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Development of a next generation DNA sequencing-based multi detection assay for detecting and identifying *Leishmania* parasites, blood sources, plant meals and intestinal microbiome in phlebotomine sand flies

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ABSTRACT

Leishmaniasis is a disease caused by *Leishmania* parasites transmitted by phlebotomine sand flies (Diptera: Psychodidae). Human infections with different *Leishmania* species cause characteristic clinical manifestations; cutaneous or visceral leishmaniasis. Here we describe the development and application of a Miseq Next GenerationSequencing (NGS)-based Multi Detection Assay (MDA) designed to characterize metagenomics parameters pertinent to the sand fly vectors which may affect their vectorial capacity for *Leishmania*. For this purpose, we developed a MDA by which, DNA fragments were amplified through polymerase chain reactions (PCR) and then sequenced by MiSeq/NGS. PCR amplification was achieved using some published and some new primers designed specifically for identifying *Leishmania* spp. (ITS1), sand fly spp. (cytochrome oxidase I), vertebrate blood (Cytochrome *b*), plant DNA ribulose-1,5-bisphosphate carboxylase large subunit gene (rbcL), and prokaryotic micobiome (16 s rRNA). This MDA/NGS analysis was performed on two species of wild-caught sand flies that transmit different *Leishmania* spp. in two ecologically distinct, but geographically neighboring locations. The results were analyzed to identify, quantitate and correlate the measured parameters in order to assess their putative importance in the transmission dynamics of leishmaniasis.

1. Introduction

Phlebotomine sand flies are the vectors of leishmaniasis, which affects large populations in many regions of the world (Alvar et al., 2012; Desjeux, 2004). The leishmaniases are a group of diseases caused by intracellular protozoan parasites of the genus *Leishmania*. Leishmaniasis manifests as three main clinical forms: cutaneous, muco-cutaneous and visceral leishmaniasis (CL, MCL & VL, respectively) (WHO/TDR, 2010). Phlebotomine sand fly females contract *Leishmania* parasites during blood-feeding on infected human or animal hosts and transmit the pathogens during subsequent blood meals (Killick-Kendrick, 1999; Ready, 2013). Many phlebotomine sand fly species are suspected of transmitting *Leishmania* parasites in different parts of the world (Killick-Kendrick, 1990). Sand fly species are identified morphologically based on differences in internal cuticular structures including pharynx, cibarium and spermathecae of females and external genitalia for males (Lewis, 1982).

Parts of Israel and the Palestinian Authority are endemic to three species of *Leishmania* that cause CL and VL in humans. *L. major* causes CL in large tracts of southern Israel as well as the Jordan Valley. This is a zoonosis transmitted by *Phlebotomus papatasi* with sand rats (*Pasmmomys obesus*) serving as the main reservoir hosts. Tristram's jirds (*Meriones tristrami*) and social voles (*Microtus guentheri*) have also been found naturally infected (Faiman et al., 2013; Hamarsheh et al., 2007; Jaffe et al., 2004; Schlein et al., 1984). CL caused by *L. tropica* is emerging in populated areas of the Galillee and the West Bank due to encroachment of rock hyraxes (*Procavia capensis*), the reservoir hosts, upon human habitation (Jacobson et al., 2003; Svobodova et al., 2006; Talmi-Frank et al., 2010)

With the advent of PCR-based molecular diagnostic techniques, several studies have described methods for detecting and identifying *Leishmania* parasites (Akhoundi et al., 2017; el Tai et al., 2000), sand fly blood meals (Abbasi et al., 2009), sand fly plant meals (Abbasi et al., 2018; Lima et al., 2016). Identification involves amplicon identification using restriction fragment length polymorphisms (RFLP) or (where possible) DNA sequencing.

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DNA barcoding is based on sequencing species-specific DNA sequences obtained through PCR using universal (Gariepy et al., 2012; Gonzalez et al., 2018; Lima et al., 2016). DNA barcoding of the mitochondrial gene Cytochrome C Oxidase Subunit I (COI) was demonstrated to be sequence specific for different animal species including insects and sand flies (Barrera et al., 2017; Dokianakis et al., 2018; Waugh, 2007).

Identification of the sources of sand fly blood meals can facilitate the incrimination of reservoir hosts and sand fly vectors. In the past, different approaches were used for blood-meal identification including immunological (Beier et al., 1988) and DNA based methods (Abbasi et al., 2009; Carvalho et al., 2017; Gebresilassie et al., 2015). Applying NGS technology is essentially similar but at least two orders of magnitude more sensitive than Sanger DNA sequencing.

While blood is only consumed by female sand flies, both sexes ingest plant derived sugar meals as energy sources (Schlein and Jacobson, 1999). Therefore, the structure of plant communities can influence the spatial and temporal distribution of sand fly populations as well as the transmission dynamics of leishmaniasis (Ready, 2013). Given the potential importance of plant meals in the epidemiology of leishmaniasis, there have been remarkably few studies conducted to identify the specific plant species that sand flies feed upon (Lima et al., 2016; Schlein and Yuval, 1987). Sand flies tend to feed on concentrated sugar solutions such as nectar or honeydew (sweet secretion of homopteran insects) (Cameron et al., 1995; Wallbanks et al., 1991). However, they also consume plant sap by piercing leaves and stems with their needle-like mouthparts (Schlein and Muller, 1995). Importantly, sand fly plant meals are typically DNA-poor making their detection and characterization by PCR and conventional DNA sequencing rather challenging (Junnila et al., 2011; Lima et al., 2016). Recently we designed new chloroplast DNA-specific PCR primers for identifying plant material ingested by phlebotomine sand flies, using highly sensitive and accurate MiSeq NGS (Abbasi et al., 2018)

