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Wide diversity of parasites in *Bombus terrestris* (Linnaeus, 1758) revealed by a high-throughput sequencing approach

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Running tittle: Parasite diversity in bumblebees

Originality-Significance Statement

High-throughput sequencing technologies enable the rapid and simultaneous identification of multiple pathogens across large numbers of samples, which *a priori* makes them comprehensive and cost-effective methods to assess parasite diversity. Here we used one of these platforms (Ion PGM ™ System) to evaluate its performance in *Bombus terrestris* specimens that were previously PCR-identified as positive for trypanosomatids (Leishmaniinae). This is the first time that the performance of classical protocols (either specific PCR amplification or amplification with broad-range primers plus Sanger sequencing) and Ion PGM sequencing are compared for investigating parasite diversity in bumblebees.

Summary

Assessing the extent of parasite diversity requires the application of appropriate molecular tools, especially given the growing evidence of multiple parasite co-occurrence. Here we compared the performance of a next-generation sequencing technology (Ion PGM ™ System) in twelve *Bombus terrestris* specimens that were PCR-identified as positive for trypanosomatids (Leishmaniinae) in a previous study. These bumblebees were also screened for the occurrence of Nosematidae and Neogregarinorida parasites using both classical protocols (either specific PCR amplification or amplification with broad-range primers plus Sanger sequencing) and Ion PGM sequencing. The latter revealed higher parasite diversity within individuals, especially among Leishmaniinae (which were present as a combination of *Lotmaria passim, Crithidia mellificae* and *Crithidia bombi*), and the occurrence of taxa never reported in these hosts: *Crithidia acanthocephali* and a novel neogregarinorida species. Furthermore, the complementary results produced by the different sets of primers highlighted the convenience of using multiple markers to minimize the chance of some target organisms going unnoticed.

Altogether, the deep sequencing methodology offered a more comprehensive way to investigate parasite diversity than the usual identification methods and provided new insights whose importance for bumblebee health should be further analysed.

Introduction

The drastic decline of insect populations is a matter of serious concern worldwide (Lebuhn et al., 2013; Hallmann et al., 2017). Although these losses have been attributed to multiple agents, the main causes of this decay are the degradation of the habitat due to urbanisation and intensive agricultural practices, the use of pesticides and fertilisers, and a battery of biological factors among which are the spread of native and exotic parasites (Goulson et al., 2015; Meeus et al., 2018; Sánchez-Bayo and Wyckhuys, 2019). The identification of these organisms, whose expansion in the environment is mostly driven by the sharing of foraging resources, often rely on classical techniques such as the microscopic examination of the samples (Plischuk et al., 2011; Murray et al., 2013; Graystock et al., 2015) and/ or their PCR amplification, usually followed by direct Sanger sequencing of the amplicons (Plischuk et al., 2011; Glenny et al., 2017; Jabal-Uriel et al., 2017). Although the latter is increasingly replacing microscopy due to its greater sensitivity and ability to differentiate morphologically similar organisms, it can also introduce severe biases in the analyses of prevalence and diversity of parasites. For instance, generic primers are usually designed within conserved regions of the genomes and therefore can amplify different taxa. When these occur at similar frequencies in a sample, the electropherograms exhibit overlapping peaks that may prevent the assignment of the species. On the other hand, Sanger sequencing reveals the occurrence of the predominant species, so parasites that are present at lower loads or have less amplification success (e.g. due to the occurrence of mutations in the priming sites or the sequence composition of the amplicon) can go easily undetected. These drawbacks can be partially alleviated by adding a cloning step prior to sequencing (Maharramov et al., 2013; Gómez-Moracho et al., 2014; Ravoet et al., 2015; Cameron et al., 2016), which is expensive and time consuming, or by performing specific multiplex PCR assays (Martín-Hernández et al., 2007; Xu et al., 2017; Bartolomé et al., 2018; Tripodi et al., 2018), which allow the detection of a limited number of targets per reaction.

Although these methods have permitted the finding of several trypanosomatid (Bartolomé et al., 2018; Tripodi et al., 2018) and nosematid species (Li et al., 2012) in *Bombus* specimens, their technical limitations urge the use of more powerful tools to better assess the extent of parasite diversity. Nowadays, the most comprehensive and cost-effective way to investigate this matter at a large scale is to apply high-throughput sequencing methods, which have much greater depth and resolution than Sanger sequencing (Kulski, 2016)

and enable the simultaneous identification of multiple pathogens across large numbers of samples without having to clone the amplicons.

