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Species-specific diagnostics of *Apis mellifera* trypanosomatids: A nine-year survey (2007–2015) for trypanosomatids and microsporidians in Serbian honey bees

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ABSTRACT

In this study, honey bees collected in Serbia over 9 consecutive years (2007–2015) were retrospectively surveyed to determine the prevalence of eukaryotic gut parasites by molecular screening of archival DNA samples. We developed species-specific primers for PCR to detect the two known honey bee trypanosomatid species, *Crithidia mellificae* and the recently described *Lotmaria passim*. These primers were validated for target specificity under single and mixed-species conditions as well as against the bumblebee trypanosomatid *Crithidia bombi*. Infections by *Nosema apis* and *Nosema ceranae* (Microsporidia) were also determined using PCR. Samples from 162 colonies (18 from each year) originating from 57 different localities were surveyed. *L. passim* was detected in every year with an overall frequency of 62.3% and annual frequencies ranging from 38.9% to 83.3%. This provides the earliest confirmed record to date for *L. passim* and the first report of this species in Serbia. *N. ceranae* was ubiquitous, occurring in every year and at 95.7% overall frequency, ranging annually from 83.3% to 100%. The majority of colonies (60.5%) were co-infected with *L. passim* and *N. ceranae*, but colony infections by each species were statistically independent of one another over the nine years. Although *C. mellificae* and *N. apis* have both been reported recently at low frequency in Europe, neither of these species was detected in Serbia. These results support the hypothesis that *L. passim* has predominated over *C. mellificae* in *A. mellifera* during the past decade.

1. Introduction

Trypanosomatids are increasingly being recognized as one of the more prevalent parasitic microorganisms in managed western honey bees (*Apis mellifera* L.) around the world (e.g. vanEngelsdorp et al., 2009; Runckel et al., 2011; Cornman et al., 2012; Morimoto et al., 2013; Ravoet et al., 2013; Schwarz et al., 2015a; Daughenbaugh et al., 2015; Hartmann et al., 2015) and have been correlated with increased colony mortalities in Europe and the USA (reviewed in Schwarz et al., 2015b). Two species of Trypanosomatidae are currently recognized from *A. mellifera*, *Crithidia mellificae* Langridge and McGhee, 1967 and *Lotmaria passim* Schwarz, 2014. The former was considered prevalent in *A. mellifera* when it was described and repeatedly isolated during the 1960s and 1970s. Thereafter, a gap in knowledge of the trypanosomatid species communities living in honey bees occurs until 2008, when nucleotide sequences of trypanosomatids from honey bees were beginning to be isolated and accessioned in GenBank. Although these sequences were typically denominated as *C. mellificae*, in-depth molecular and ultrastructure analyses determined that the majority of these actually belonged to a new species, *L. passim* (Schwarz et al., 2015a). Molecular methods are necessary to distinguish *C. mellificae* from *L. passim* as the morphology of their cells is impossible to discriminate with 100% certainty (Schwarz et al., 2015a). The relative distribution of these two species largely remains unknown, in part due to the lack of species-specific tools for molecular diagnosis.

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Another parasitic microorganism of honey bees is the microsporidian *Nosema ceranae*, which has been found abundantly in *A. mellifera* from the territory of Serbia (Stevanovic et al., 2011, 2013). From 2008 to 2012, Stevanovic et al. (2013) found continually high prevalence of *N. ceranae* in Serbian honey bee colonies (73–98%). *N. ceranae* has been present in Serbia since at least the year 2000 and is not regarded as an emergent pathogen in this region. Due to the absence of any molecular evidence that its congener *Nosema apis* has ever been abundant in Serbia, there is no support that *N. ceranae* has displaced *N. apis* within Serbian honey bees (Stevanovic et al., 2011). This is in contrast to honey bee microsporidia populations from other areas of Europe where both species have been documented, typically with predominance of *N. ceranae* over *N. apis* (Klee et al., 2007; Martín-Hernández et al., 2007) or less commonly predominance of *N. apis* over *N. ceranae* (Forsgren and Fries, 2013).