Several studies on the intestinal microbiome of sand flies showed that the most abundant bacterial species were Gram negative soil and plant pathogenic bacteria (Dillon et al., 1996; Gouveia et al., 2008; Hillesland et al., 2008; Sant'Anna et al., 2012). While the source of these infections has not been determined with certainty, sand fly larvae develop in the soil feeding on composting organic material. Thus, larval food is replete with soil bacteria, some of which may transcend metamorphosis into the gut of the adult stage. Moreover, adult sand flies routinely feed upon exposed plant sugar sources such as honeydew or nectar and these may be contaminated with different bacteria and fungi. Importantly, the presence and species composition of a prokaryotic microbiome in sand fly female guts was shown to be a crucial factor in determining their susceptibility to *Leishmania* infections (Kelly et al., 2017; Louradour et al., 2017).

Next Generation Sequencing (NGS) is a high throughput, highly sensitive sequencing method that allows mass sequencing of DNA fragments encompassing whole genomes (Besser et al., 2018; Bonk et al., 2018; de Paz et al., 2018; Mardis, 2013; Zhu et al., 2017). MiSeq NGS can also be applied to metagenomics studies analysing a vast array of genetic information from many organisms derived from one sample. Importantly, NGS provides not only qualitative but also quantitative data for each amplicon species while avoiding the tedious cloning steps required for traditional sequencing. The results include myriad sequences of each amplicon, reflecting its abundance in the original sample.

The main aim of the current study was to adapt a comprehensive DNA based method that utilizes NGS for the detection and identification of *Leishmania* species, the identification of blood and plant meals as well as the bacterial microbiome in individual wild-caught sand flies. In addition, we included cytochrome oxidase subunit I (COI) to facilitate the identification of the host sand fly species. This multi-detection assay (MDA) produced a vast array of DNA sequences that were analyzed as one batch with a computerized workflow designed using available bioinformatics programs.

2. Materials and methods

2.1. Sample collection

Sand flies were collected in CL endemic foci in Israel and the Palestinian Authority using CO_2 baited traps. Only blood-fed female sand flies were selected for this study. A total of 34 sand flies were collected in Sde Eliyahu in the Northern Jordan Valley (32°25′55.99″N 35°30′45.64″E), an agricultural area, which is endemic for *L. major* transmitted by *Ph. papatasi* (Faiman et al., 2013). A further 31 sand flies were collected in Tubas (32°19′4.42″N 35°22′14.92″E); a Palestinian town endemic for *L. tropica* transmitted by *Ph. sergenti*. Sde Eliyahu and Tubas are only 20 km from each other but ecologically very distinct.

Sand flies were brought to the laboratory on ice, washed in a dilute household detergent solution followed by 1% sodium hypochlorite and a final wash in distilled water. The sand fly carcasses were kept in DNA lysis buffer.

2.2. DNA extraction

Sand flies were macerated individually in a micro-centrifuge tubes in 100 µl lysis buffer (100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 0.2% mercaptoethanol, 2% hexadecytrimethlammonium bromide) containing 200µg Proteinase K (Sigma-Aldrich, St. Louis, USA). The solution was kept at 60 °C for 2 h followed by phenol extraction and ethanol precipitation. Extracted DNA was suspended in 100 µl TE buffer (1 mM Tris-HCl, 0.1 mM EDTA) and kept at −20 °C.

2.3. NGS primers

Five different sets of primers were used in this study (Table 1): 1- *Leishmania*-specific ITS1-PCR. 2- Vertebrate Cytochrome *b* for blood meal identification. 3- Cytochrome c oxidase subunit I (COXI) for identifying the sand fly species. 4- 16S rRNA primers that amplify

Table 1
Primer sequences of the five PCR systems used for sand fly NGS analyses.

PCR system	Purpose	Primers' names and their nucleotide sequence	PCR Tm (°C)	Ref.
Leishmania ITS1	Parasite ID	**LITSR:** CTGGATCATTTTCCGATG **L5.8S:** TGATACCACTTATCGCACTT	56	(el Tai et al., 2000)
Cyt b	Blood meal ID	**Cyto1:** CCATCAAACATCTCAGCATGATGAAA **Cyto32:** AACTAGGTCTGTCCCAATG	56	(Abbasi et al., 2009)
COXI	Sand fly sp. ID	**COID1:** GGAACTGGGTGAACAGTTTATCCCCC **COIR1:** ATGTTGATAAAGAATAGGATCTCCTCC	53	Un-published
16S rRNA	Microbiome ID	**Direct:** CCTACGGGNGGCWGCAG **Reverse:** AGGACTACHVGGGTATCTAATCC	60	(Klindworth et al., 2013)
Plant *rbcL*	Plant meal ID	**rbcLD:** TATGTAGCTTAYCCMTTAGACCTTTTTGAAGA **rbcLR:** GCTTCGGCACAAAAKARGAARCGGTCTC	53	(Abbasi et al., 2018)

bacterial DNA for microbiome characterization. 5- Plant ribulose-1,5-bisphosphate carboxylase large chain (*rbcL)* was used as a marker for plant meals. All primers were synthesised with a specific sequence added at the 3′ end: forward primer overhang adaptor (TCGTCGGCA GCGTCAGATGTGTATAAGAGACAG), or reverse overhang adaptor (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) based on Nextera, Illumina DNA library preparation kit (Illumina,San Diego,USA)

2.4. Polymerase chain reaction (PCR)

All PCR reactions were performed in a total reaction volume of 25μl using ready-mix PCR kits (Syntezza, Jerusalem, Israel). The nucleotide sequences of primers used in the five PCR reactions as well as the melting temperatures are summarized in Table 1. The temperature profile started at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 30 s at the specified annealing temperature, and 72 °C for 1 min, and concluded with an elongation step at 72 °C for 10 min.

