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Phylogenetic analysis of the trypanosomatid parasite *Lotmaria passim* in honey bees (*Apis mellifera)* in Poland

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Abstract

Introduction: *Lotmaria passim* (*L. passim*) is a single-celled flagellate which colonises the bee gastrointestinal tract and is highly prevalent in honey bees. This parasite is associated with colony losses. Honey bee (*Apis mellifera*) colonies were sampled from five apiaries in the north-eastern part of Poland for the phylogenetic analysis of *L. passim*. **Material and Methods:** Each apiary consisted of approximately 60 bee colonies, of which 20 were randomly selected. Samples of 60 differently aged worker bees were collected from each colony and pooled. A total of 100 bee colonies from five apiaries were examined. Protozoa of the Trypanosomatidae family were identified by PCR. *L. passim* was detected in 47 (47%) of the samples. The 18S ribosomal (r) RNA amplicons of *L. passim* were sequenced by a commercial service. Their sequences were analysed with BLASTN and noted to be compatible with the GenBank sequences of this region of the organism's genome. A sequence analysis was performed using the BioEdit Sequence Alignment Editor and Clustal W software. **Results:** The amplicon sequences of *L. passim* were 100% homologous with the sequences deposited in GenBank under accession numbers KM066243.1., KJ684964.1 and KM980181.1. **Conclusion:** This is the first study to perform a phylogenetic analysis of *L. passim* in Polish honey bees. The analysis demonstrated high levels of genetic similarity between isolates of *L. passim* colonising apiaries in the north-eastern region of Poland.

Keywords: bee parasite, *Lotmaria passim*, *Apis mellifera*, Trypanosomatidae, phylogenetic analysis.

Introduction

Honey bees (*Apis mellifera*) stand out as highly efficient pollinators crucial for facilitating plant reproduction. Over the past decade, there has been a notable surge in bee mortality rates reported in apiaries worldwide. Multiple factors contributing to bee mortality have been pinpointed, encompassing climate change, pesticide application and pathogenic infections (3, 11, 17). Bee colonies are most frequently infected by parasites, such as *Varroa destructor* mites, microsporidian *Nosema* spp. and trypanosomatid *Lotmaria passim* (15); viruses including deformed wing virus, acute bee paralysis virus, chronic bee paralysis virus and black queen cell virus; and bacteria, among which are *Paenibacillus larvae* and *Melissococcus plutonius*.

Lotmaria passim is one of the most widespread trypanosomatids in bee colonies (15). These single-celled flagellates belonging to the Trypanosomatidae family are obligatory parasites known to infect mammals, insects and plants. In insect hosts, these pathogens establish colonies in the digestive tract (7). Protozoa in insects are found across various taxa, predominantly parasitising the mid- and hind-guts of their hosts (7). These particular protozoa compromise the behaviour, fitness, immune responses and even the physiology of bees. Furthermore *L. passim* infestations can lead to honey bee population declines in the field (4, 13).

Castelli *et al*. (2) found that *L. passim* was widespread in honey bees and affected the health of bee colonies. *Crithidia mellificae (C. mellificae)*, another common trypanosomatid bee parasite, was not detected. The report by Castelli *et al.* (2) showed that *L. passim*

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had been present in Uruguay, Argentina and Chile at least since 2007. Similar results were reported by Gómez-Moracho *et al.* (5), who also showed that *L. passim* and *C. mellificae* significantly reduced the lifespan of infected bees. In their research, honey bees were artificially infected with *C. mellificae* and *L. passim*, and infected bees died earlier than bees in the control groups. The survival rate of bees infected with *L. passim* and *C. mellificae* was reduced relative to that of the control bees. Another finding of the investigation by Gómez-Moracho *et al.* (5) was that *L. passim* was more pathogenic than *C. mellificae.*

The prevalence of these protozoa in apiaries is currently being investigated. Sequences of different loci of the *L. passim* genome, including small subunit ribosomal (r) 18S RNA, have been deposited in GenBank from locations around the world. These genes have been logged from other trypanosome species in addition to *L. passim*, and an investigation of sequences by Schwarz *et al.* (15) noted high similarity among species, but interspecific sections which allowed one species to be discriminated from another were also observed.

