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Short Communication

PCR-specific detection of recently described *Lotmaria passim* (Trypanosomatidae) in Chilean apiaries

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ABSTRACT

The recently described trypanosome *Lotmaria passim* is currently considered the most predominant trypanosomatid in honey bees worldwide and could be a factor in honey bee declines. For a specific and quick detection of this pathogen, we developed primers based on the SSU rRNA and gGAPDH genes for the detection of *L. passim* in Chilean honey beehives. PCR products amplified and sequenced for these primers shared 99–100% identity with other sequences of *L. passim*. The designed primers were specific and we were able to detect a high prevalence (40–90%) of *L. passim* in bee hives distributed throughout Chile. Our described PCR-based method offers a feasible and specific detection of *L. passim* in any honey bee samples.

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1. Introduction

Trypanosomatids are unicellular flagellates and obligate parasites that infect different groups of hosts including mammals, plants and insects (Camargo, 1999; Barret et al., 2003; Maslov et al., 2013). Insect's trypanosomes are found in the different taxa parasitizing mainly in the mid- and hind-guts of their hosts (Maslov et al., 2013). Trypanosomes found in hymenopteran species, especially in honey and bumble bees have become more relevant in recent years since they have shown to have negative effects on behavior, physiology, fitness and the immune system of these pollinators (Brown et al., 2003; Gegear et al., 2005; Runckel et al., 2011; Ravoet et al., 2013; Schwarz and Evans, 2013). Furthermore, recent works have recognized the trypanosome *Crithidia mellificae* Langridge and McGhee 1967 as a possible cause of honey bee declines in field surveys in the United States and Europe (Cornman et al., 2012; Ravoet et al., 2013). However, a current report has described *Lotmaria passim* (Schwarz et al., 2014) as a new species infecting honey bees (Schwarz et al., 2015a). Many isolates initially identified as *C. mellificae* were reclassified as *L. passim*, thus making this species the most predominant trypanosomatid in honey bees worldwide, in contrast to *C. mellificae* which is currently considered infrequent (Schwarz et al., 2015a). This suggests that *L. passim* is more important than we thought. Recently, Ravoet et al. (2015) were able to differentiate between *C. mellificae* and *L. passim* by fragment length polymorphism in the internal transcribed spacer 1 (ITS1) with primers previously used in trypanosome species on mammals (Maia Da Silva et al., 2004). However, there are no specific primers to detect *L. passim* in honey bee samples without culture or cloned material, which both imply an expensive, time-consuming process. Nowadays, many DNA sequences of *L. passim* from different countries have been deposited in the Genbank. These sequences are represented by different loci including small subunit ribosomal RNA (SSU rRNA) and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH). Sequenced sections of these genes showed high similarity among trypanosome species, but interspecific sections which allowed one species to be discriminated from another were also observed (Schwarz et al., 2015a). Since molecular methods are required to accurately diagnose some of the more common pathogen species associated with colony collapse disorder (CCD), including trypanosome species (Schwarz et al., 2015b); and furthermore, because it is essential to have an accurate, robust, repeatable and sensitive diagnostic method for specific detection and identification of *L. passim*, we designed specific primers based on SSU rRNA and gGAPDH as reference regions for the specific detection of *L. passim* in honey bees. We then used this PCR-based method to determine the prevalence of *L. passim* in Chilean bee hives since that the beekeeping is an important activity in the country, not only because it provides pollination services for fruit and seed

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production, but also, because honey bee product exports, mainly honey and pollen.

2. Material and methods

2.1. Field sampling and DNA extraction

Since that the mostly of apiaries (75%) are concentrated in central and south-central Chile (Velis et al., 2009), a group of 50 worker bees were collected from spring 2014 to fall 2015 from 189 beehives located in the Metropolitan (RM, 33°26′S–70°39′W), O'Higgins (VI, 34°10′S–70°43′W), Maule (VII, 35°25′S–71°39′W), Biobío (VIII, 36°46′S–73°03′W), and Araucanía (IX, 38°44′S–72°35′W) regions. Ten honey bees were crushed and homogenized in a stomacher bag with 10 mL of phosphate-buffered saline (PBS 1X) for 90 s at high speed in a Stomacher 80 Lab Blender (Seward, London, UK). The samples were centrifuged at 10,000*g* for 15 min. The supernatant (8 mL) was discarded and 200 μL of the precipitated extract was used for DNA extraction. The extract was ground in 1.5 mL microcentrifuge tubes using sterile plastic pestles with 350 μL of CTL buffer following the E.Z.N.A Insect DNA Kit's instructions (OMEGA, Bio-Tek Inc., Atlanta, GA, USA) for genomic DNA extractions. The DNA was quantified (Infinite 200 PRO NanoQuant, Tecan Group Ltd., Männedorf, Switzerland) and stored at −80 °C.