In the majority of European countries, including Serbia and its bordering countries, no surveys of honey bee trypanosomatids have been conducted, neither using microscopy nor molecular tools. Given the indications of possible involvement of trypanosomatids on honey bee health (Cornman et al., 2012; Ravoet et al., 2013), and a continuing worldwide problem of high colony losses among managed *A. mellifera* colonies (Goulson et al., 2015; Lee et al., 2015), it is important to conduct molecular screening for *C. mellificae* and *L. passim* among Serbian honey bees. To achieve this, DNA samples of bees collected from Serbian apiaries during a 9-year period (2007–2015) were analyzed using newly designed primers for each of the honey bee trypanosomatid species. In addition, all samples had been initially screened for *Nosema* species and obtained results are used to assess whether microsporidia and trypanosomatid infections co-occur in *A. mellifera*.

2. Material and methods

2.1. DNA isolation from samples

Archival honey bee DNA samples stored at −20 °C at the Department of Biology, Faculty of Veterinary Medicine, University of Belgrade originated from bees that had been sent by beekeepers over 9 years (2007–2015) from 57 locations in Serbia (Fig. 1). Subspecies and haplotype of these bees were not determined, but previous investigations revealed that honey bees in Serbia are mostly hybrids of *A.m. carnica* and *A.m. macedonica* comprising seven mitochondrial haplotypes (Muñoz et al., 2012; Nedić et al., 2014). Bees were primarily intended for examination of *Nosema* spp. infection and follow-up species identification. Sixty adult workers were sampled per colony and used for the extraction of DNA using DNeasy Plant Mini Extraction Kit (Qiagen, Hilden, Germany) as described in Stevanovic et al. (2013). All DNA samples were immediately screened for the microsporidia *N. apis* and *N. ceranae*. Among 1192 DNA samples stored in our archive, 18 samples per year were randomly chosen (162 DNA samples in total) for molecular screening of trypanosomatids. Eight apiary locations were sampled in two different years and the apiary owned by authors of this work (at "Beograd" locality) was sampled in five different years (Table S1).

DNA was extracted from axenic cultures of *C. mellificae* (strain 30862) and *L. passim* (strain BRL) as described previously (Schwarz et al., 2015a). DNA from lab-reared *Bombus impatiens* bumble bees infected with *C. bombi* was used for validation of trypanosomatid primer specificity. Active infections of putative *C. bombi* promastigotes were confirmed by microscopic visualization of fresh fecal drops. Bee abdomens were individually homogenized using a FastPrep FP120 cell disrupter (Qbiogene, Carlsbad, CA) in 2% (w/v) hexadecyltrimethylammonium bromide (CTAB) buffered with 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 0.2% (v/v) 2-mercaptoethanol, 50 μg proteinase K (Promega, Madison, WI) and 5% (v/v) RNase cocktail (Life Technologies, Carlsbad, CA). DNA was isolated with standard phenol:chloroform:isoamyl alcohol (25:24:1) phase separation followed by alcohol precipitation.

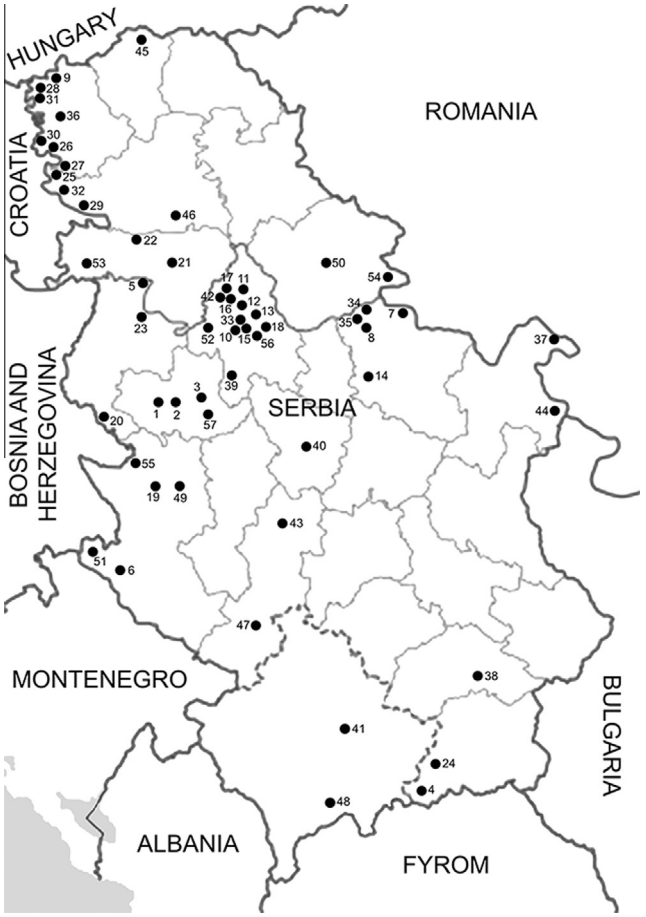

Fig. 1. Map of sampling locations (location tags correspond to those in Table S1).