The aim of this study was to confirm and determine the phylogenetic position of *L. passim* in the bee colonies sampled, identify this parasite's genome in Poland and compare the results with *L. passim* isolates from other countries deposited in GenBank.

Material and Methods

Five apiaries from the north-eastern region of Poland (53°47′N, 20°30′E) (4U, 4L, 5U, 5L and 6L) were selected for the research. Each apiary consisted of approximately 60 bee colonies, and 20 colonies were randomly selected from each. Samples of 60 differently aged worker bees were collected in each hive and pooled. The samples were homogenised, and genomic DNA was isolated from each homogenate with the Genomic MiniKit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. The isolated DNA was stored in test tubes at a temperature below −20°C until analysis. The following primers were used: *Lotmaria passim cytochrome b* gene (LpCytb)_F1 5′-cGAAGTgCaCATATATGCTTtAC-3′ and LpCytb_R 5′-gcCAaAcACCaATaACtGGtACt-3′. These primers produced a 247-base-pair (bp) amplicon (19). The presence of *L. passim* was determined by PCR with the use of HotStarTaq *Plus* Polymerase (Qiagen, Hilden, Germany) and the HotStarTaq *Plus* Master Mix Kit (Qiagen). The reaction mixture was 20 µL in volume and consisted of 10 µL of HotStarTaq *Plus* Master Mix (1 unit HotStarTaq *Plus* DNA Polymerase, 1× PCR Buffer constituted of 1.5 mM of MgCl and 200 µM of each deoxynucleotide triphosphate), 0.1 µL of LpCytb_F1 (0.5 µM) primer, 0.1 µL of LpCytb_R (0.5 µM) primer, 2 µL of CoralLoad Concentrate (Qiagen), 4.8 µL of RNase Free water and 3 µL of template DNA (100 ng). The reaction was carried out in an Eppendorf Mastercycler thermal cycler (Hamburg, Germany) under the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, primer annealing at 55°C for 45 s and elongation at 72°C for 60 s. After the completion of the last PCR cycle, a final extension step was conducted at 72°C for 10 min. The products of the PCR were separated by electrophoresis in 2% agarose gel (ABO, Gdańsk, Poland) in 1× tris, acetic acid and ethylenediaminetetraacetic acid (MP Biomedicals, Irvine, CA, USA) at 5 V/cm. The size of the obtained products was evaluated by comparison with the GeneRuler 100 bp Ladder Plus molecular size marker (Fermentas, Vilnius, Lithuania). Midori Green (NIPPON Genetics, Düren, Germany) was added to the gel at 0.5 µg/mL to visualise the resulting DNA fragments of *L. passim.* The electrophoresis results were archived using the GelDoc gel documentation system (Bio-Rad, Hercules, CA, USA). Amplicons were obtained with the Clean-Up kit (A&A Biotechnology), and when purified, were sequenced by Genomed (Warsaw, Poland). Sequencing results were compared with the 18S rRNA sequences of nucleotides deposited in GenBank at the National Center for Biotechnology Information with the use of BLASTN version 2.2.18 (1). A sequence analysis was performed using the BioEdit Sequence Alignment Editor and Clustal W software version 2.0. (6). The evolutionary history was inferred using the unweighted pair group method with arithmetic means (18). Evolutionary distances were computed using the maximum composite likelihood method (21) and were expressed as the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 (22).

Results

Lotmaria passim was detected in 47 (47%) of the colonies. Referring positive results for individual apiaries to all positive results, *L. passim* was identified only in 2 samples (4.26%) in group 4U, in group 4L it was identified in 12 samples (25.53%), in group 5U in 14 samples (29.78%), in group 5L in 10 samples (21.28%), and in group 6L *L. passim* was present in 9 (19.15%) of bee colonies.