2.5. Miseq NGS library preparation and sequencing

Was performed as per illumina's Miseq protocol for 16S metagenomic sequencing library preparation (Klindworth et al., 2013). For Each sand fly (Tot: 65), five amplicons were generated using the specific primers (Table 1). Thereafter, amplicons from each sand fly were pooled and purified using AMPure XP beads (Beckman Coulter, California, United States) according to the manufacturer's protocol. The purified pooled amplicons underwent 8 cycle-PCR reactions to anneal Illumina sequencing adaptors and dual-index barcodes with unique sequences for identifying amplicons derived from each sand fly (Illumina, 2013). The barcodes tagged amplicons were purified once again with 0.6x volume of beads (AMPure XP beads) in order to eliminate primer-dimers produced during the reactions. After the last purification step, equal volumes of pooled amplicons from each sand fly, were pooled in one tube. The pooled amplicons were analyzed for DNA quality, concentration and band sizes using an Agilent 2200 TapeStation (Agilent Technologies, CA, USA). Library sequencing was performed with an Illumina MiSeq machine using the 500 cycle MiSeq reagent kit (version 2) with paired 250 bp reads, according to the manufacturer's instructions.

2.6. Bioinformatics analysis

Raw Illumina sequencing data were generated from the PCR amplicons as FASTQ files of read1 (forward) and read2 (reverse) for every sample. These sequence reads were uploaded to Galaxy platform (https://usegalaxy.org/) for further processing and analyses (Enis Afgan et al., 2016). Initially raw sequences were filtered for quality control at a phred score of 20, followed by merging forward and reverse reads, the different amplicons were selected according to their specific nucleotide sequence and their specific length. Sequence reads displaying at least 97% homology were selected using BLAST analysis to determine the number of reads homologous to any of the five markers used.

3. Results

3.1. Association between blood and plant meal sources, Leishmania infections and gut microbiome

Fig. 1 depicts the relative quantity of marker DNA sequences (*Leishmania* – ITS1, Blood-meals – vertebrate Cyt *b*, bacterial microbiome – 16S and plant meals - *rbcL*) in wild caught sand flies from different CL foci. A clear inverse correlation is evident between *Leishmania* parasite load and mammalian blood. On average, infected sand flies had less blood in their guts (= high ITS1 and lower number of Cyt b reads). Further evidence documenting this inverse correlation was obtained by plotting parasite load and blood meal concentration individually for each sand fly (Fig. 2). This observation probably reflects the fact that large volumes of blood werefound in recently-fed sand flies, in which *Leishmania* parasites had only a short time to multiply.

There was no significant correlation between *Leishmania* parasite load and either plant or microbiome load. Lack of correlation was maintained, even when sand flies were analysed individually (Fig. 3).

3.2. Microbiome analysis

The microbial species in the four groups of sand flies are shown in Fig. 4. There was no correlation between the presence of any bacterial species and *Leishmania* infection (Fig. 4). The main bacterial species, found in all groups were*: Mesoplasma entomophilum* and *Pseudomonas* spp. In group 1 (uninfected sand flies Tubas), *M. entomophilum* had the highest number of reads in 3 sand flies while 3 *Pseudomonas* spp. (*Pseudomonas fragi*, *P. lundensis*, *P. plecoglossicida*) were abundant in 11 sand flies. Two infected sand flies from the same area (group 2: *Leishmania* infected *Ph. Sergenti,* Tubas) showed a high number of reads of the human pathogen *Stenotrophomonas maltophilia*. The most abundant bacterial species was *P. fragi*, identified in 17 sand flies in Group 2.

Analysis of infected and non-infected *Ph. papatasi* from the agricultural areas in Sde-Eliyahu showed different microbial community structure than other groups. Bacterial species such as *Escherichia albertii* were found in 9 sand flies of Group 3 (*Ph. papatasi*, Sde Eliyahu). *Acinetobacter lwoffii* that is commonly associated with skin infections, was found in 16 and 14 sand flies of group 3 and 4 respectively.

3.3. Identification of sand fly species

Based on the cytochrome oxidase subunit I (COXI) primers (Table 1) we identified the species of sand flies (Supplementary data, Table 1). Most were of the abundant CL vector species in the region, i.e. *Ph. sergenti* in Tubas and *Ph. papatasi* in Sde-Eliyahu. Additional sand fly species were: *Ph. perfiliewi* and *Ph. tobbi*. *All obtained COX1 reads belonged to one sand fly species, so molecular identification based in COX I PCR, can be used instead of or in addition to morphological identification.*

3.4. Blood meal analysis

Complete DNA-sequence analyses for all sand flies; concerning the quantitative identification of blood meal type and infection by *Leishmania* species are seen in tables 1–3 in Supplementary data. Figs. 5 and 6 indicate that human beings were the most common blood hosts for *Ph. sergenti* and *Ph. papatasi*. A significantly higher percentage of sand flies fed on human blood and these sand flies contained more blood compared to sand flies that had fed on other animals. The only exception to these findings were the L. *tropica* infected sand flies from Tubas where hyraxes represented the most common blood host (Fig. 5, Table 1 Supplementary data).

