Molecular Diagnostics of the Honey Bee Parasites Lotmaria passim and Crithidia spp. (Trypanosomatidae) Using Multiplex PCR

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Molecular diagnostics of the honey bee parasites *Lotmaria passim* and *Crithidia* spp. (Trypanosomatidae) using multiplex PCR

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Trypanosomatidae are obligate parasites of mainly invertebrate hosts, although some species such as *Leishmania* species and *Trypanosoma* species can also cause human diseases. A species that occurs in honey bees, *Crithidia mellificae* Langridge and McGhee, primarily occurs in the rectum of its host, but little is known about the effect on infected individuals (Schwarz et al. 2015). There was little research on *C. mellificae* since its first description by Langridge & McGhee (1967), until Runckel et al. (2011) revealed the presence of *C. mellificae* in commercial beekeeping operations transporting bees among Mississippi, California, and South Dakota. The lack of research on *C. mellificae* is surprising because the related *C. bombi* Gorbunov is known to have serious effects on bumble bee health. For example, *Bombus* (Hymenoptera: Apidae) colonies that were founded by infected queens were shown to be 40% less fit, displaying smaller colony sizes and producing fewer reproductive males (Brown et al. 2003). Bumble bee workers infected with *C. bombi* show increased handling time while foraging for nectar, visiting fewer flowers than their uninfected counterparts in timed studies (Otterstatter et al. 2005).

Ravoet et al. (2013) found that, in addition to *Varroa destructor* Anderson and Trueman, the presence of *C. mellificae* and the microsporidian *Nosema ceranae* (Fries et al.) are predictive markers of winter mortality for honey bees in Belgium, but little else has been documented on *C. mellificae*'s effects on honey bees. Efforts to characterize the effects of this pathogen have also been complicated by taxonomic confusion among the difficult-to-identify trypanosomatids. In 2015, a new species of trypanosome parasite of honey bees was identified, *Lotmaria passim* Schwarz, from honey bee colonies in Maryland, USA (Schwarz et al. 2015). Since its description, *L. passim* has also been detected in Belgium, Japan, and Switzerland (Ravoet et al. 2015). However, much of the past literature on trypanosomatid infections has assumed that *C. mellificae* was the only trypanosomatid species afflicting honey bees (Schwarz et al. 2015).

Schmid-Hempel & Tognazzo (2010) developed a small subunit (SSU) primer set that will amplify, via polymerase chain reaction (PCR), DNA from *Crithidia* species, and that will work for *L. passim* as well other trypanosomes. Recently, Ravoet et al. (2015) developed a molecular diagnostic technique to distinguish *L. passim* from *C. mellificae* using amplicon size polymorphisms in the ribosomal DNA first internal transcribed spacer region (ITS1). Although this technique allowed for species-level identification of some infections, less than 30% of their samples were successfully amplified (Ravoet et al. 2015), inspiring the development of a more universally robust method. The purpose of this study was to develop a molecular diagnostic technique to distinguish *L. passim* from *C. mellificae* using a PCR primer set specific for *L. passim* and *Crithidia* species in a multiplex PCR with the highly conserved nuclear SSU gene.

Honey bees were collected from 2013 to 2014 from the Hawaiian Islands of Hawaii (Big Island), Lanai, Kauai, Molokai, Oahu, and Maui, as well as from American Samoa. Samples were preserved in 70 to 100% ethanol. DNA was extracted from individual honey bees by using a salting-out protocol with in-house reagents (Sambrook & Russell 2001). Samples of *C. bombi* and *C. expoeki* Schmid-Hempel and Tognazzo were obtained from infected bumble bees, with trypanosomatid identity verified through sequencing (GenBank accession numbers KU937107 and KU937108, respectively). Positive controls for *C. mellificae* and *L. passim* were obtained from type strains (30254 and PRA-422, respectively) deposited at the American Type Culture Collection (ATCC, Manassas, Virginia), extracted with the same methods as for the honey bee samples.