The sequences of all *L. passim* amplicons were subjected to a phylogenetic analysis, for which the final dataset comprised 325 positions. Thirty-five samples contained *L. passim* DNA (35 samples) which was 100% homologous in sequence with GenBank sequences AB716358.1, AB860145.1, GU321191.2, KJ684956.1, KJ684957.1, KM066241.1, KM066248.1, KM066249.1 and KM980181.1. The amplicons of two samples were characterised by 97.97–99.19% compatibility with the amplicon sequences deposited in GenBank under accession numbers KJ684963.1, KJ684964.1, KM066243.1 and KM066250.1. The remaining ten analysed samples had 100% homologous sequences with GenBank record number MG494247.1. Details are presented in Fig. 1.

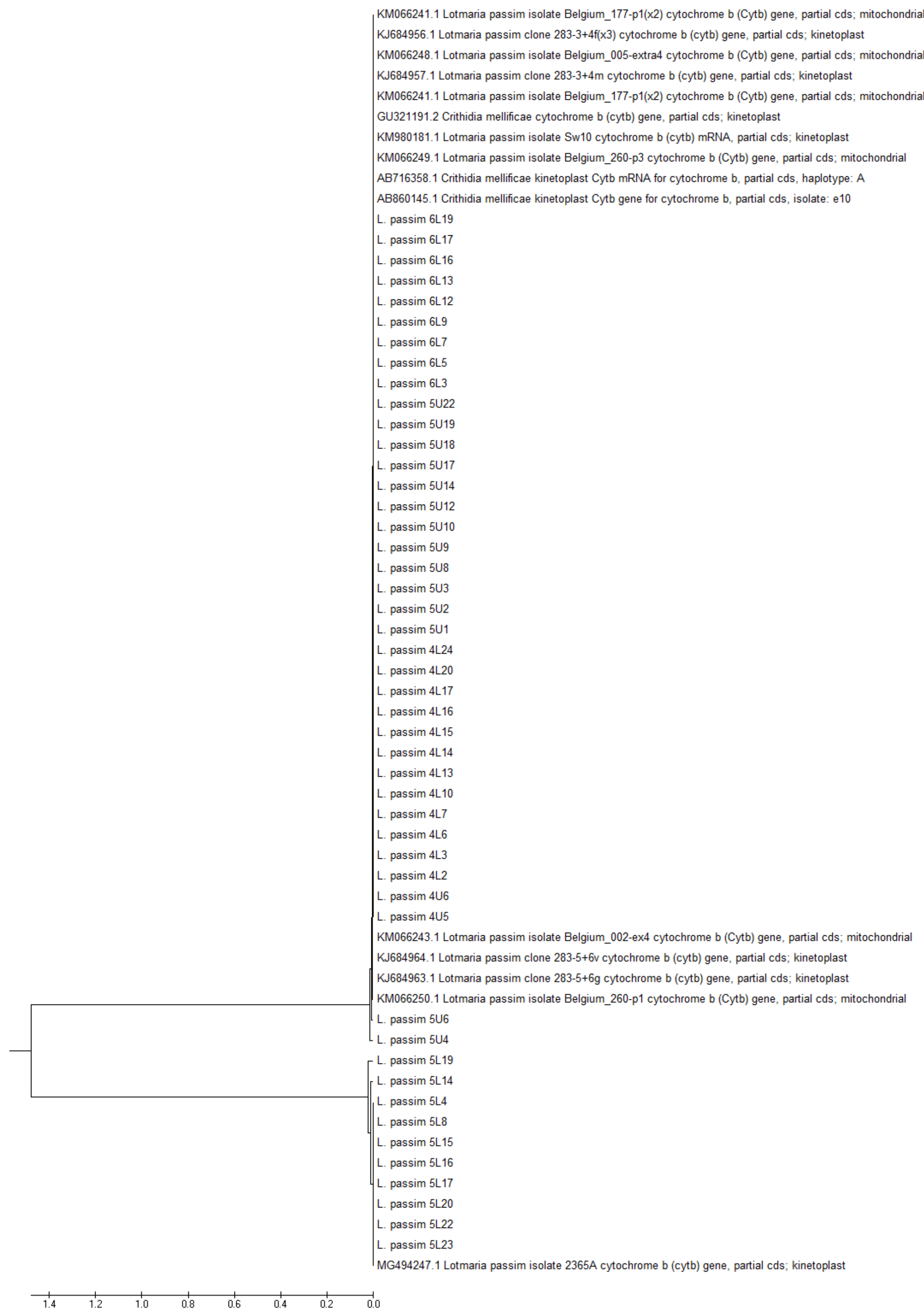

Fig. 1. Evolutionary relationships between taxa of honey bee protozoa parasites isolated from pooled worker bee samples yielding 47 nucleotide sequences from five areas of north-eastern Poland. Evolution was inferred using the unweighted pair group method with arithmetic means. The optimal tree is shown with a total branch length of 0.17974936, expressed in the same units as the evolutionary distances used to infer the phylogenetic tree. Distances were computed using the maximum composite likelihood method and are expressed as the number of base substitutions per site. The first+second+third+noncoding codon positions were analysed and all positions containing gaps and missing data were eliminated. The final dataset contained 325 positions

Discussion

In the present study, *L. passim* was detected in 47% of bee colonies in the examined apiaries. The majority of the obtained amplicon sequences were 100% homologous with *L. passim* amplicons (KJ684957.1, KM066241.1., KM066248.1 and KM066249.1) deposited in GenBank (10, 14, 16, 17). A separate, large proportion

of the samples were 100% homologous with the sequence under GenBank accession number MG494247.1 (23). It should be noted that protozoa of the Trypanosomatidae family colonise not only bees, but also other insects like bumblebees, hive beetles and wasps. Several research studies reported on the presence of *L. passim* in insect pollinators around the world. For example, Nanetti *et al*. (10) investigated the presence of *L. passim*, *C. mellificae*, deformed wing virus and Kashmir bee virus in the small hive beetle (*Aethina tumida*). They showed the presence of these honey bee pathogens in hive beetles collected from free-flying colonies. *Lotmaria passim* colonise the guts of various insects like honey bees, bumblebees, solitary bees and wasps (8, 10, 15), to varying extents: Tripodi *et al.