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Novel multiplex PCR reveals multiple trypanosomatid species infecting North American bumble bees (Hymenoptera: Apidae: *Bombus*)

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A B S T R A C T

Crithidia bombi and *Crithidia expoeki* (Trypanosomatidae) are common parasites of bumble bees (*Bombus* spp.). *Crithidia bombi* was described in the 1980s, and *C. expoeki* was recently discovered using molecular tools. Both species have cosmopolitan distributions among their bumble bee hosts, but there have been few bumble bee studies that have identified infections to species since the original description of *C. expoeki* in 2010. Morphological identification of species is difficult due to variability within each stage of their complex lifecycles, although they can be easily differentiated through DNA sequencing. However, DNA sequencing can be expensive, particularly with many samples to diagnose. In order to reliably and inexpensively distinguish *Crithidia* species for a large-scale survey, we developed a multiplex PCR protocol using species-specific primers with a universal trypanosomatid primer set to detect unexpected relatives. We applied this method to 356 trypanosomatid-positive bumble bees from North America as a first-look at the distribution and host range of each parasite in the region. *Crithidia bombi* was more common (90.2%) than *C. expoeki* (21.3%), with most *C. expoeki*-positive samples existing as co-infections with *C. bombi* (13.8%). This two-step detection method also revealed that 2.2% samples were positive for trypanosmatids that were neither *C. bombi* nor *C. expoeki.* Sequencing revealed that two individuals were positive for *C. mellificae*, one for *Lotmaria passim*, and three for two unclassified trypanosomatids. This two-step method is effective in diagnosing known bumble bee infecting *Crithidia* species, and allowing for the discovery of unknown potential symbionts.

1. Introduction

Two species in the genus *Crithidia* (Kinetoplastea: Trypanosomatidae), *C. bombi* and *C. expoeki*, are common parasites of bumble bees (Hymenoptera: Apidae: *Bombus* spp.). *Crithidia bombi* has been known since the 1980s (Lipa and Triggiani, 1980), whereas *C. expoeki* was only recently discovered through molecular means (Schmid-Hempel and Tognazzo, 2010). Both species have a cosmopolitan distribution, and yet surveys of *Crithidia* in bumble bees that employ molecular approaches lump the two species together, which is an impediment to understanding more about the distributions and pathologies of these two organisms (Cordes et al., 2012; Tripodi et al., 2014). Studies that have identified the *Crithidia* species infecting bumble bees, have employed sequencing (Gallot-Lavallée et al., 2016; Gamboa et al., 2015) which is expensive and unrealistic for large scale surveys.

Distinguishing the two species morphologically is difficult due to their small size and the variable appearance of each stage of their complex lifecycles, although they can be easily differentiated through DNA sequencing (Schmid-Hempel and Tognazzo, 2010). However, DNA sequencing can be expensive, particularly for large surveys or diagnostic efforts with hundreds or thousands of individuals to assess. A polymorphism in the ITS1 region has been used to distinguish the two species, as ITS-1 is 35–40 bp shorter in *C. expoeki* than in *C. bombi* (Popp and Lattorff, 2011; Schmid-Hempel and Tognazzo, 2010). However, differences < 50 bp are difficult to accurately distinguish on commonly used agarose gels. The ITS1 region within *Lotmaria passim* ranges from 347 to 365 bp (see Genbank accessions KP133024 and KP132994), overlapping that of *C. bombi*, which ranges from 364 to 373 bp (see Genbank accessions GU321144 and GU321127) and rendering these indistinguishable without sequencing. Additionally, ITS1 exhibits size polymorphisms within both *C. mellificae* and *L. passim,* and it is likely that increased sampling of trypanosomatid species would reveal fragment sizes that would result in diagnoses that confound uncharacterized species with expected ones (Stevanovic et al., 2016). In order to reliably and inexpensively diagnose *C. bombi* and *C. expoeki* infections for a large-scale bumble bee survey, we developed a multiplex PCR protocol using species-specific primers coupled with a novel trypanosomatid-universal primer set that can additionally detect

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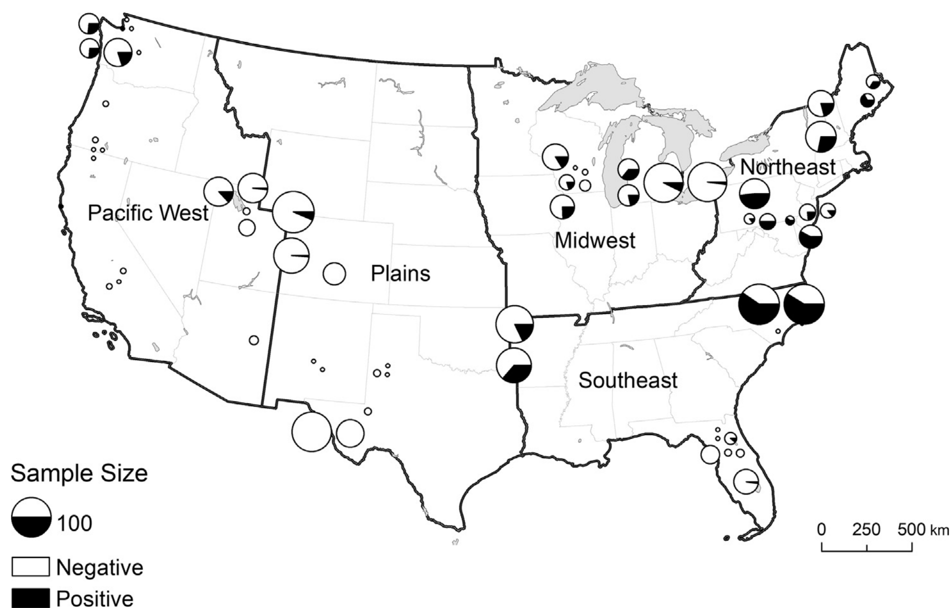

