Plant-feeding phlebotomine sand flies, vectors of leishmaniasis, prefer *Cannabis sativa*

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Blood-sucking phlebotomine sand flies (Diptera: Psychodidae) transmit leishmaniasis as well as arboviral diseases and bartonellosis. Sand fly females become infected with *Leishmania* parasites and transmit them while imbibing vertebrates' blood, required as a source of protein for maturation of eggs. In addition, both females and males consume plant-derived sugar meals as a source of energy. Plant meals may comprise sugary solutions such as nectar or honeydew (secreted by plant-sucking homopteran insects), as well as phloem sap that sand flies obtain by piercing leaves and stems with their needle-like mouthparts. Hence, the structure of plant communities can influence the distribution and epidemiology of leishmaniasis. We designed a next-generation sequencing (NGS)–based assay for determining the source of sand fly plant meals, based upon the chloroplast DNA gene ribulose bisphosphate carboxylase large chain (*rbcL*). Here, we report on the predilection of several sand fly species, vectors of leishmaniasis in different parts of the world, for feeding on *Cannabis sativa*. We infer this preference based on the substantial percentage of sand flies that had fed on *C. sativa* plants despite the apparent "absence" of these plants from most of the field sites. We discuss the conceivable implications of the affinity of sand flies for *C. sativa* on their vectorial capacity for *Leishmania* and the putative exploitation of their attraction to *C. sativa* for the control of sand fly-borne diseases.

Cannabis sativa | leishmaniasis | next-generation sequencing | phlebotomine sand flies | plant feeding

Phlebotomine sand flies (Diptera: Psychodidae) transmit leishmaniasis, bartonellosis, and several arboviruses that infect humans (1, 2). The leishmaniases comprise a group of diseases with diverse clinical manifestations afflicting millions of humans, typically in impoverished regions of the world (3). Only female sand flies feed on blood, which is required for the maturation of their eggs (1). Consequently, sand fly females contract *Leishmania* (Kinetoplastida: Trypanosomatidae) infections when they ingest infected blood and transmit the parasites during subsequent blood meals.

In addition to the females consuming blood, both male and female sand flies regularly ingest plant-derived sugar meals as a source of energy. Because of the dependence of sand flies on plant meals, the structure of plant communities can affect the spatial and temporal distribution of sand fly populations (4). Moreover, certain plants upon which sand flies feed can shorten their life or reduce their capacity for transmitting leishmaniasis (5, 6).

Sand fly plant meals may comprise nectar, honeydew, and/or phloem sap. While nectar and honeydew are readily accessible on the surface of plants, in flowers, and in extrafloral nectaries, to reach the phloem, sand flies pierce leaves and stems with their needle-like mouthparts (7–10).

Although phloem sap is highly nutritious and generally lacks toxicity, only a restricted range of homopteran insects (Hemiptera: Sternorrhyncha) feed exclusively on phloem sap. Some of these obligatory phloem-sucking insects concentrate scarce essential amino acids from phloem by excreting the excess sugary solutions in the form of honeydew (11). The specific types of sugars and their relative concentrations in honeydew can be used to incriminate honeydew consumers (7–9), while the plant sources of phloem-feeding insects can be identified by plant-specific PCR followed by DNA sequencing (12). The utility of PCR-based approaches for identifying plants consumed by phlebotomine sand flies has been demonstrated in several studies (13, 14). Most of these studies employed the ribulose bisphosphate carboxylase large chain (*rbcL*) gene, which is an accepted marker for phylogenetic and barcoding studies of plants (15).

State-of-the-art, next-generation sequencing (NGS) is much more sensitive than Sanger DNA sequencing and can facilitate the acquisition of vast amounts of genomic data, from complex samples, in one reaction mixture (16–19). We developed an NGS-based multidetection assay for wild-caught sand flies designed to identify *Leishmania* infections, blood and plant meals, as well as characterize their gut microbiome. Here, we report on the plant feeding habits of several sand fly species,

Significance

Sand fly females suck blood from vertebrate animals, including humans, and thereby transmit *Leishmania* parasites and arboviruses. In addition, both sand fly sexes consume plant-derived sugar meals. Therefore, the structure of plant communities can influence the transmission dynamics of sand fly-borne diseases. Our findings demonstrate that, in proportion to their abundance, *Cannabis sativa* plants were consumed by sand flies much more frequently than expected (i.e., *C. sativa* is probably highly attractive to sand flies). We discuss the conceivable influence of *C. sativa* on the transmission of *Leishmania* and its potential utility for sand fly control.