The increasing evidence of trypanosomatid co-occurrence in pollinators (Ravoet et al., 2015; Bartolomé et al., 2018; Tripodi et al., 2018; Bartolomé et al., 2020) prompted us to evaluate the performance of one of these massive parallel sequencing technologies (Ion PGM ™ System; Thermo Fisher Scientific Inc.) in the twelve trypanosomatid-positive bumblebees detected in Jabal-Uriel et al. (2017). These positive samples, which were identified as such by PCR amplification (Meeus et al., 2010), were also screened for the presence of nosematids and neogregarines using both classical protocols (Jabal-Uriel et al., 2017) and next generation sequencing.

Here we report the results of this evaluation that can be considered as a test for future large-scale studies.

Results and Discussion

The use of Ion PGM sequencing allowed the detection of up to four species of trypanosomatids per individual (Table 1), which contrasts with the finding of *Crithidia bombi* Lipa and Triggiani, 1988 as the only species identified by direct Sanger sequencing in the former analysis of these samples (Jabal-Uriel et al., 2017). This discordance is likely due to the fact that *C. bombi* is the most abundant trypanosomatid in bumblebees and masks the presence of other species poorly represented in the PCR amplification products, as shown by the "clean" electropherograms displayed by these samples (Supplemental Figure 1).

Trypanosomatids appeared as a combination of three species - *Lotmaria passim* Evans and Schwarz, 2014, *Crithidia mellificae* Langridge and McGhee, 1967 and *C. bombi* – (Table 1). This is in good agreement with previous results from honeybee and bumblebee studies (Bartolomé et al., 2018; Bartolomé et al., 2020), and stresses the importance of applying next generation technologies to get a better overview of the parasite diversity in individual samples. This is further supported by the first detection of *Crithidia acanthocephali* Hanson and McGhee, 1961 in bumblebees, a protozoan that was recently found to be rather frequent in *Apis mellifera* Linnaeus, 1758 colonies following the same Ion PGM protocol (Bartolomé et al., 2020). A revision of taxonomic ambiguities by DNA barcoding (Boucinha et al., 2020) proved that the sequences of

this trypanosomatid were identical to those of *Crithidia flexonema* Wallace et al., 1960, so from now on both names are used for this taxon.

Two of the samples, B13.67 and B13.80, presented an *SSU* exclusive allele with a single nucleotide difference (A at position 61; Supplemental Figure 2) with respect to the sequences of *Crithidia expoeki* Schmid-Hempel and Tognazzo, 2010 and *C. acanthocephali*/ *C. flexonema*, and two with respect to those of *C. bombi*. Considering the low levels of divergence displayed by the *SSU*-locus across trypanosomatid species (Cepero et al., 2015), it was impossible to ascertain whether this sequence represented a polymorphic variant of any of the former species or corresponded to a new taxon (Trypanosomatidae sp.; Tsp in Table 1; GenBank accession numbers MW001676 and MW002685 in Supplemental Figure 2).

This issue brings up the question about the importance of the selection of genes and primers in the outcome of pathogen surveys. In this case, the use of two loci (*SSU* and *RPB1*) allowed the identification of several species of trypanosomatids, although the two markers showed different sensitivities. *RPB1* enabled the detection of *L. passim*, *C. mellificae*, *C. bombi* and *C. acanthocephali/ flexonema*, whereas *SSU* revealed the presence of *L. passim*, *C. bombi* and the above-mentioned Trypanosomatidae sp., which illustrates the convenience of using multiple markers to minimize the chance of some target organisms going unnoticed (Supplemental Table 1). It must also be noted that this strategy also permitted to discard the presence of trypanosomatids in one of the samples initially identified as positive (B13.140) with the primers of Meeus et al. (2010).

With respect to neogregarines, the PCR-Sanger sequencing protocol detected the presence of *Apicystis bombi* (Liu, Macfarlane and Pengelly, 1974) in only two of the samples (B13.48 and B13.83), whereas the use of a different set of primers combined with Ion PGM sequencing permitted the detection of this species in all the specimens investigated (Table 1), as well as the identification of a new neogregarine taxon recently found in honeybees (GenBank accession number MN031271; Bartolomé et al., 2020).

The PCR detection of microsporidians revealed the occurrence of *Nosema bombi* Fantham and Porter, 1914 in just one individual (B13.83; Table 1); this result was confirmed by Ion PGM sequencing, which also enabled the identification of *Nosema ceranae* Fries, Feng, da Silva, Slemenda and Pieniazek, 1996 in this and another sample (B13.67, Table 1). It should be pointed out that although the presence of both parasites

is regularly reported in *Bombus* spp. (Plischuk et al., 2009; Gamboa et al., 2015; Cameron et al., 2016; Sinpoo et al., 2019), the description of different *Nosema* species co-occurring in single bumblebees is rather rare (Li et al., 2012).