Fig. 1. Map of the United States showing the locations (N = 65) across five regions from which bumble bees were collected and the results of the initial trypanosomatid screening. The size of each pie chart is scaled to the sample size of bees screened at each location; within the charts, white indicates negative, black indicates positive.

unexpected relatives. We then applied this two-step method to a sample of 356 trypanosomatid-positive bumble bees from North America as a first-look at the distribution and host range of each parasite in this area.

2. Materials and methods

2.1. Sampling and DNA extraction

As part of a larger survey, 1728 bumble bee samples were collected from 65 sites across the continental United States, including samples from 18 states across five regions (Midwest, Northeast, Pacific West, Plains, and Southeast, Fig. 1). Bumble bees were collected from flowers using aerial nets, held and transported in liquid nitrogen or on dry ice, and then frozen at −80 °C until DNA extractions were conducted. DNA was extracted from the abdominal viscera of individual bees using TriSure Reagent (ThermoFisher Scientific, Waltham, MA) and a guanidine-thiocyanate back extraction buffer per manufacturer's instructions. DNA extracts were stored at −20 °C until use in PCR.

2.2. Primer design

Extracts were initially screened for the presence of *Crithidia* and other trypanosomatids using the universal trypanosomatid primers CB-SSUrRNA-F2 and CB18SR2 (Table 1). The primer CB18SR2 was designed with the Primer 3 plug-in (Untergasser et al., 2012) within Geneious v.6.1.8 (Biomatters, Auckland, NZ) to amplify an approximately 580 bp region of 18S small subunit rRNA from a broad range of trypanosomatids in the genera *Blastocrithidia, Blechomonas, Crithidia, Lotmaria, Herpetomonas, Lafontella, Leptomonas, Novymonas, Paratrypanosoma, Sergeia, Wallaceina,* and *Zelonia* using sequences available on Genbank. Cytochrome-b (Cyt-b) sequences of *C. bombi, C. expoeki, C. mellificae, L. passim,* and other relatives were obtained from Genbank, and fragment-size differentiated, species-specific primers were developed for *C. bombi* (CB279F/R, 279 bp product) and *C. expoeki* (CE163F/R, 163 bp product) using Primer 3 (Table 1, Fig. A1). An additional primer set (Crith18SF/R, 470 bp product) was developed using a highly conserved region of 18S that could serve as a positive control for the presence of either *Crithidia* species in the multiplex reaction (Table 1). This region is conserved among many trypanosomatid species, thus it can also serve as a means by which new parasite-host associations could be discovered. In addition to the *in silico* analysis of sequence data, the generality of this primer set and the specificity of the species-specific primers were verified by testing the multiplex on samples of honey bees infected with *L. passim*.

2.3. PCR diagnosis and sequencing

Reactions consisted of 1 μL of DNA extract, 0.8 μM of each primer, 1.3X buffer, 2 mM MgCl2, 0.8 mM total dNTPs and 1 unit of *Taq* polymerase, with molecular-grade water to bring the solution to a total volume of 25 μL. Each reaction series also included positive controls for each *Crithidia* of interest and a negative control consisting of molecular-grade water. Thermal-cycling conditions for the initial screening were an initial denaturation of 94 °C for 2 min, followed by 10 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, then 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 45 s and a final elongation step of 72 °C for 5 min. For the multiplex reaction, conditions were an initial denaturation of 94 °C for 3 min, followed by 40 cycles of 94 °C for 45 s, 57 °C for 45 s, 72 °C for 60 s, and a final elongation step of 72 °C for 7 min. Amplification products were separated on 2% agarose gels, stained with 2.5X GelRed solution (Biotum, Fremont, CA) and visualized under UV light (Bio-Doc-It, UVP, Upland, CA). For the initial screening, samples exhibiting a band at ~580 bp were determined to be trypanosomatid-positive and were subjected to multiplex reaction. In the multiplex reaction, samples exhibiting a band at ~470 bp were diagnosed as trypanosomatid-positive, those with a band at 279 bp were diagnosed as *C. bombi*-positive, and those with a band at 163 bp were diagnosed as *C. expoeki*-positive (Fig. 2). Ten samples screened as negative were also included in the multiplex reaction test to verify its specificity.

Samples positive for trypanosomatids, but negative for either of the species-specific primer sets in the multiplex were sequenced for a ~680 bp region of 18S or a ~425 bp region of Cyt-b (Table 1). Representative samples of trypanosomatid, *C. bombi,* and *C. expoeki* PCR-positives (N = 25) were also sequenced using the primers developed here. All sequenced samples were enzymatically purified with ExoSap-It (Affymetrix, Sunnyvale, CA) and sent for sequencing in both directions to Eton Bioscience (San Diego, CA). All sequences over 200 bp were deposited in GenBank.

2.4. Phylogenetic analyses

Bayesian analysis was used to examine potential relationships

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