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Fig. 1. Compound scheme comprising a map showing the countries where sand flies were sampled and representative photographs of the sampling sites. The endemic sand fly and *Leishmania* species, as well as the diseases they cause, are also indicated. The leaf icon denotes the presence of *C. sativa* DNA in plant-fed sand flies.

important vectors of leishmaniasis in different parts of the world (Fig. 1).

Results and Discussion

Initially, we performed laboratory experiments to ascertain that plant DNA would be detectable in plant-fed sand flies. We performed PCR using primers for *rbcL* on insectary-reared sand flies that had access (24 h) to freshly cut branches and leaves. Of the eight flies offered *Pistacia lentiscus*, 50% (2) of the males and 75% (3) of the females were positive for *rbcL* compared with 100% (four males and four females) that had consumed *Salvia judaica* (Fig. 2). Since the plants were intact, were not parasitized by homopteran insects (that secrete honeydew), and had no flowers, we were confident that plant feeding was achieved by probing of leaves and/or stems. We assume that the source of the plant DNA detected in sand flies was from plant cells damaged by their mouthparts as they pierced plant tissues to reach the phloem.

In northern Ethiopia (Fig. 1), we used *rbcL* PCR to identify plants consumed by wild-caught *Phlebotomus orientalis*, the local vector of visceral leishmaniasis (VL) (20). We extracted DNA from 804 sand flies, achieved *rbcL* amplification in 65 of them, and sequenced the *rbcL* amplicons from 36 (Table 1 and *SI Appendix*, Fig. S1). The derived *rbcL* sequences implicated mostly common plants such as sesame (*Sesamum indicum*), the salient crop cultivated in that region, as well as various wild tree species from the family *Fabaceae*. In addition, among the plant-fed sand flies, we were surprised to find four that had consumed *Cannabis sativa,* which was never observed at the study site (*SI Appendix*, Table S1).

Following these preliminary studies, we developed and began using an NGS-based multidetection assay on wild-caught sand flies. We found that a very high proportion of the sand flies in several active leishmaniasis foci in different parts of the world fed on plants, frequently preferring exotic plants (in locations where such plants were available) over the endemic flora. We also discovered that sand flies often fed upon multiple plants of different species. Surprisingly, *C. sativa rbcL* was identified in a significant proportion of the flies trapped in four of five field sites (Fig. 1 and Table 1).

Although, on its own, *rbcL* does not identify most plants to the species level, *C. sativa rbcL* is highly specific. Pairwise alignment of *C. sativa* with other members of the family Cannabaceae

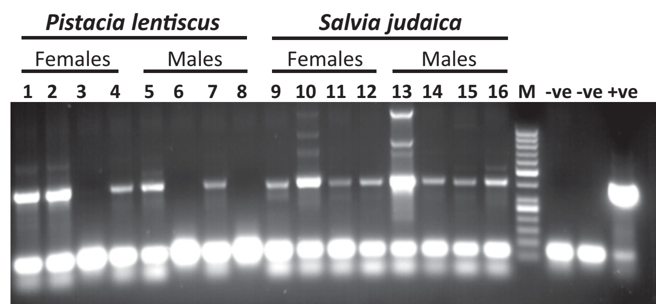

Fig. 2. Agarose gel electrophoresis of sand fly plant meals. Fresh young branches of two different plants (*P. lentiscus* and *S. judaica*) were introduced into cages with 1-d-old laboratory-reared *Ph. orientalis* sand flies, vectors of VL in Sudan and northern Ethiopia (2). The sand flies had not fed before the experiment. Twenty-four hours later, we extracted DNA from individual sand flies, performed *rbcL* PCR, and found that 13 of 16 were positive for plant DNA. Results confirmed that sand fly plant meals are detectable by PCR, at least when they feed on phloem sap (i.e., macerate some cells to reach the sap and ingest DNA from these cells).

showed that *C. sativa rbcL* sequences were always distinct from other members, including the closely related genus, *Humulus*. Furthermore, previous studies showed that *rbcL* sequence analyses could even distinguish between different *C. sativa* strains (21). To verify these findings, we constructed a network of sand fly-derived *C. sativa rbcL* haplotypes that confirmed them to be reliably species-specific (Fig. 3*A*). The haplotype network comprised *rbcL* sequences from *C. sativa* (137 nt) consumed by sand flies collected in different countries. Most (21) sequences from all locations were identical and fell within the central circle, which also included a *C. sativa* reference sequence from the GenBank (NC_027223). Unique haplotypes were found in some of the locations, but the number of point mutations separating these from the main haplotype was significantly smaller than the closest outgroup (*Humulus*) (Fig. 3*A*). Based on these findings, we employed *rbcL* PCR and NGS for all of the subsequent analyses.