2.2. Primer design and identification of trypanosomatid species

Species-specific primers were designed at polymorphic regions (lower-case bps) of the *cytochrome b* gene (*Cytb*) between validated *L. passim* (GenBank KJ684960) and *C. mellificae* (GenBank KJ684951) sequence accessions. *L. passim*: LpCytb_F1 (5′-cGAAGTg CaCATATATGCTTtAC-3′) and LpCytb_R (5′-gcCAaAcACCaATaACtGG tACt-3′). *C. mellificae*: CmCytb_F (5′–AGTtTGAgCtGTtGGaTTTgTt–3′) and CmCytb_R (5′–AACCtATtACaGGcACaGTTGC–3′). Verification of trypanosomatid DNA in templates during primer validation was performed with universal trypanosomatid primers previously published (vanEngelsdorp et al., 2009).

PCR was performed using a Multigene Gradient Thermal Cycler (Labnet International Inc.) in 20 μL volumes containing 1 × PCR buffer (Kapa Biosystems), 200 μM dNTP (Kapa Biosystems), 0.5 mM $MgCl_2$ (Kapa Biosystems), 0.3 μM of each primer, 0.5 U of Taq polymerase (Kapa Biosystems) and 1 μL of template DNA. The PCR parameters for *L. passim* amplification were: an initial DNA denaturation step of 2 min at 95 °C, followed by 40 cycles of 30 s at 95 °C (denaturation), 30 s at 55 °C (primer annealing) and 20 s at 72 °C (DNA extension), and terminated with a final extension step of 2 min at 72 °C. The same PCR conditions were used for *C. mellificae* amplification, except the annealing temperature was validated from 55 to 59 °C. Amplification products were

separated on a 1.5–1.8% agarose gel stained with ethidium bromide and visualized under UV light. Commercial O'RangeRulerTM (Fermentas) or TrackIt (Invitrogen, Carlsbad, CA) 100 bp DNA ladders were used as size markers. Positive controls were developed to ensure quality control of PCR assays using column purified (QIAprep; Qiagen) recombinant plasmids (pGEM-T Easy; Promega) containing primer amplicons derived from pure, axenic reference cell cultures of either *L. passim* or *C. mellificae* maintained in the laboratory of R. Schwarz. Negative and positive control reactions were run in tandem with all survey samples.

2.3. Identification of Nosema species

Nosema species in all samples were identified immediately upon sample arrival. Species determination was carried out either by duplex PCR with species-specific primers (321APIS-FOR/REV and 218MITOC-FOR/REV) designed by Martín-Hernández et al. (2007) or by PCR-RFLP method (with nos-16S-fw/rv primers) as described in Stevanovic et al. (2011).

2.4. Statistics

The relative proportions of *L. passim* and *N. ceranae* infections in bees were tested for independence with two-tailed Fisher's exact test per McDonald (2014).

3. Results

Species-specific primers targeted polymorphic sites in a mitochondrial DNA locus of the cytochrome b gene (*Cytb*), shown previously to phylogenetically distinguish species and strains of honey bee trypanosomatids (Schwarz et al., 2015a). The *L. passim* primer pair (LpCytb_F1 + LpCytb_R) included 13 polymorphic sites unique to *L. passim* vs. *C. mellificae* and produced a 247 bp amplicon (Fig. 2). Primers targeting *C. mellificae* (CmCytb_F + CmCytb_R) included 12 polymorphic sites unique to *C. mellificae* vs. *L. passim* and produced a 140 bp amplicon (Fig. 2). Neither of these primer pairs cross-reacted with non-target honey bee trypanosomatid DNA derived from pure cell line cultures, nor did they cross-react with a related species that infects bumblebees, *C. bombi* (Fig. 2). Serial dilutions of mixed-species templates that included both *L. passim* and *C. mellificae* confirmed each primer was species-specific under disproportional scenarios, where DNA from one or the other species predominated. Non-specific amplification did not occur under these mixed-species conditions, out to a maximum 1000-fold difference tested (10^{-1} vs. 10^{-4}; Fig. 2). Universal trypanosomatid primers confirmed that trypanosomatid DNA was present and suitable for PCR amplification in all templates used during primer validation.