3.5. Plant meal analysis

There were no specific differences in plant source feeding profiles among infected or un-infected sand flies (Fig. 7). The main plant meal sources were found to represent those available in the two study areas. The common plant families consumed by sand flies in both study areas were *Fabaceae* and *Cupressaceae*, with selective feeding on other plant families that were only available in one of the study areas such as *Solanum* in Sde Eliyahu and *Malus* in Tubas (Fig. 7).

4. Discussion

We developed a powerful, highly sensitive, MiSeq NGS-based multi-detection assay (MDA) to identify and characterize metagenomics parameters associated with phlebotomine sand flies, the vectors of

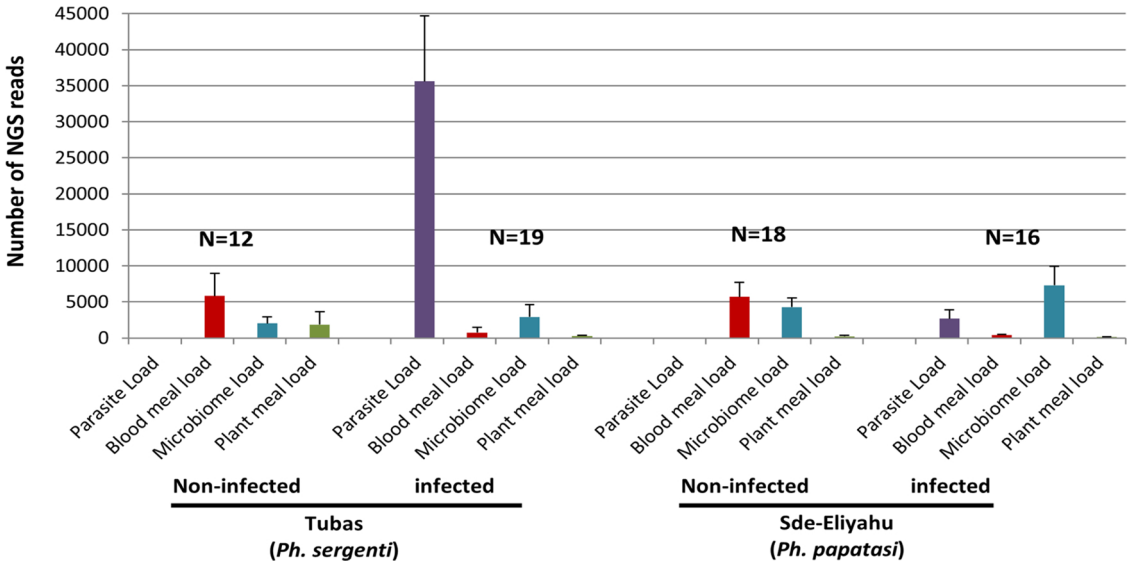

Fig. 1. Mean number of NGS reads per sand fly per amplicon. Included are amplicons for: *Leishmania* parasites (ITS1), Blood host (vertebrate Cyt *b*), Prokaryotic microbiome (16S) and plant-meal source (*rbcL*).

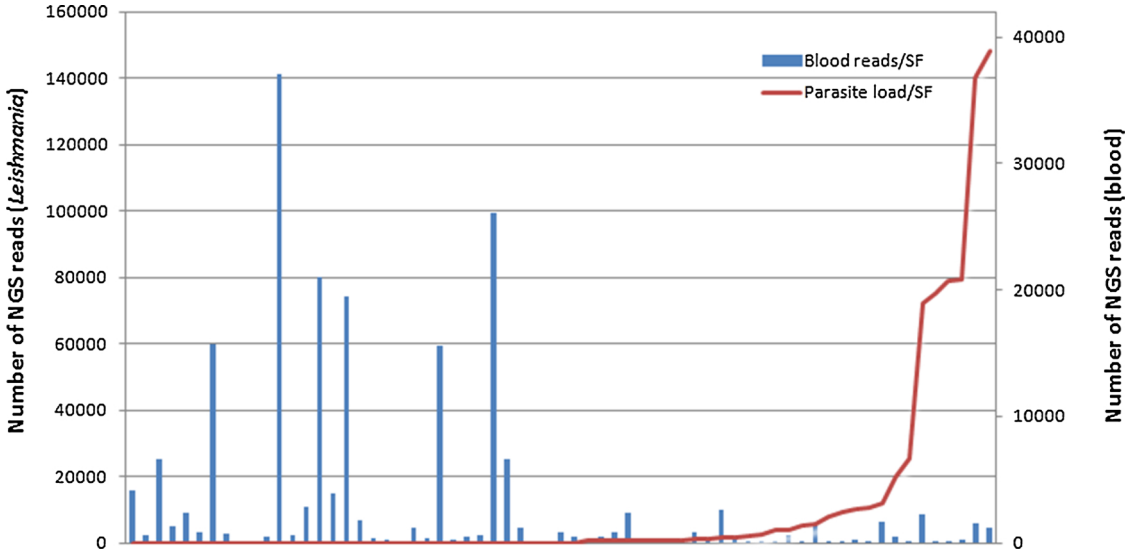

Fig. 2. Graph depicting an apparent inverse correlation between *Leishmania* infection intensity (red curve) and the quantity of blood in individual sand flies (blue bars). This observation probably reflects the fact that large volumes of blood are found in recently-fed sand flies where the *Leishmania* parasites had shorter times to multiply.