Amplification of trypanosome DNA was done using the SSU PCR primers CB-SSUrRNA-F2 and B4 (Schmid-Hempel & Tognazzo 2010), which yield an approximately 716 to 724 bp product. A volume of 2 mL of extracted DNA was used for PCR, and the remainder of the reaction mixture followed Taylor et al. (1997). The PCR temperature profile followed Schmid-Hempel & Tognazzo (2010). Amplicon verification was conducted by gel electrophoresis using 2% agarose gels, and PCR products were visualized using a BioDoc-it™ Imaging System (UVP, Inc., Upland, California). Positive samples were purified and concentrated with VWR centrifugal devices (VWR, Radnor, Pennsylvania) and sent to Eurofins Genomics (Huntsville, Alabama) for direct sequencing in both directions.

Sequences were aligned visually (GenBank accession numbers KU499926, KU499927), and a BLAST search (National Center for Biotechnology Information) was conducted with Geneious 6.0.3 (Auckland, New Zealand). Additional sequences were downloaded from GenBank for molecular phylogenetic analysis to confirm that the positive samples were *L. passim* (Fig. 1). Bayesian phylogenetic analysis was conducted with the MrBayes (Ronquist & Huelsenbeck 2003) plug-in within Geneious with 100,000 burn-in and 1,000,000 replications using a GTR+G model based on Akaike's Information Criterion results from jModelTest v2.1.3 (Darriba et al. 2012). *Leishmania donovani* (Laveran and Mesnil) (GenBank GQ332356) was used as the outgroup taxon.

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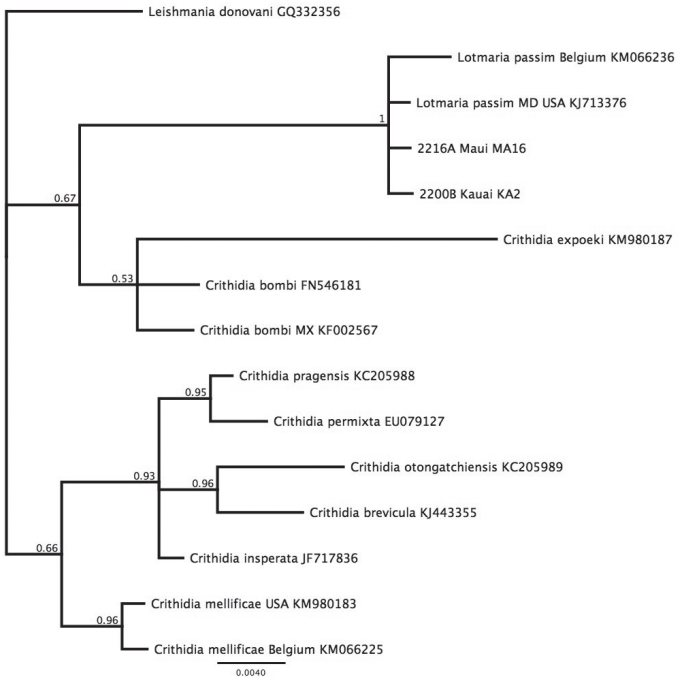

Fig. 1. Bayesian molecular phylogenetic tree showing relationship of 2 Hawaiian *Lotmaria passim* positive samples relative to other trypanosomes from GenBank for a 608 bp region of the rDNA SSU gene.

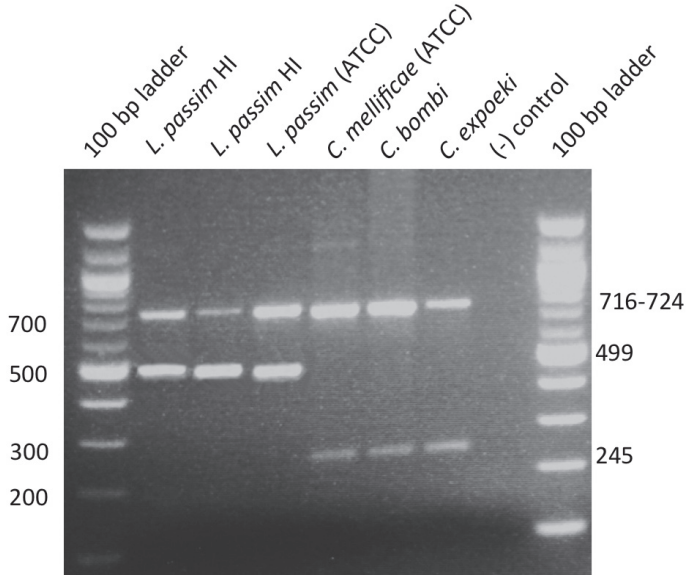

Fig. 2. Multiplex PCR gel showing the 716 to 724 bp amplicon for *Lotmaria passim* and *Crithidia* species, the *L. passim* specific 499 bp amplicon, and the *Crithidia* specific 245 bp amplicon.