When these primer pairs were tested on honey bee (*A. mellifera*) DNA extractions, amplicons produced in reactions containing the

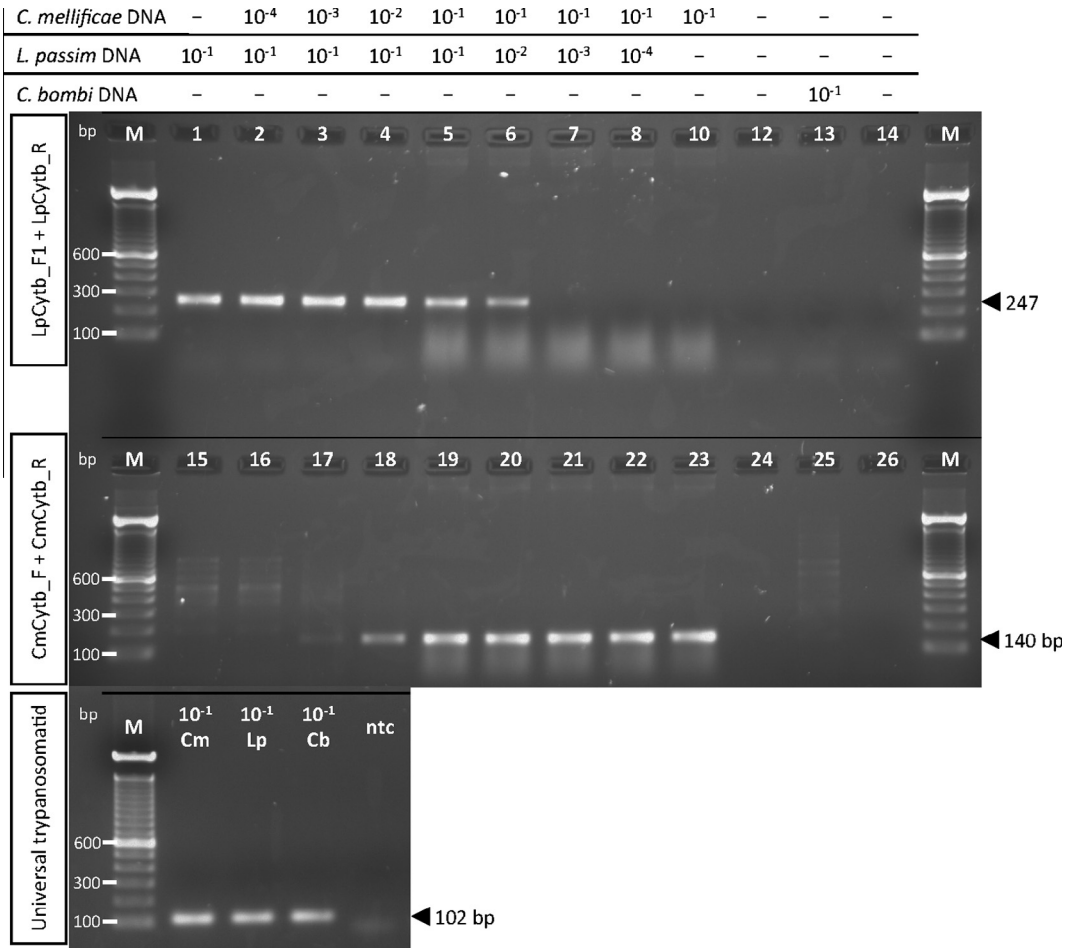

Fig. 2. Trypanosomatid primers tested against single and mixed species DNA templates from *Crithidia mellificae* (Cm), *Lotmaria passim* (Lp), and *Crithidia bombi* (Cb) with traditional PCR. DNA dilutions (10^{-1}–10^{-4}) from *L. passim* (strain BRL) and/or *C. mellificae* (strain 30862) were used. Amplicons of *cytochrome b* DNA from *L. passim* (LpCytb_F1 + LpCytb_R) and *C. mellificae* (CmCytb_F + CmCytb_R) are indicated. Universal trypanosomatid primers amplified a fragment of *28S rRNA* from all species of trypanosomatids. Thermal cycling conditions used 55 °C for the annealing step and 30 rounds of PCR amplification. Gel is 1.8% agarose stained with ethidium bromide. M = 100 bp marker.