2.2. Primer design

Specific primers to detect *L. passim* were designed based on the small subunit ribosomal RNA (SSU rRNA) and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) reference sequences recently reported in the GenBank (accessions KM066228, KJ713376, KJ713378 and KM066224, KJ713349 KJ713356 for SSU rRNA and gGAPDH genes, respectively) (Schwarz et al., 2015a). Furthermore, other sequences of trypanosome species detected on bees (*C. mellificae* and *Crithidia bombi*) were also aligned using the ClustalW2 tool to determine heterogeneous regions in SSU rRNA and gGAPDH genes from which putative primers were designed for PCR. The reference sequences of *L. passim* were checked by AmplifX 1.5.4 software (http://crn2m.univ-mrs.fr/pub/amplifx-dist) and the best primer pair was designed considering the size of the DNA fragment, the stability of the PCR reaction, and a similar melting temperature (Tm) between primer pairs. We also looked for a pair in which no primer dimer occurred in the PCR reaction. To determinate the primer's specificity alignment, a group of putative primers were checked online using Primer-Blast at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) using specific annealing parameters in Trypanosomatidae and Apidae families in the nucleotide reference (nr) database. Thus, the primer pairs Lp2F (5′-AGGGATATTTAAACCCATCGAA-3′) and Lp2R (5′-ACCACAAGAGTACGGAATGC-3′), as well as, the primers Lp-gF (5′-TTGCGAAGAGCTCGCCTGAGGT-3′) and Lp-gR (5′-TCGCCGTGTAGGAGTGAATGGTC-3′) were selected and fragments of 459 and 402 bp were amplified *in silico* conditions using AmplifX software within the SSU rRNA and gGAPDH genes, respectively.

2.3. Primers specificity in PCR conditions

To test the specificity of the primers Lp2F/R and Lp-gF/R, PCR reactions were conducted for each primer pair using DNA extracted from *Apis mellifera* and bumble bees (*Bombus terrestris* L.) that had previously proven to be positive to *C. mellificae* (Belgium isolate, accession KU096059) and *C. bombi* Lipa and Triggiani 1988 (Chilean isolate, accession KU096060), respectively. We also tested the primers specificity in a possibly mixed infection in different bee hosts. In this case, we mixed 25 ng/μL DNA of *L. passim*-infected honey bees with DNA of *C. mellificae*-infected honey or *C. bombi*-infected bumble bees. Other previously published primers used to detect *C. mellificae* (TrypanF1: 5′-GTTGACGGAATCAACCAAACAAAT-3′, TrypanR: 5′-GCGTCAGAGGTGAAATTCTTAGACC-3′) (Cox-Foster et al., 2007) and *C. bombi* (CB-SSUrRsNA-F2: 5′-CTTTTGACGAACAACTGCCCTATC-3′, CB-SSUrRsNA-B4: 5′-AACCGAACGCACTAAACCCC-3′) (Schmid-Hempel and Tognazzo, 2010) were also tested for their specificity in *L. passim*-infected honey bee samples.

2.4. PCR, nucleotide sequencing and phylogenetic analyses

PCR reactions with primer pairs Lp2F/R, Lp-gF/R, TrypanF1/R1 and CB-SSUrRsNA-F2/B4 were performed in a volume of 25 μL per sample containing 1 × PCR buffer, 2 mM $MgCl_2$, 250 μM dNTP, 0.4 μM of each primer, 1 U *taq*DNA polymerase (Invotrogen™, Life Technologies, Carlsbad, CA, USA), and 25 ng/μl of DNA. The thermal profile of PCR's 35 cycles (MultiGene™ Gradient Thermal Cycler, Labnet International Inc., Woodbridge, NJ, USA) included a denaturalization at 94 °C for 30 s (3 min for the first cycle), alignment at 60 °C for 20 s, extension at 72 °C for 45 s, and final extension at 72 °C for 7 min. PCR products were run in 1% (wt/vol) agarose gels with 0.04 μl/ml of GelRed™ nucleid acid stain (Biotium Inc., Hayward, CA, USA) in a TBE buffer at 80 V for 45 min. The amplicons were observed and photographed in a UV light transilluminator.

To verify that the designed primers were specific to *L. passim*, and at the same time, to estimate their relationship with other trypanosome species, the amplified DNA fragments were purified and directly sequenced in both directions (Macrogen, Seoul, South Korea). The sequences similarities were determined by the Basic Local Alignment Search Tool (BLAST, NCBI) and compared online for sequence homologies (Clustal W2) with other sequences in the European Bioinformatics Institute database (EBI, Cambridge, UK). To determine the phylogenetic relationships between Apidadae trypanosomatid isolates, a multiple and concatenated alignment of SSU rDNA and gGAPDH sequences (amplified by Lp2F/R and Lp-gF/R) was performed using ClustalX 2.0 (Larkin et al., 2007). At the same time, a phylogenetic tree was constructed with Seaview 4.0 (Gouy et al., 2010) using the neighbor-joining method with 1000 bootstraps for *L. passim* reported in the United States (accessions KJ713376, KJ713378, KJ713349 and KJ713356), Belgium (accessions KM066228 and KM066224) and Chile. Other trypanosomatids related to bees, such as *C. bombi* (accessions GU321194 and GU321192), *Crithidia expoeki* Schmid-Hempel and Tognazzo, 2010 (accessions GU321195 and GU321193) and *C. mellificae* (accessions KJ713366 and KJ713342) in the United States and Belgium (accessions KM066226 and KM066211) were also included. *Crithidia permixta* Maslov and Lukes 2009 (accessions EU079127 and EU076607) was used as the root of the tree.

A Chi-square test was run to compare the *L. passim* infections detected in Chilean honey bee colonies in different regions.

3. Results and discussion

Primer pairs Lp2F/R and Lp-gF/R were able to detect and amplify *L. passim* directly from honey bee samples after DNA extraction (Fig. 1A). We also showed that these primers could not amplify other trypanosomes, such as *C. mellificae* and *C. bombi* (Fig. 1A). On the contrary, we found that previously published primers which are currently used to detect *C. mellificae* and *C. bombi* also amplified *L. passim* present in honey bee tissues (Fig. 1A). In fact, sequences of PCR products amplified from honey bee samples by primers TrypanF1/R1 (*C. mellificae*) (accessions KT252554 and

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