In an initial assessment of natural enemy impact on this invasive pest, Ruberson et al. (2012, Appl. Entomol. Zool. 48: 3–13) found only sporadic predation of adults, only one adult parasitized by the tachinid *Phasia robertsonii* (Townsend), and one adult infected with the fungus *Beauveria bassiana* (Balsamo) Vuillemin. Since that report, the egg parasitoid *Paratelenomus saccharalis* (Dodd) has been documented parasitizing *M. cribraria* eggs in soybean and kudzu in many areas of its expanded range (Gardner et al. 2013, J. Entomol. Sci. 48: 355–359; Medal et al. 2015, Florida

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Entomol. 98: 1250–1251), and epizootics *B. bassiana* have been reported in kudzu bug populations infesting kudzu and/or soybeans in Georgia (Gardner and Olson 2016, J. Entomol. Sci. 51: 325–328), South Carolina (Seiter et al. 2013b, J. Entomol. Sci. 49: 326–330), Tennessee (Britt et al. 2016, J. Entomol. Sci. 51: 321–324), and North Carolina (Lahiri and Reisig 2016, J. Integr. Pest. Manag. 7:14).

Previous studies have shown that *B. bassiana* isolates are present within soil regardless of the soil type (Harrison and Gardner 1991, J. Entomol. Sci. 26: 360–366; Shapiro-Ilan et al. 2002, Environ. Entomol. 32: 187–195). The observed epizootics in kudzu bug populations suggest that *B. bassiana* inoculum is present in those soils and epizootics build with favorable environmental conditions. In the study presented herein, our objective was to quantify *B. bassiana* from kudzu soils and molecularly identify strains of *B. bassiana* from various locales.

Soil from seven kudzu patches located in Union, Mecklenburg, Cabarrus, and Gaston counties in North Carolina was collected in 3×1–m^2 areas toward the periphery of the patches (Table 1). All kudzu patches were located next to a roadway, except for Patch 7 (Union Co., Highway 74 site), which was next to a medical office building. Collection dates were in September 2014, March 2015, and June 2015. Ten sampling cores (diameter: 6.985 cm) were taken at random within the areas using a soil corer. Cores were transferred into 4-L buckets for transport. To insure that cross-contamination did not occur, the corer was cleaned using a sodium hypochlorite solution after each sample. Once the buckets were transported to the lab, soil cores were pooled together in plastic containers (30.5×15.25 cm; 1,000 g/container) with cores from corresponding patches.

Soil cores were allowed to air-dry for 48 h to eliminate bacteria and nematodes as variables in the study. Sterile distilled water (SDW) was used to rehydrate the soil (5% v/v). To quantify fungal densities in soil, five 1-g samples of soil from each patch were individually added to 9 mL of SDW and vortexed for 60 s, serially diluted (10^{-1}, 10^{-2}, and 10^{-3}), and then pipetted (250 μL) onto selective media for *B. bassiana* and closely related fungal species (Doberski and Tribe 1980, Trans. Br. Mycol. Soc. 74: 95–100). Serial dilutions were conducted on 1, 3, and 7 d post-rehydration of soil. Plates were incubated for 7 to 10 d at 25°C after which fungal colony-forming units (CFUs) were enumerated. *Beauveria bassiana* was identified by colony color and morphology. Colonies were subsampled from the Doberski and Tribe (1980) media and inoculated on Sabouraud's dextrose agar plus 1% yeast extract (SDAY) at 24 ± 1°C throughout the experiment.

CFU counts were analyzed as a randomized block design and were subjected to analysis of variance using the general linear models procedure (PROC GLM, SAS 2009, SAS Institute, Cary, NC). Before analysis, the numerical CFU data were square-root transformed. All significant ($P = 0.05$) differences among means were separated using the Tukey Studentized range.

The fungi cultured on SDAY were harvested and placed in a desiccator. Twenty milligrams of dry fungal mass were added to a microcentrifuge tube containing glass beads (0.5 mm) (Sarstedt Inc., USA, Newton, NC) with 650 μL SDW with 0.01% (v/v) Tween 80™ (Sigma-Aldrich, St. Louis, MO). The dry fungal cells were mechanically disrupted using a bead beater (Genie Disruptor, Scientific Industries, Bohemia, NY). DNA extraction was conducted using E.Z.N.A.® HP Fungal DNA kit (Omega Bio-Tek, Norcorss, GA) per manufacturer's directions.