The highest proportion of *C. sativa*-fed sand flies (70%) was found near Tubas in the Palestinian Authority ($\chi^2 = 17.673$, df = 6, $P = 0.007104$; Figs. 2 and 3*B* and Table 1), and more sand flies from Tubas consumed *C. sativa* than any other (single) plant ($\chi^2 = 17.136$, df = 3, $P = 0.0006625$). Importantly, the sand flies tested were not a random group; they were all blood-fed females selected for identifying the source of blood in their guts. This bias may also partially explain the extremely high (61%) *Leishmania tropica* infection rate detected in these *Phlebotomus sergenti* females (Fig. 3*B* and Table 1).

Kfar Adumim is located about 100 km south of Tubas in the arid Judean Desert. Here, we collected sand flies near private homes with lush gardens. *C. sativa* was a significant plant food source for both male and female *Ph. sergenti* (Table 1; 35% for females, 29% for males), while other domestic plants comprised the bulk of the remaining meals (Fig. 4*A*). We collected the sand flies during late summer when the seasonal wild vegetation had mostly dried up (photograph in Fig. 1). Therefore, it is conceivable that plants growing in irrigated gardens comprise important sugar sources for sand flies in desert areas. Several authors have postulated that irrigated gardens can promote sand fly breeding by increasing the humidity in the soil where phlebotomine larvae develop (23). Our findings suggest that, in deserts, the availability of sugar sources near human residences surrounded by irrigated gardens may also be important for supporting dense sand fly populations.

In addition to *rbcL*, plant meals of sand flies from Kfar Adumim were analyzed with maturase K (*matK*) and ATP synthase (ATPS). Results confirmed that *rbcL* was much more sensitive than *matK* and more specific than ATPS (*SI Appendix*, Fig. S2). These findings reassured us of the dependability of *rbcL* for identifying *C. sativa* even without using additional markers.

In our analyses, we also examined the number of NGS *rbcL* reads for different plants. Results were highly variable, with many flies feeding on several plants (*SI Appendix*, Fig. S3*A*). Quantitation based on the number of reads was not used to compare sand fly plant meals from different locations because this parameter was found to be largely uninformative due to its dependence on the time since the consumption of plant meals (i.e., DNA degradation), as well as the efficiency of sand fly DNA preservation and extraction.

Sde Eliyahu is a collective agricultural settlement (kibbutz) in the Jordan Valley, surrounded by cultivated vegetable gardens, plantations, and orchards. Several plant families representing mostly cultivated species were identified as plant meal sources of phlebotomine sand flies. However, *C. sativa* DNA was not detected in any of the sand flies collected in Sde Eliyahu (*SI Appendix*, Fig. S3*B*). Notably, Sde Eliyahu is an active focus of zoonotic cutaneous leishmaniasis (CL) caused by *Leishmania major* and transmitted by *Phlebotomus papatasi* (24). We assume that the absence of *Cannabis* meals was attributable to the nonexistence of these plants in the region. However, since Sde Eliyahu was the only field site where *Ph. papatasi* predominated, we cannot be certain that it was not due to an innate aversion of *Ph. papatasi* to *Cannabis*.

The Bura field site in Kazakhstan is a zoonotic focus of CL caused by *L. major* and transmitted by *Phlebotomus mongolensis*. Ecologically, the site is a northern subzone desert where mostly seasonal plants were growing. We noted wild *C. sativa* growing in ditches along the dirt tracks crisscrossing the site. Interestingly, sand flies avoided many of the common plants (e.g., *Haloxylon*), but *Cannabis* comprised a frequent source of plant meals (Fig. 4*B*). The *C. sativa rbcL* sequences from sand flies captured in