* (23) analysed 357 trypanosomatid-positive samples in bumblebees and their sequencing revealed only one sample positive for *L. passim*. In our earlier research we also found *L. passim* in bumblebees (8). This research proves that this protozoan is commonly found in the environment and indicates that phylogenetic analyses should be carried out to test the difference in the strength of its affinity for individual species.

Sensitive PCR methods support the detection and identification of *L. passim* in honey bees. Yamamoto *et al.* (25) reported on the presence and phylogenetic position of *L. passim* in Japan. They analysed its prevalence in six regions and detected this parasite in 16.7%–66.7% of honey bees. In addition, they performed phylogenetic analysis and assigned all obtained isolates to the *L. passim* clade. The homologous sequences came from Japan (KP133004, KP133017, KP133018 and KP133035), Switzerland (KP132995, KP132999, KP133000 and KP133003) and Belgium (KP133021, KP133031, KP133033 and KM980191–KM980194). This study also showed that *L. passim* infects various apiaries in Poland. The bees tested were 47% infected with *L. passim*. Similar results were obtained by Quintana *et al.* (12), who analysed the prevalence of *L. passim* in honey bees in Argentina. They obtained similar results to those of our study: 41% of the samples tested positive for this pathogen. *Lotmaria passim* was also identified in other parts of the world including Hawaii and American Samoa (20), and in Poland in wild bees (8).

Conclusion

This is the first study to perform a phylogenetic analysis of *L. passim* in Polish honey bees (*Apis mellifera*). The analysis demonstrated high levels of genetic similarity between *L. passim* sequences isolated from parasites colonising apiaries in the north-eastern region of Poland. However, ten amplicon sequences of the 18S rRNA *cytb* gene were phylogenetically distant from the remaining amplicons identified in the studied bees. Published data being generally scarce, further research is needed to analyse the genetic material of *L. passim* colonising Polish apiaries and to prevent the possible spread of this protozoan.

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