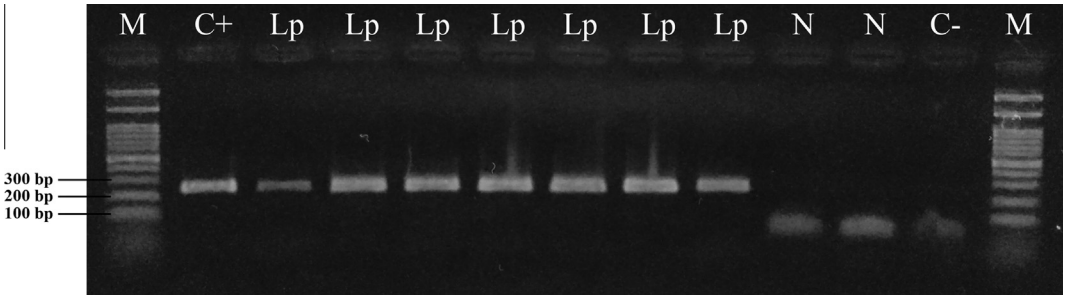

Fig. 3. Detection of *Lotmaria passim* (Lp) in DNA homogenates of *Apis mellifera* samples from Serbia. PCR amplicons were produced in reactions containing primer pair LpCytb_F1 and LpCytb_R. Lanes: M, 100 bp ladder; C+, positive *L. passim* control 10^{-5}; C−, negative control; Lp, PCR products of samples that correspond to *L. passim*; N, PCR product of *L. passim*-free sample. Gel is 1.5% agarose stained with ethidium bromide.

LpCytb_F1 and LpCytb_R primer pair matched the 247 bp size expected for *L. passim* (Fig. 3). Samples with this result were considered positive for *L. passim*. No honey bee DNA samples in this study produced amplicons in reactions containing the CmCytb_F and CmCytb_R primer pair (not shown). Such samples were considered negative for *C. mellificae*.

Summarized results from molecular screening of archival honey bee DNA samples for the presence of trypanosomatids and microsporidia are given in Table 1 with detailed results according to location and colony in Table S1. Analyses of all samples (n = 162) over a 9-year period (2007–2015) from 57 localities in Serbia revealed only one trypanosomatid species, the recently described *L. passim*. It was detected in 101 (62.3%) samples from 44 localities. Analyses of 18 samples per year revealed the following annual frequencies of *L. passim:* 13 samples (72.2%) in 2007, 7 (38.9%) in 2008, 13 (72.2%) in 2009, 11 (61.1%) in 2010, 15 (83.3%) in 2011, 15 (83.3%) in 2012, 7 (38.9%) in 2013, 12 (66.7%) in 2014 and 8 (44.4%) in 2015. The other honey bee trypanosomatid species, *C. mellificae*, was not detected in any of the honey bee DNA samples.

In the case of microsporidia, only *N. ceranae* was recorded, and no *N. apis* or mixed *N. apis*/*N. ceranae* infections were detected. The prevalence of *N. ceranae* was very high as it was found in 155 samples (95.7%), and no location in Serbia was found without at least one colony infected with *N. ceranae* (Table S1).

A majority of colonies (60.5%, n = 98) were co-infected with *L. passim* and *N. ceranae*, originating from 43 locations throughout Serbia, including Belgrade (Table S1). Infections with only *N. ceranae* and not *L. passim* occurred in 35.2% of colonies (n = 57) from 32 locations (Table 1). Rarely (1.9%, n = 3) colonies were infected with *L. passim* without also being infected with *N. ceranae*, one colony from 2010 (Ripanj) and two colonies from 2012 (Ugljare and Belgrade). However, over the 9 years surveyed, proportions of *L. passim* and *N. ceranae* infections in *A. mellifera* were statistically independent of one another (Fisher's exact test $P = 0.427$).