Table 1. Summary of the sand flies analyzed for plant DNA by NGS or PCR

Study area	Species	No. of sand flies*	*Cannabis*/plant DNA	No. (%) of infected/*Leishmania* spp.
Sheraro[†]/Ethiopia	*Ph. orientalis*	804	4/36	N/D
Tubas/West Bank	*Ph. sergenti*	31	16/24	19 (61.3)/*L. tropica*
Kfar Adumim/West Bank	*Ph. sergenti*	72 F48 M	22/62 F13/45 M	0
Sde-Eliyahu/Israel	*Ph. papatasi*	33	0/30	15 (45)/*L. major*
Bura/Kazakhstan	*Ph. mongolensis*	211	35/102	22 (10.4)/*L. major* or *L. turanica*
Camacari/Brazil/urban	*Lu. longipalpis*	94	18/48	5 (5.3)/*L. infantum*
Camacari/Brazil/rural		44	10/19	7 (15.9)/*L. infantum*

F, female; M, male; N/D, not done.

*Apparent discrepancies between sample sizes shown here and those depicted in figures were caused by some sand flies having consumed meals from two or more different plants.

[†]Assayed by PCR and Sanger DNA sequencing, which is less sensitive than NGS (*SI Appendix*, Fig. S1).

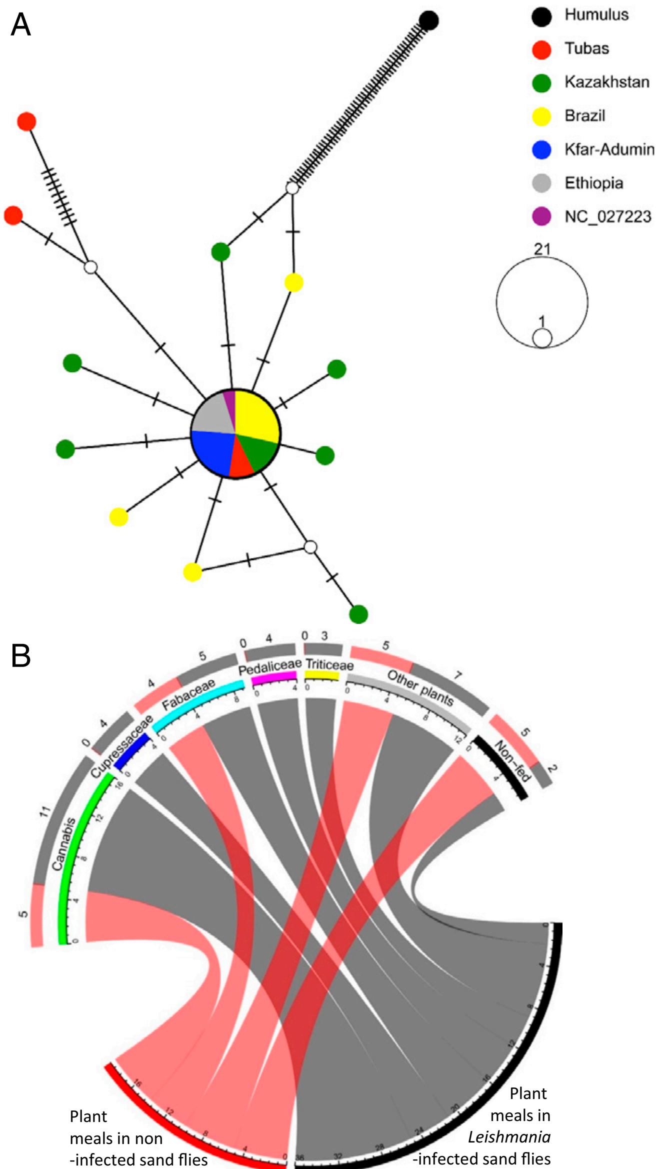

Fig. 3. NGS-based plant meal analyses. (*A*) Haplotype network of *C. sativa rbcL* sequences (137 nt) from sand flies collected in different countries. The large circle in the center represents a cluster of 21 identical sequences found in sand flies from five collection sites as well as a *C. sativa* reference sequence from the GenBank (NC_027223). The smaller colored circles represent unique haplotypes found only in one collection site. The small white circles represent inferred median haplotypes, and the dashes depicted perpendicularly on the connecting edges indicate single-nucleotide polymorphisms (SNPs)/point mutations. Note that the number of SNPs separating the closest outgroup (*Humulus*) is much higher than any of the *C. sativa* haplotypes. (*B*) In Tubas, 56 plant meals (bottom half of the circle) were consumed by 24 *Ph. sergenti* females (top of the circle). Sixteen flies had fed on *C. sativa*. Nineteen females (gray shading) were infected with *L. tropica*. There was no significant difference in *C. sativa* feeding between *Leishmania*-infected and noninfected sand flies ($\chi^2 = 2.25$, df = 1, $P = 0.1336$).