leishmaniasis. This Miseq MDA enabled us to simultaneously determine the sequences of five different amplicons including: (1) Insect cytochrome oxidase I to identify the sand fly species. (2) Vertebrate cytochrome *b* gene to identify vertebrate blood hosts. (3) The plant chloroplast gene, ribulose bisphosphate carboxylase large chain (*rbcL*) to identify sources of plant meals. (4) *Leishmania* ITS1 amplicon sequences to identify *Leishmania* species infecting sand flies. (5) The 16S amplicon sequences to assess the composition of the prokaryotic microbiome in sand fly guts.

The advantage of NGS technology stems from its capability to sequence all amplicons regardless of their relative quantity in the sample. Thus, in addition to being a highly sensitive diagnostic method, NGS has the advantage of being a quantitative method as well (Dunne et al., 2012; Kelly et al., 2017). Characterization of the composition of the microbiome depends on this trait and, in our study, quantitation was important also for estimating the relative amounts of different plants as well as blood meal sources.

The parameters addressed by our MDA are all of potential relevance for the transmission of leishmaniasis. Firstly, in most foci only one or, at most two, sand fly species transmit human leishmaniasis and any epidemiological investigation should incriminate these vectors (Killick-Kendrick, 1990; Ready, 2013). We designed new primers for sand fly species identification that amplify the cytochrome oxidase I gene of all sand fly species in our region (Table 1. Abbasi, unpublished, and GeneBank accession numbers: JX105037 to JX105043). The species composition we detected was generally consistent with previous studies in these regions (Faiman et al., 2013; Sawalha et al., 2003). *Ph. sergenti* was identified as the principal species infected with *L. tropica* in Tubas while *Ph. papatasi* was infected with *L. major* in Sde Eliyahu (Table 1 Supp. Data). This finding supports accepted knowledge of vector-parasite specificities as well as previous studies in our region (Jaffe et al., 2004; Ready, 2013).

Identification of blood sources is crucial for understanding the transmission cycles of *Leishmania* spp. Previous studies identified blood meal sources using serological or PCR-based techniques. Although these approaches were useful at the time, they lacked specificity and

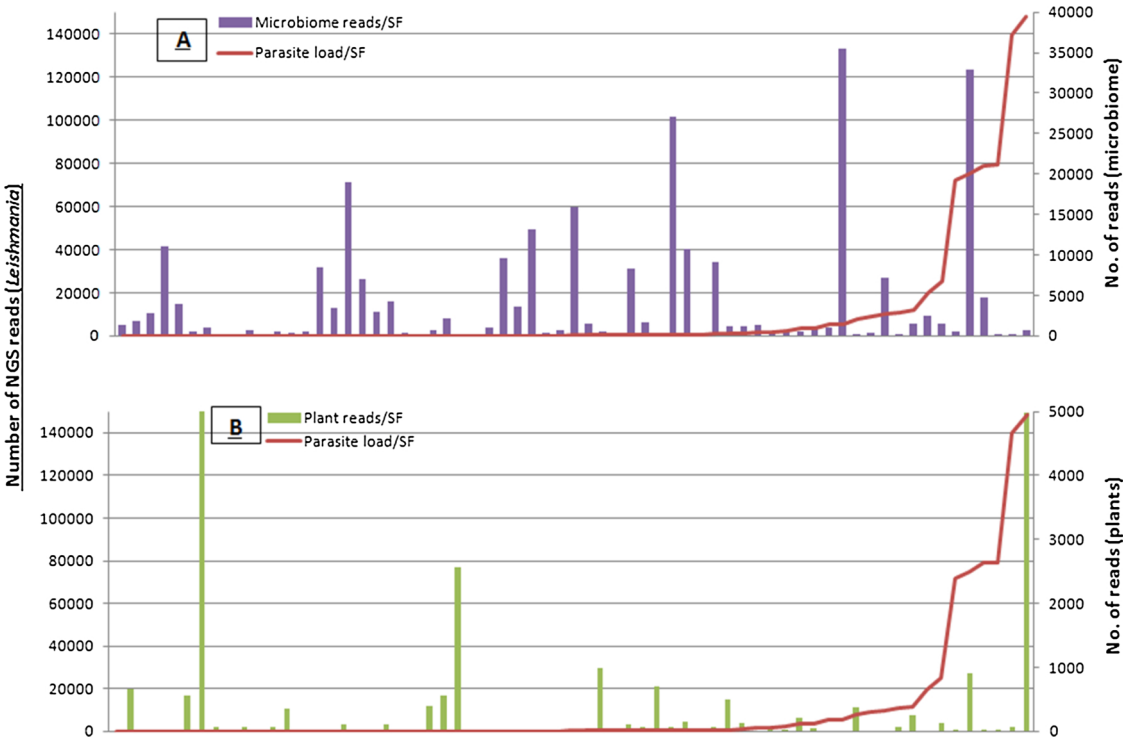

Fig. 3. Lack of observable correlations between the *Leishmania* parasite loads in sand flies and (**A**) Intensity of Microbiome infections, or (**B**) Quantity of plant DNA.