Table 1
Summarized annual honey bee colony sampling in Serbia and infection status with *Lotmaria passim* and/or *Nosema ceranae* from 2007 to 2015.[a]

Year[b]	*L. passim* infection only	*N. ceranae* infection only	Co-infection	Uninfected
2007	0	5	13	0
2008	0	11	7	0
2009	0	5	13	0
2010	1	4	10	3
2011	0	3	15	0
2012	2	2	13	1
2013	0	11	7	0
2014	0	6	12	0
2015	0	10	8	0
Total	3 (1.9%)	57 (35.2%)	98 (60.5%)	4 (2.5%)

[a] All samples tested negative for *N. apis* and *C. mellificae*.
[b] 18 colonies were sampled each year using 60 adult bees per colony.

4. Discussion

Up to now, no information regarding the status of honey bee trypanosomatids has been published for Serbia nor for its bordering countries. In addition, the confirmation that an apparently emergent trypanosomatid species now occurs in honey bees in addition to a historically recognized species (Schwarz et al., 2015a) motivated the molecular screening of archival honey bee samples from Serbian apiaries during the last nine years (2007–2015), which were initially collected for microsporidia screening. This study provides the first long-term survey (>1 year) for each of the trypanosomatid parasites known to infect honey bees and provides the first record of *L. passim* in Serbia.

L. passim was consistently present in Serbian honey bees every year since 2007 at moderate to high prevalence (38.9–83.3%). This frequency and the absence of *C. mellificae* in these samples corroborates the current hypothesis (Schwarz et al., 2015a; Ravoet et al., 2015; Arismendi et al., 2016) that *L. passim* is a common parasite in *A. mellifera* and is now the dominant trypanosomatid species in adult worker bees. Arismendi et al. (2016) found *L. passim* at similar moderate to high prevalence rates, ranging from 44% to 92% across different regions of Chile within a one year survey. Although they did not survey for *C. mellificae* and thus the status of this species in Chile remains unknown, they provide the only other study we are aware of that uses validated primers specific to *L. passim* that do not cross-react with *C. mellificae* and can provide comparable, species-specific survey data to our survey from Serbian honey bee colonies. Despite the trend that *L. passim* predominates *C. mellificae* based on currently available data, this limited number of studies does not warrant the assumption that prior surveys for trypanosomatids using universal trypanosomatid primers or unconfirmed metagenomic assemblies should be ascribed as *L. passim*. In fact, *C. mellificae* has been confirmed recently using sequence analysis in Belgium by Ravoet et al. (2015) from not only *A. mellifera* but also miner bees (*Osmia* spp.). Thus, there remains a need to explore the current and historical status of this species in different geographic areas and hosts.

The discovery of *L. passim* in samples of *A. mellifera* collected in Serbia dating from 2007 establishes the earliest record of this species so far (on a global scale), one year earlier than previously known. Prior to this study, the earliest confirmed report of *L. passim* is represented by nucleotide sequence accessions GU321191.2 and GU321196.1 archived at GenBank. Although these sequences, isolated from *A. mellifera* collected in Switzerland during 2008, are erroneously identified as *C. mellificae*, Schwarz et al. (2015a) published a corrected taxonomic identification, confirming that these sequences, along with all other previously accessioned trypanosomatid sequences from honey bees were in fact derived from *L. passim*. To date, the only confirmed molecular reports of *C. mellificae* in *A. mellifera* have been from Belgium, where it occurs infrequently (Ravoet et al., 2015). Although the

history of honey bee trypanosomatids may never be fully realized, results from the current study improve historical understanding of this species and further add that there is no evidence *L. passim* has co-occurred with *C. mellificae* in Serbia between 2007 and 2015. Other researchers with access to archived honey bee samples (tissue or purified nucleic acids) are encouraged to follow the approach demonstrated here to shed light on the emergence of *L. passim* and the history of trypanosomatid parasite species community dynamics in bees.