Kazakhstan were the most polymorphic, with six unique haplotypes representing *C. sativa* strains (Fig. 3*A*).

The municipality of Camaçari, Bahia state, Brazil, covers ~785 km^2; some areas are urbanized and others are industrial parks, while the rest remain essentially rural. We collected *Lutzomyia longipalpis*, the local vector of VL for studies pertaining to this disease's transmission. Areas we refer to as urban comprised mostly single-family homes with small yards and gardens (photograph in Fig. 1). The rural areas were sparsely populated, but we collected sand flies relatively close to houses. NGS identified *C. sativa rbcL* DNA as well as several other salient plant families upon which sand flies had fed (Fig. 5). Findings largely concurred with previous studies, demonstrating a preference of *Lu. longipalpis* for cultivated gardens over the endemic vegetation (9, 14). Interestingly, circumstantial evidence

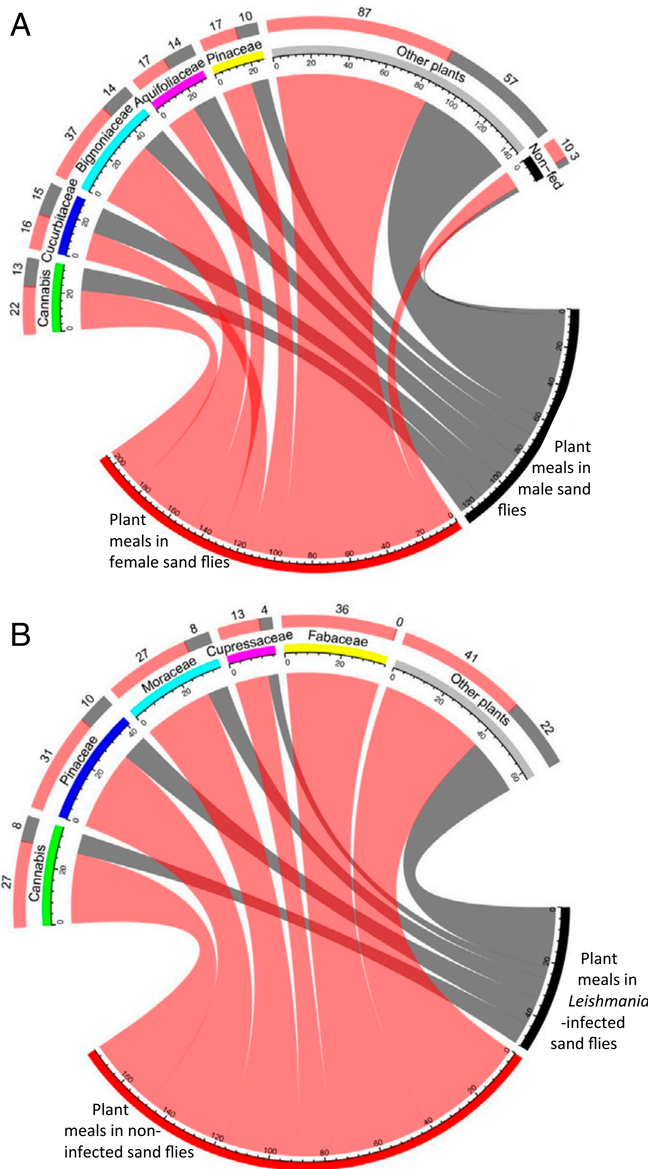

Fig. 4. Sand fly plant meal analyses based on *rbcL* PCR and NGS. (*A*) In Kfar Adumim, 62 female (pink shading) and 45 male (gray shading) *Ph. sergenti* had fed on plants. Of these, 22 females and 13 males had fed on *C. sativa*. Feeding percentages of males and females were not significantly different ($\chi^2 = 2.2857$, df = 1, $P = 0.1306$). (*B*) In Bura, Kazakhstan, an analysis of 102 female sand flies that had fed on plants was performed. Thirty-five had fed on *C. sativa*. Twenty-two sand flies were infected with *L. major* and/or *Leishmania turanica*. There was no significant difference in *C. sativa* feeding rates between infected and noninfected sand flies ($\chi^2 = 2.1304$, df = 1, $P = 0.1444$). We also detected DNA from coniferous plants in some of the sand flies. However, since there were no conifers growing close to the field site, we attribute this finding to wind-blown pollen grains adhering to the sand flies externally. The pollen of gymnosperms contains chloroplasts, making them detectable by *rbcL* PCR/NGS (22).