Table 1. Kudzu soil collection sites in Mecklenburg, Union, Cabarrus, and Iredell counties, North Carolina.

Patch	Location	GPS Coordinates
1	Mecklenburg County	N 35° 11′ 12.084″, W 80° 55′ 58.008″
2	Gaston County	N 35° 16′ 26.292″, W 81° 1′ 34.248″
3	Gaston County	N 35° 13′ 33.996″, W 81° 8′ 10.248″
4	Mecklenburg County	N 35° 29′ 10.86″, W 80° 51′ 32.292″
5	Cabarrus County	N 35° 11′ 12.192″, W 80° 55′ 57.864″
6	Cabarrus County	N 35° 24′ 22.896″, W 80° 36′ 39.06″
7	Union County	N 34° 58′ 23.268″, W 80° 30′ 44.424″

In order to screen for virulent *B. bassiana* strains, amplification of the chitinase gene, Bbchit-1, was conducted using previous described amplification conditions (Fang et al. 2005, Appl. Environ. Microbiol. 71: 363–370). To identify strain-specific isolates from soil samples, polymerase chain reaction (PCR) was conducted using internal transcribed spacer (ITS; ITS1: 5′–TCC GTA GTG AAC CTG CGG–3′; ITS4: 5′–TCCTCCGCTTATTGATATGC–3′) primer sets, and using previously described amplification conditions (Rehner and Buckley 2005, Mycologia 97: 84–98). Electrophoresis using a 1% agarose gel was conducted with both gradient and conventional PCR products; different DNA ladders were used. Gel products were excised using an E.N.Z.A® Gel Extraction Kit. Products (Omega Biok-tek, Norcross, GA, USA) were then subjected to PCR cleanup using DNA Clean & Concentrator™ (Zymoresearch, Irvine, CA). Products were sequenced from both directions using SeqRegular (Sanger Sequencing, Eton Bioscience, Inc., Raleigh, NC) using primer sets ITS1 and ITS4. Sequence data were aligned using Clustal Omega (EMBL-EBI, Hinxton, Cambridge, UK), and a BLAST search was conducted of the National Center for Biotechnology Information (NCBI) nucleotide database.

Kudzu soil sampled from different seasons of the year during which kudzu bugs are active (e.g., autumn, spring, and summer) showed no significant differences in mean CFUs recovered from soil ($P = 0.32$). Also, there was not a significant interaction between the season and patch that was sampled ($P = 0.77$). However, a higher mean number of CFUs were observed in fall and spring sampling months. This is likely due to high relative humidity levels maintained within the kudzu canopy during those seasons. Additionally, direct or indirect ultraviolet radiation could reduce CFUs during the summer months. Mean CFUs were consistent across kudzu patch samples, except for the Union County patch, which suggests more inoculum in the soil in that location (Table 2). However, there were no significant differences among mean CFU quantities in kudzu soil ($P = 0.14$), with the exception of the Highway 74-East site. Here, the kudzu patch was within close proximity to sprinklers that were active through most of the spring, summer, and fall months, which could be the reason for high inoculum present in the soil compared to other kudzu patches. Overall, diversity of *B. bassiana* strains was observed in all samples from kudzu patches, indicating that these strains are present in the soil. Lahiri et al.

Table 2. Mean ± SD colony-forming units (CFUs) isolated from soil sampled from kudzu patches by season sampled.

Patch	CFUs per Gram of Soil*			Recovered *Beauveria bassiana* Strains
	Spring	Summer	Fall	
1	1.5 ± 0.49a	1.8 ± 2.11a	4.2 ± 2.87a	Voucher WA51510
2	1.6 ± 0.94a	1.3 ± 0.63a	1.6 ± 0.91a	ABNB6, Kudzu
3	2.8 ± 0.74a	1.3 ± 0.25a	3.0 ± 2.23a	ABNB6, 33A, Bb3
4	2.0 ± 1.01a	1.4 ± 0.22a	10.9 ± 2.22a	Kudzu, Isolate 2 voucher BB48, 5A
5	1.3 ± 0.50a	1.4 ± 0.41a	0.00 ± 0.00a	Sb4
6	5.4 ± 2.07a	1.5 ± 0.45a	3.5 ± 2.50a	ABNB6, 33A, NZD-mf92
7	48.5 ± 4.57a	1.8 ± 0.15a	12.1 ± 3.87a	ABNB6, 33A, Bb12

* Means within columns followed by the same letter are not significantly different (Tukey's Studentized range, $P = 0.05$).