The infection of honey bees with one parasite may affect their susceptibility to infection by another parasite, either by increasing or decreasing host susceptibility to co-infection (reviewed in Evans and Schwarz (2011) and Schwarz et al. (2015b)). We found that *L. passim* and *N. ceranae* co-infections occurred in honey bee colonies at a fairly high rate (60.5%) over the 9 years surveyed. Despite this frequency, there was no detectable correlation between the rate at which they infected honey bee colonies based on a test of independence. The ubiquity of *N. ceranae* infections in this region and the rarity of colonies uninfected with both *N. ceranae* and *L. passim* (2.5%), however, could make statistical correlations difficult to detect. Testing relationships between these two species in future studies from areas where *N. ceranae* is less pervasive will improve the statistical power for tests of independence, and could reveal important insight into their interspecific infection dynamics. The rarity of colonies infected solely with *L. passim* and not *N. ceranae* does suggest it is unlikely *L. passim* reduces honey bee susceptibility to *N. ceranae* infection. Although the interspecific dynamics between these two common gut parasites remains to be determined, their combined effects on their host may be synergistic. Ravoet et al. (2013) reported that honey bee colonies were more likely to succumb to winter mortality when concurrently infected with *N. ceranae* and trypanosomatids (negative synergy). Physiological impacts for interactions between these species have been identified via alterations of immune response in honey bees (Schwarz and Evans, 2013) and are likely factors involved. This along with the reported abundance of trypanosomatid and microsporidian parasites shown here in Serbia as well as in honey bee colonies from the USA (e.g. vanEngelsdorp et al., 2009; Runckel et al., 2011; Cornman et al., 2012), Japan (Morimoto et al., 2013), China (Yang et al., 2013), and Turkey (Tozkar et al., 2015) suggest further work is needed to understand dynamics among these species and their hosts. In addition, different subspecies of honey bees should be taken into account in future surveys of trypanosomatids and analyses of their impact on hosts.

The *cytochrome b* gene was selected for species-specific primer design because it is known to have 11–12% interspecific variability yet is highly conserved within species (<1% intraspecific variation) based on two described strains each of *L. passim* and *C. mellificae* (Schwarz et al., 2015a). These primers were carefully designed and optimized for use in either traditional PCR, as shown here, or in quantitative PCR by keeping amplicon size under 250 base pairs. Validation of this second methodology was beyond the scope of this study but is part of an ongoing study for future publication. A different approach to molecular species-level diagnosis of trypanosomatids uses a single primer pair designed to amplify fragments of varying lengths at the internal transcribed spacer region-1 (ITS-1) locus (Ravoet et al., 2015). This approach indiscriminately amplifies bee trypanosomatids (i.e., *L. passim*, *C. mellificae*, *C. bombi*, *Crithidia expoeki*) and relies on species-specific fragment length polymorphisms. The ITS region of *L. passim* and *C. mellificae* is highly variable, with a reported 33.5–35.3% interspecific variability and up to 2% intraspecific variability (Schwarz et al., 2015a). Although the size difference between amplicons generated with these universal trypanosomatid primers is readily distinguishable by gel electrophoresis between *C. mellificae* and *L. passim* (~140 bp), it has not been tested on mixed-species conditions and may be biased to one species over the other, producing a single amplicon from the more abundant species in a sample or from the species whose template to primer match has the fewest polymorphisms. In addition, the high variability of this locus warrants caution as other species (e.g., *C. bombi*, *C. expoeki*) and novel strains may result in overlapping fragment lengths that make species-level diagnosis unreliable without sequence confirmation.

Our primers specifically targeting *C. mellificae* for PCR diagnostics are the first of their kind, while PCR diagnostic primers for the specific detection of *L. passim* have just been reported by Arismendi et al. (2016). These primers target different loci, SSU rRNA (459 bp) and gGAPDH (402 bp). Although these primers were not tested under mixed-species conditions, they did not cross-react with pure *C. mellificae* nor *C. bombi* templates. We did not use these primers because we had already developed and gathered data with our own. All of these primers will nonetheless be valuable to empirically and accurately determine the identity of trypanosomatids in bees. This is very important because, as noted previously (Schwarz et al., 2015a; Arismendi et al., 2016), many sequence accessions in public databases (e.g. GenBank) are misidentified and continue to lead to unintentionally spurious reports on trypanosomatid species (Cersini et al., 2015) that further muddy our understanding of the emergence and distribution of these microorganisms.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2016.07.001.

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