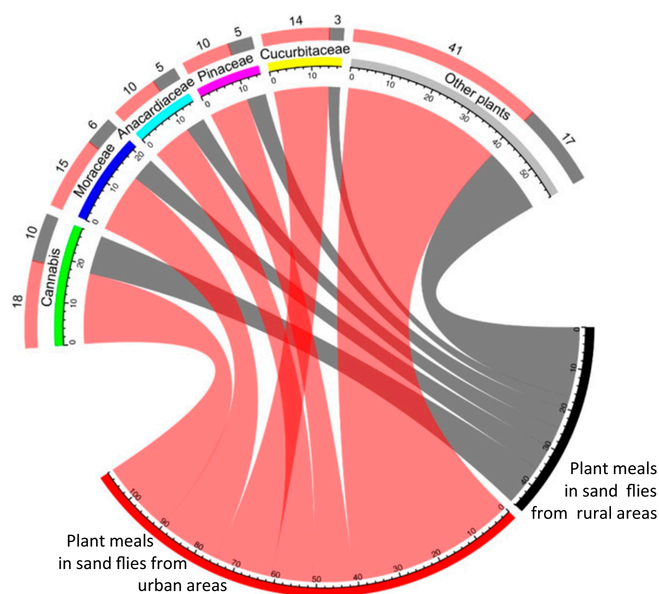

Fig. 5. NGS-based sand fly plant meal analyses based on *rbcL* from Camaçari, Brazil. Sand flies were collected in urban and rural habitats. In the urban area, 48 females had fed on plants, including 18 that consumed *C. sativa*. In the rural habitats, 19 females had fed on plants, including 10 on *C. sativa*. There was no difference in *C. sativa* feeding rates between *Leishmania*-infected and uninfected sand flies ($\chi^2 = 2.3143$, df = 1, $P = 0.1282$).

has led to the hypothesis that exotic plants in urban areas may have played a role in the urbanization of VL in Brazil during the past two to three decades (25).

The attraction of sand flies to specific plants was documented in several field studies (9, 14, 26). Since our findings resulted from surveys rather than experiments, we could not assess the relative attraction of sand flies to common plants. Presumably, because cultivation of *C. sativa* is illegal in the countries where we worked, we did not see *Cannabis* plants in any of the sampling sites except for Kazakhstan, where *C. sativa* shrubs grew endemically. Therefore, we conclude that *Cannabis* comprised but a small fraction of the available sugar sources in any particular habitat and that its ample representation among sand fly plant meals signifies bona fide attraction.

We can only speculate on the putative benefits, other than carbohydrate fuel, gained by sand flies feeding on *C. sativa*. Although cannabinoids can be toxic to some insects (27), a CB1/CB2 cannabinoid receptor agonist protected *Drosophila melanogaster* fruit flies from paraquat toxicity (28). Certain insect species feed upon the highly nutritious *C. sativa* pollen (29), but sand flies possess sucking mouthparts suitable for imbibing liquids only (30) and do not ingest solid food. *C. sativa* synthesizes phytocannabinoids [e.g., tetrahydrocannabinol (THC), cannabidiol] that exert a mostly beneficial influence on humans and most classes of animals by activating G protein-coupled endocannabinoid receptors (31). However, insects apparently lack cannabinoid receptors, as evidenced by the failure of THC to activate G proteins in insect tissues and the absence of cannabinoid receptor orthologs in the *D. melanogaster* genome (32).

Phytocannabinoids from *C. sativa* exhibited antimicrobial activity against some bacteria and fungi (33). Since thriving gut microbiomes are crucial for the development of *Leishmania* infections in the sand fly gut (34, 35), a microbicidal effect could harm *Leishmania* infections. Lastly, partially characterized compounds from *C. sativa* exhibited potent antileishmanial activity in vitro (36).

Pollinator insects, including mosquitoes, are attracted to plants that emit volatile molecules such as terpenes (37–39). Sixty-eight volatile compounds were detected and partially characterized in pollen and vegetative parts of *C. sativa* by GC-MS (40).