At any rate, it is important to emphasise that the PCR detection of a parasite in a host does not necessarily mean that it is infecting it, especially in the case of sympatric pollinators that share ecological niches and may ingest multiple pathogens deposited onto the flowers (Durrer and Schmid-Hempel, 1994; Ruíz-González et al., 2012; Graystock et al., 2015; Figueroa et al., 2019). However, regardless of their role as incidental carriers or targets of infection - whose evaluation requires the conduction of controlled experiments -, the presence of parasites in bees may contribute to spread the infection to other susceptible hosts (Evison et al., 2012; Ruíz-González et al., 2012). In this sense, improving their detection with next generation sequencing technologies might be of great help not only to refine the assessment of the levels of prevalence and parasite diversity, but also to inspire further research evaluating the potential impact that these agents may have on their hosts.

Experimental Procedures

Samples and classical protocols of parasite identification

All trypanosomatid-positive isolates (Supplemental Table 2) detected in Jabal-Uriel et al. (2017) were used to test the performance of Ion PGM sequencing for the assessment of parasite diversity. These were PCR-identified using the SEF/ SER primers of Meeus et al. (2010) and assumed to be *C. bombi* after Sanger-sequencing the amplicons of three samples (B13.39, B13.80 and B13.168).

The detection of other parasite families was also based on a preliminary PCR-screening using broad-range primers – specifically those published by Tay et al., (2005) to identify microsporidia and those designed by Meeus et al. (2010) to detect neogregarines -, followed by a further analysis to determine the species in positive samples (Jabal-Uriel et al., 2017); *Nosema* spp. were assigned by applying the PCR protocols described by Martín-Hernández et al. (2007) and Plischuck et al. (2009) to reveal the presence of *N. ceranae* and/ or *N. apis*, and that of *N. bombi*, respectively, whereas the two samples positive for neogregarines (B13.48 and B13.83) were Sanger-sequenced and identified as *A. bombi*.

To perform the second part of the experiment (Ion PGM sequencing), the concentration of each DNA isolate was first measured with Nanodrop 2000 (Thermo Fisher Scientific) and set at final concentration of approximately 40 ng/ µl for PCR-amplification with different pairs of broad-range primers.

Primers and PCR amplification for further Ion PGM sequencing

Universal primers amplifying the greatest possible number of nosematid, trypanosomatid and neogregarine species were designed with Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) after identifying conserved regions within the alignments of Nosematidae (*small-subunit ribosomal DNA* - or *SSU* - and *Actin*), Leishmaniinae (*SSU* and *RPB1- RNA polymerase II large subunit RPB1-*) and Neogregarinorida (*SSU*) sequences available in GenBank. Amplicons were required to be shorter than 300 bp for subsequent Ion-PGM sequencing (Table 2).

PCR reactions were performed in 20 µl volume containing: 10.4 µl of H_2O, 4 µl of 5X Phusion HF Buffer (Thermo Fisher Scientific), 0.4 µl of dNTP mix 10 mM, 2 µl of each primer 5 µM, 0.2 µl of Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and 1 µl of DNA. Cycling conditions were set according to manufacturer's instructions and consisted of an initial denaturalization at 98 °C for 30 s, followed by 35-45 cycles of 98 °C for 10 s, 60-66 °C for 30 s (Table 2) and 72 °C for 10 s, and a final extension of 8 min at 72 °C. Negative controls were included in every PCR reaction, which was carried out twice to avoid biases caused by the stochastic amplification of different DNA molecules. The resulting amplicons were first checked by 2% agarose gel electrophoresis and then pooled into a single sample per *Bombus* specimen and marker, respectively; these were purified with Agentcourt AMPure XP (Beckman Coulter) and quantified with Qubit 2.0 (Thermo Fisher Scientific) prior to Ion PGM sequencing.

Ion PGM sequencing

Library preparation and sequencing

Amplicon Libraries were prepared at the Fundación Pública Galega de Medicina Xenómica with the Ion Plus Fragment Library Kit and the Ion Xpress Barcode Adaptors 1-96 Kit (Thermo Fisher Scientific Inc.) according to the manufacturer protocol ("Prepare Amplicon Libraries without Fragmentation Using the Ion Plus Fragment Library Kit", Publication Number MAN0006846 Rev. B.0). Briefly, an equimolar pool of purified amplicons was prepared and a total of 100 ng of amplified DNA per sample was used for library

preparation. The Ion Plus Fragment Library Kit was used to end-repair the amplicons, ligate them to the Ion Xpress Barcode Adaptors and nick-repair to complete the linkage between the adapters and the inserts. Final barcoded libraries were quantified using the Ion Library TaqMan Quantitation Kit and an equimolar pool of twelve samples was prepared at a final concentration of 50 pM. Template preparation and chip loading were performed on the Ion Chef System (Thermo Fisher Scientific Inc.), whereas sequencing was carried out on the Ion PGM Sequencer using an Ion 318™ Chip v2 (Thermo Fisher Scientific Inc.), which can deliver up to 5 million reads per run, with an average length of 200-300 base pairs (Kulski, 2016).