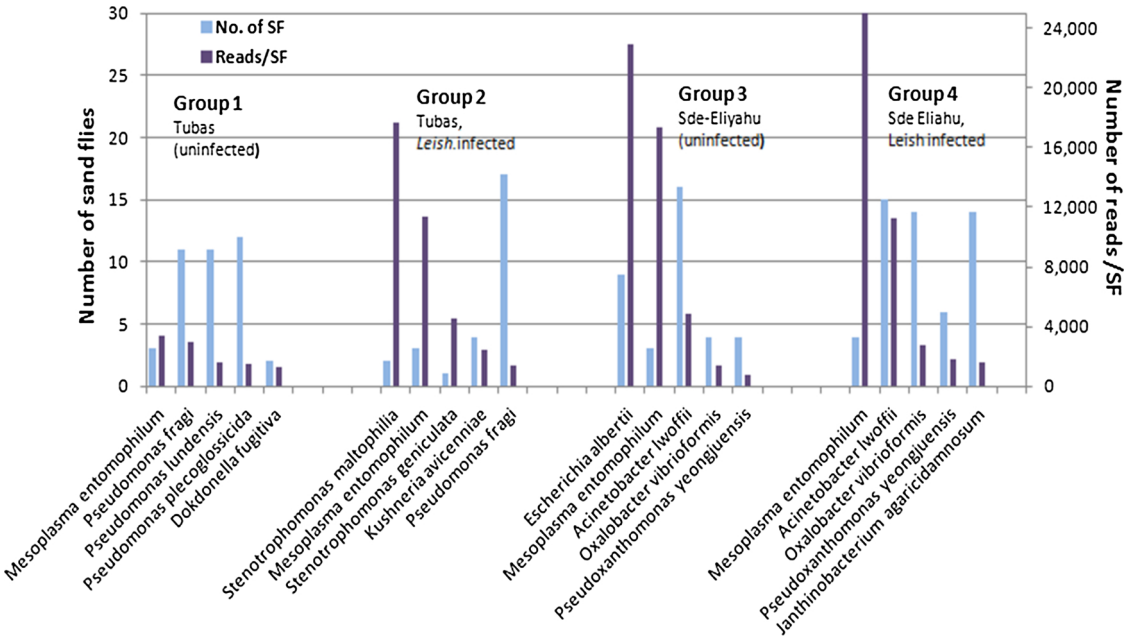

Fig. 4. The number of infected sand flies (light blue bars) and the average number of reads per sand fly infected with the five most abundant microbial species identified in wild-caught sand flies.

sensitivity. Moreover, both serological and PCR based techniques were limited to identifying only animal species anticipated to inhabit the region (Abbasi et al., 2009; Beier et al., 1988; Blackwell et al., 1994; Gebresilassie et al., 2015; Kent, 2009). In the current study, NGS-based blood meal analysis identified blood hosts even when these were not known to inhabit the area (Figs. 5, 6).

In the Tubas area, *Leishmania* infected sand flies were most frequently engorged on rock hyrax blood (Fig. 5, Table 2, Supp Data). Hyraxes are the main reservoir hosts of *L. tropica* in our region (Svobodova et al., 2006; Talmi-Frank et al., 2010), and an outbreak of CL, caused by *L. tropica* was reported in Tubas at the time of sand fly collection (I. Salah, personal communication). Similarly, blood of *Meriones tristrami* and *Microtus guentheri,* reservoir hosts of *L. major* in Sde Eliyahu (Faiman et al., 2013) was identified in several *Ph. papatasi* females captured in Sde Eliyahu (Fig. 6).

We identified, humans as an important blood source for both *Ph. papatasi* and *Ph. sergenti*. In most cases, meals comprising human blood were quantitatively larger than other blood meals (calculated by the number of specific amplicon reads) (Figs. 5, 6). Based on these results we infer the probable preference of both sand fly species for human