Two samples of Hawaiian honey bees, one from Maui and the other from Kauai were *L. passim* based on the molecular phylogenetic analysis. These samples formed a common clade with *L. passim* sequences from Belgium (GenBank KM066236) and Maryland, USA (KJ713376).

Using DNA sequences of *L. passim* from Hawaiian honey bees, along with sequences of *Lotmaria* and *Crithidia* species from GenBank (Fig. 1), PCR primers were designed to only amplify *L. passim* or *Crithidia* species by aligning the DNA sequences using Geneious software. A region of the alignment was found that had nucleotide polymorphisms between *L. passim* and *Crithidia* species. From this region, we selected the PCR primer L.passim18S-F, (5'-AGGGATATTTAAACCCATCGAAAATCT-3'), which has 4 conserved nucleotide differences between *L. passim* sequences and *Crithidia* species sequences, with 3 nucleotide differences occurring at the 3' end of the primer. The primer yields a 499 bp product in conjunction with primer CBSSU rRNA B4. A second PCR primer was designed to only amplify *Crithidia* species. This primer, designated as C. mel 474-F (5-TTTACGCATGTCATGCATGCCA-3"), has 4 nucleotide differences relative to *L. passim*, with 2 of them due to a 'TG' insertion in *L. passim* that does not occur in *Crithidia*. This PCR primer combined with CBSSU rRNA B4 results in a 245 bp amplicon. Both the L.passim18S-F + CBSSU rRNA B4, and C. mel 474-F + CBSSU rRNA B4 amplicons were confirmed by DNA sequencing of 3 positive samples for *L. passim* and 1 for *C. mellificae*. Multiplex PCR was done as outlined above with a PCR temperature profile consisting of holding the samples for 2 min at 94 °C, then 40 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension of 72 °C for 5 min.

The multiplex PCR with primers CBSSU rRNA P2, CBSSU rRNA B4, L.passim18S-F, and C. mel 474-F yielded a 716 to 724 bp product for *L. passim* and *Crithidia* species, a 499 bp product for only *L. passim*, and a 245 bp product for *Crithidia* species (Fig. 2). The multiplex PCR diagnostic for *L. passim* was evaluated on 96 worker honey bees collected from 6 Hawaiian Islands, and 6 honey bees collected from American Samoa. In total, 14 samples from Hawaii (Big Island), Kauai, Lanai, Maui, and Molokai, and 1 sample from American Samoa were positive for *L. passim,* and none were positive for *Crithida* species based on the molecular diagnostic technique.

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Summary

Lotmaria passim Schwarz (Trypanosomatidae) is a recently described trypanosome parasite of honey bees in the continental United States, Europe, and Japan. We developed a multiplex PCR technique using PCR primers specific for *L. passim* or *Crithidia* species to distinguish *L. passim* from *C. mellificae* Langridge and McGhee. We report the presence of *L. passim* in Hawaii and American Samoa for the first time. More importantly, this multiplex PCR will be a useful diagnostic technique for screening honey bee samples for the presence of this pathogenic trypanosome.

Key Words: *Apis mellifera*; molecular diagnostics; parasite; trypanosome

Sumario

Lotmaria passim Schwarz (Tripanosomatidae) es un parásito tripanosoma recientemente descrito de las abejas melíferas en el territorio continental de los Estados Unidos, Europa y Japón. Hemos desarrollado una técnica de PCR múltiple usando cebadores de PCR específicos para *L. passim* o *Crithidia* spp. para distinguir *L. passim* de *C. mellificae*. Reportamos la presencia de *L. passim* en Hawai y Samoa Americana por primera vez. Más importante aún, este PCR multiplex será una técnica de diagnóstico útil para la revisión de muestras de abejas de la miel y la presencia de este tripanosoma patógeno.

Palabras Clave: *Apis mellifera*; diagnóstico molecular; parásito; tripanosoma

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