Data analysis

Raw reads were processed with the Ion Torrent Suite software, which sorted the data according to the barcodes assigned to each sample (Supplemental Figure 3). Afterwards, these were transformed into fasta files providing the number and sequence of the haplotypes obtained for each amplicon in a sample, as well as the number of reads on each direction. The latter was used as a control to discriminate between genuine variants and sequencing errors, as this technology tends to produce indel-strand asymmetries (Bragg et al., 2013). Thus, when a haplotype was obtained in just one direction, or when the difference between the number of forward and reverse reads was very large, it was considered artefactual. The assignment of reads to a specific amplicon was based on the sequences of the primers, which were removed from further analyses. These sequences were then aligned with Bioedit (Hall, 1999) and identified by means of BLASTn, using as search sets both the nucleotide collection (nt) and whole-genome-shotgun contigs (wgs).

All sequences obtained unintentionally (mostly from bacteria and pollen) were manually removed from further analyses.

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Table legends

Table 1. Comparison of PCR-Sanger or specific PCR *vs*. PCR-Ion PGM results. In the case of Ion PGM sequencing, the presence of a trypanosomatid or nosematid species in a sample was determined by a positive result (+) in any of the two markers used for these parasites. Ca: *Crithidia acanthocephali*; Cb: *Crithidia bombi*; Cm: *Crithidia mellificae*; Lp: *Lotmaria passim*; Tsp: Trypanosomatidae sp.; Nb: *Nosema bombi*; Nc: *Nosema ceranae*; Ab: *Apicystis bombi*; Nsp: Neogregarinorida sp.; NA: not available.

Table 2. Primers used for PCR amplification (Ion PGM sequencing). Ta= annealing temperature, bp= base pairs.

Supplemental Material

315 Supplemental Table 1. Ion PGM sequencing results per locus.

316

317 Supplemental Table 2. Sampling information of the *B. terrestris* specimens analysed in this study

318

319 Supplemental Figure 1. Sanger-sequencing traces of *Crithidia bombi* obtained from bumblebee samples

320 B13.39, B13.80 and B13.168.

321

322 Supplemental Figure 2. Alignment of Trypanosomatidae sp. detected in specimens B13.67 and B13.80 with

323 other trypanosomatid sequences harbouring 99% similarity at the *SSU* gene.

324

325 Supplemental Figure 3. Filtering process of the Ion PGM reads.

	Trypanosomatids						Neogregarines			Nosematids		
	Sanger	Ion PGM					Sanger	Ion PGM		Specific PCR	Ion PGM	
Specimen	Cb	Ca	Cb	Cm	Lp	Tsp	Ab	Ab	Nsp	Nb	Nb	Nc
B13.33	NA		+	+	+			+	+			
B13.35	NA		+	+	+			+				
B13.37	NA	+	+	+	+			+				
B13.39	+		+	+	+			+				
B13.44	NA	+	+	+	+			+	+			
B13.48	NA		+	+	+		+	+	+			
B13.61	NA		+	+	+			+	+			
B13.67	NA		+	+	+	+		+	+			+
B13.80	+		+	+	+	+		+	+			
B13.83	NA		+	+	+		+	+		+	+	+
B13.140	NA							+	+			
B13.168	+		+	+	+			+				

Target DNA	Primer	Sequence	Ta	Size (bp)
Nosematid *SSU*	Nos SSU-F	TGGACTGCTCAGTAATACTCACTT	60	256
	Nos SSU-R	ACTTCCCATAACTGCCTCAGA		
Nosematid *Actin*	Nos Actin-F	AAGCYTGTGATGTBGATATYAGA	60	187
	Nos Actin-R	ATWGATCCACCAATCCAKACACT		
Trypanosomatid *SSU*	Tryp SSU-F2	GGCTACCGTTTCGGCTTTTG	66	183
	Tryp SSU-R2	CTTCATTCCTAGAGGCCGTG		
Trypanosomatid *RPB1*	Tryp RPB1-F1	GTGGCTGGAYCTGTGGGAGC	66	283
	Tryp RPB1-R1	GCCRTTGATGAACTTCGCCAC		
Neogregarine *SSU*	Neog SSU-F	GCGCGCTACACTGATACAC	64	222
	Neog SSU-R	TTGTCCGTATTGTTCACCGGA		