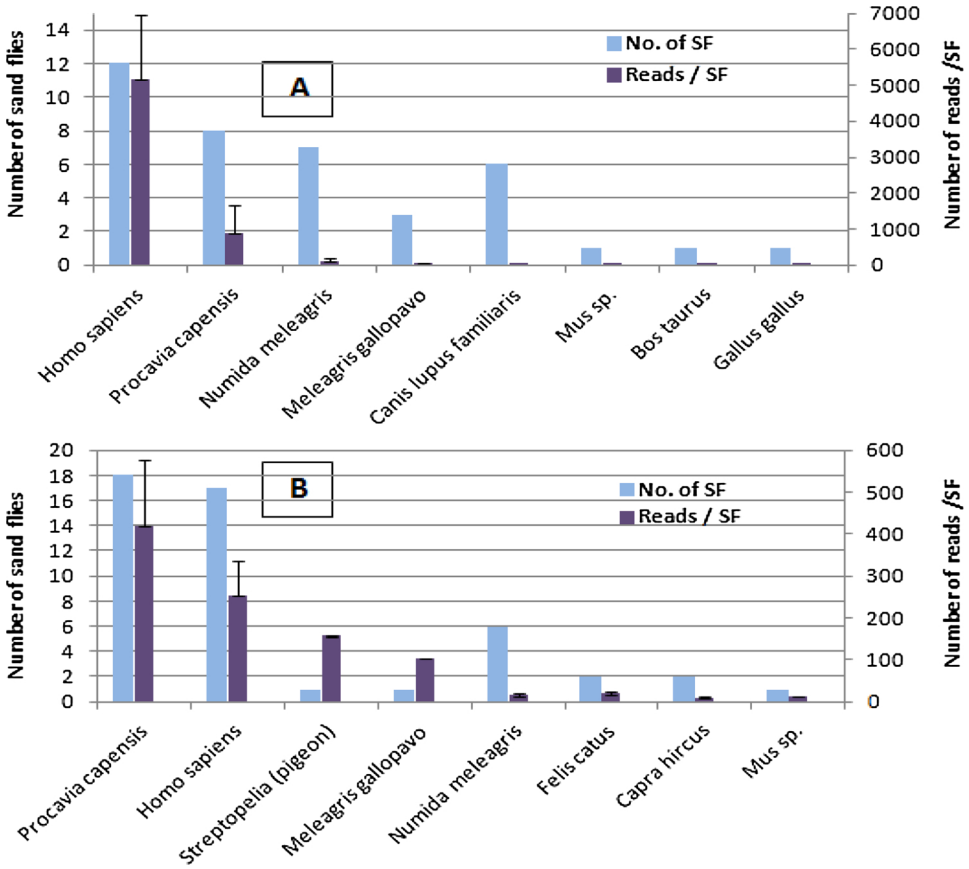

Fig. 5. Blood sources and their frequencies in *Ph. sergenti* sand flies caught in Tubas: A = non-infected, B = infected with *L. tropica*. Note preponderance of hyrax (the known host of *L. tropica*) blood in *L. tropica* infected sand flies of group B.

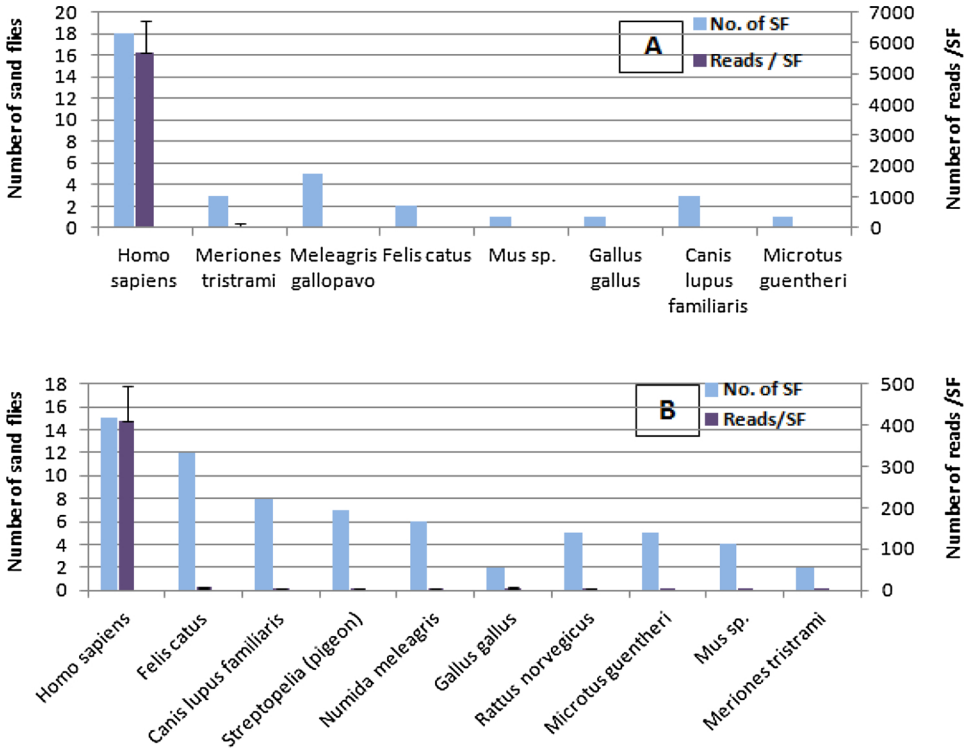

Fig. 6. Blood sources and their frequencies in *Ph. papatasi* sand flies from Sde Eliyahu: A = non-infected, B = infected with *L. major*). Note presence of *Meriones tristrami* and *Microtus guentheri* (reservoir hosts of *L. major* in Sde Eliyahu (Faiman et al., 2013) blood in 7 sand flies.