(2015, J. Entomol. Sci. 50: 69–73) reported *M. cribraria* overwintering in leaf litter samples, thus suggesting that the insect comes in contact with the soil at some point during fall, winter, and spring months. This behavior would suggest that fungal–insect contact could occur and could explain initiation of epizootics observed in kudzu and soybeans.

Forty-two isolates were molecularly identified using the ITS region and blasted against the NCBI database (Table 3). Of the 42 isolates, the most commonly

Table 3. Strains of *Beauveria bassiana* isolated from kudzu soil samples in Charlotte, NC, regional survey.

Strains	GenBank Accession Number
ABNB6	KX196303.1
33A	KU517852.1
Kudzu	KX228573.1
Bb12	KP862979.1
5A	KX255641.1
Bb3	KP862992.1
Isolate 2 voucher BB48	KT932308.1
Voucher WA51510	KU163449.1
NZD-mf92	KM278053.1
Sb4	KP862989.1

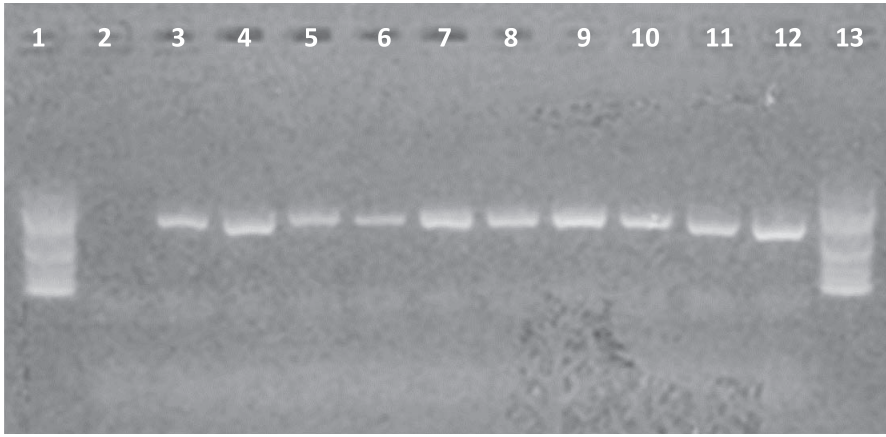

Fig. 1. Gel image. Lanes 1 and 13 contained the molecular ruler. Lane 2 contained a negative control with water. Lane 3 contained a positive control, *Beauveria bassiana* GHA strain. Lanes 4–12 contained strains 20–29, which were isolated from kudzu soil samples. These 600-Kb bands were excised, cleaned, and sequenced.

identified from colonies isolated from soil were 33A, Kudzu, and ABNB6 (Table 2). Amplification of the Bbchit gene occurred in all 42 strains; therefore, all possess this unique virulence gene (Fig. 1).

In 2016, cadavers were collected on tree bark from surrounding trees of two of the seven kudzu patches. Areas around five of the remaining kudzu patches were inspected for cadavers; however, none were seen. Fungi on the cadaver were harvested using a sterile scalpel and inoculated on SDAY at 25°C to maintain cultures, while the remainder of the fungi were added to a microcentrifuge tube containing glass beads, mechanically disrupted as previously described, and molecularly identified as previously listed.

An NCBI blast resulted in 100% identity to *B. bassiana* strain ABNB6. As of October 2016, a *B. bassiana* epizootic was observed at the Union County Highway 74-East site with both adults and nymphs showing visible mycosis (Fig. 2). Therefore, the identification of ABNB6 strain in the soil and on cadavers suggests that the insect likely was infected from an interaction at the soil level. However, this is a low sample size, mainly due to limited numbers of kudzu bugs with infections of *B. bassiana* during this study. While *B. bassiana* Kudzu strain was identified in soil and by researchers on kudzu bugs in South Carolina (Portilla et al. 2016, Insects 31: doi:10.3390/insects7030031), this suggests that there may be more than one strain infecting kudzu bugs in the southeastern United States. Further investigations should be performed to determine if there are geographical constraints of the strains, or if one appears to be more virulent that the other.

Fig. 2. Nymph and adult kudzu bugs infected with *Beauveria bassiana* at the Union County Highway 74-East site in October 2016. There had been no recorded infected adults or nymphs in the previous 2 y at this site; however, there were high inoculum amounts of *B. bassiana* present in the soil. *Beauveria bassiana* strain ABNB6 was molecularly identified as the strain that infected cadavers, as well as a strain identified in soil samples from this site.