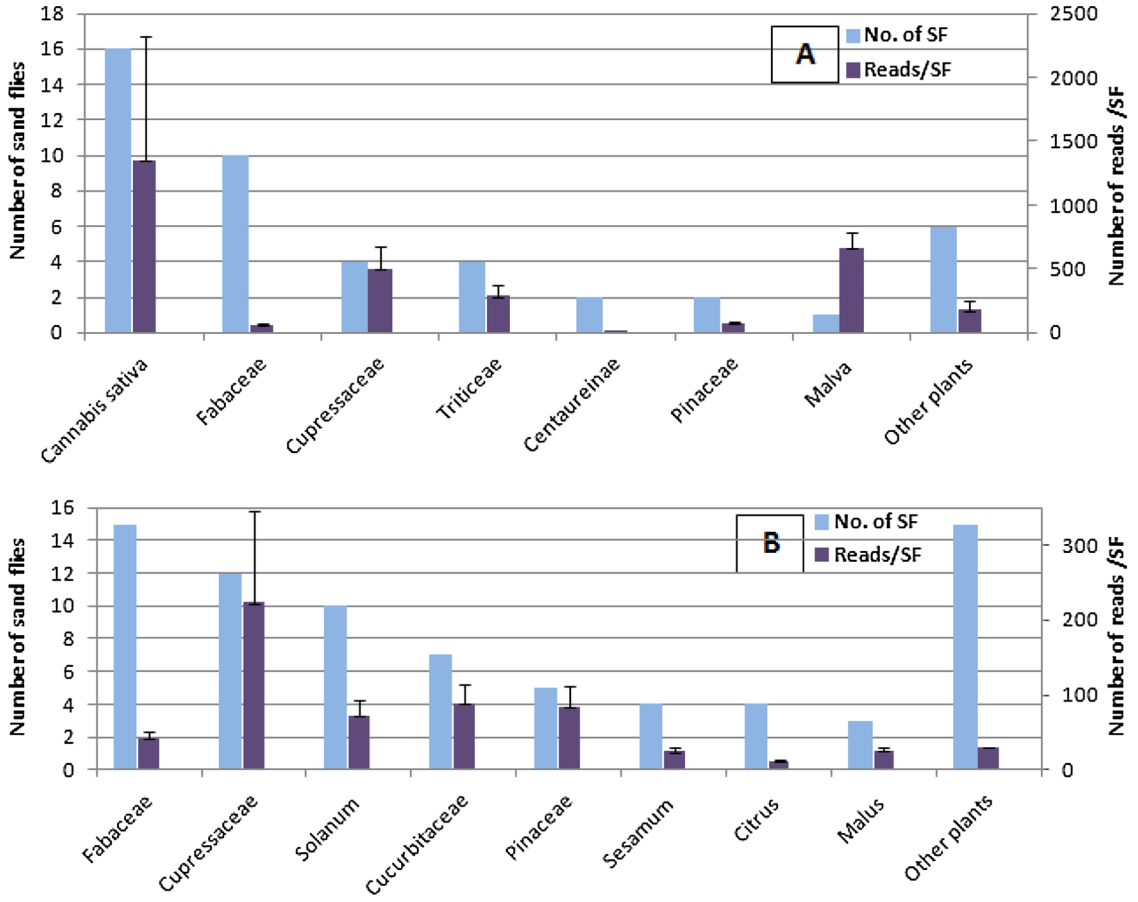

Fig. 7. Plant meal sources, relative quantities and frequency in wild-caught sand flies. Quantitation was based on the DNA sequence of *rbcL* amplicons. A = Sand flies collected from Tubas area, B = Sand flies collected from Sde Eliyahu.

blood despite the normally opportunistic feeding patterns of other sand fly species (Bravo-Barriga et al., 2016; Gonzalez et al., 2018). Our findings are entirely consistent with *Ph. papatasi* and *Ph. sergenti* being the vectors of human CL caused by *L. major* in Sde Eliyahu and *L. tropica* in tubas, respectively.

Bacterial cultures and Sanger sequence analyses of 16S rDNA amplicons were previously used for sand fly microbiome analysis (Akhoundi et al., 2012; McCarthy et al., 2011). In later studies, high-throughput DNA sequence analysis of the 16S rDNA was the method of choice, presenting more detailed information on the microbiome of sand flies (Monteiro et al., 2016).

In our study we noticed an increase in microbiome reads in most *Leishmania* positive sand flies, this finding is inconsistent with previous studies that showed an inverse relationship between microbiome diversity/intensity with *Leishmania* parasite load (da Silva and Sacks, 1987; Kelly et al., 2017). On the other hand, (Kelly et al., 2017) demonstrate a clear correlation between specific microbial species and survival of *L. infantum* in a colony-reared sand flies. The composition of sand fly midgut microbiome varied among the different sand fly groups (e.g. different species, different locations or *Leishmania* infected or non-infected).

Mesoplasma entomophilum and some *Pseudomonas* species were recorded in all sand fly groups. Other common bacterial species were *Stenotrophomonas maltophilia, P. fragi* in *L. tropica* infected *Ph. sergenti* from Tubas and *Acinetobacter* spp. in *L. major* infected *Ph. papatasi* from Sde Eliyahu. *Acinetobacter lwoffii* is known as an opportunistic component of skin flora in 25% of healthy individuals (Regalado et al., 2009)

Microbial diversity in sand flies may be partially explained by bacteria carried over from the larval stages. Larvae breed in dark humid habitats with composting organic material. In addition, adult sand flies may acquire bacteria during sugar feeding. NGS analysis revealed significant differences in bacterial species at the phylum/family level in both *Leishmania* infected and uninfected sand flies. However, there was no significant correlation between any of the parameters measured and the microbial diversity. Moreover, we did not find any correlations between the sand fly species and the composition of the microbiome. Testing of larger numbers of sand flies will be required to address such questions.

In the current study we used the *rbcL* kinetoplast DNA gene as a marker for plant species identification. PCR amplification of *rbcL* from sand flies produces only faint bands that would not be adequate for Sanger DNA sequencing. Thus, the usefulness of DNA for identifying the plant meal sources of sand flies is tremendously enhanced by applying NGS in conjunction. Although the cut-off value we used for plant identification using GenBank, was over 98%; many plant species could not be positively identified (to the species level) due to sequence similarities shared with other species from the same family. To resolve this problem it will be necessary to employ additional plant DNA barcoding markers such as maturase k gene (matk), and ATP synthase beta subunit gene (atpB). We are currently optimizing these markers to improve the accuracy of our sand fly plant meal IDs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2019.105101.

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