A novel approach for controlling blood-sucking mosquitoes and sand flies exploits their plant feeding habit by utilizing attractive toxic sugar baits (ATSBs) that emit olfactory cues to attract sand flies and mosquitoes (41, 42). Adding *C. sativa* plant extracts could augment the efficacy of ATSBs for controlling sugar-questing disease vectors.

Finally, we do not know which compound(s) emitted by *C. sativa* attract(s) sand flies and can only speculate on the possible effects of *C. sativa* molecules on their vectorial capacity for *Leishmania*. Nevertheless, our results reliably demonstrate that this specific insect–plant association is shared by several sand fly species in diverse geographical areas (Figs. 1 and 3*A* and *SI Appendix*, Fig. S4), and suggest that it may well affect the behavior of sand flies and, thereby, the transmission of leishmaniasis.

Materials and Methods

Sand Fly Collection and Identification. In the context of several independent research projects, we collected phlebotomine sand flies in several endemic foci of leishmaniasis in different parts of the world. Among these, only in Kfar Adumim was sampling of sand flies was instigated specifically for plant meal detection. Further details on sand fly species, field sites, and laboratory processing are provided in *SI Appendix, S Materials and Methods*.

PCR. Plant meals of *Ph. orientalis* sand flies captured in Ethiopia were analyzed by PCR. Further details on PCR procedures are provided in *SI Appendix, S Materials and Methods*.

NGS-Based Assays. NGS multidetection assays included genetic markers for (*i*) plant meal identification of chloroplast *rbcL* (31) and (*ii*) the *Leishmania* species *Leishmania* ITS1 (43). Two additional markers (*matK* and ATPase) were used for identifying plant meals in sand flies captured near Kfar Adumim (44) (*SI Appendix*, Fig. S2). DNA amplicon libraries were generated using NGS PCR-specific primers (*SI Appendix*, Table S2). Libraries included plant *rbcL* and *Leishmania* ITS1 PCR-amplified DNA segments. The PCR products were purified using magnetic bead kits (AMPure XP Beads Kit; Beckman Coulter), and all amplicons from each sand fly were pooled into one tube. A second PCR assay was performed for barcoding all amplicons from individual sand flies using the Nextera XT Index Kit (Illumina). Individual sand fly libraries were quantified using Qubit (Life Technologies), and their integrity was evaluated using the Agilent 2200 TapeStation (Agilent Technologies). Following this step, equal amounts of DNA from each of the samples were pooled and the mixture was sequenced by the MiSeq System (Illumina) using a 500-cycle MiSeq Reagent Kit (version 2; Illumina) with paired 250-bp reads in accordance with the manufacturer's instructions (45).

***C. Sativa rbcL* Haplotypes.** The size of the amplified *rbcL* fragments that were sequenced by Illumina NGS was 374 bp (GenBank accession numbers MH733441–MH733473), aligning with nucleotide positions 55,451–55,824 on the complete chloroplast genome of the *C. sativa* (NC_027223) reference sequence. A region of 137 bp from all different *C. sativa* haplotypes that aligned with nucleotide positions 55,469–55,605 on the reference gene was used in multiple sequence alignments for constructing the *C. sativa rbcL* haplotype network (Fig. 3*A*). The haplotype analysis was limited to 137 bp by the reliable DNA sequence obtained for the Ethiopian sand flies' *rbcL* by PCR and Sanger sequencing. The derived sequence (250 bp) aligned at nucleotide positions 55,360– 55,609 on the reference gene. The detailed haplotype multiple alignment parameters are detailed below.

Statistical Methods.

Identification of plant meals based on* rbcL *sequences. The identification of plant meals comprised two processing steps. First, the raw sequencing data were filtered to remove short reads, Illumina adapters, and reads with less than a 90% base quality score (Q20) using Trimmomatic (46). Reads that did not match the barcode accurately were discarded. After the quality check, the identification of sand fly plant feeding sources was performed by comparing the sequences of the *rbcL* gene reads with the nonredundant database from the National Center for Biotechnology Information, downloaded December 2017 (available at ftp://ftp.ncbi.nlm.nih.gov/blast/db/), using a nucleotide BLAST program (47) and the filtered databases with taxonomy identification codes from Embryophyta (taxonomy ID: 3193). Nonmatching *rbcL* reads were discarded. The assignment of taxonomic information was performed as

follows. The genus level was reached when only one hit passed the strict thresholds of 99% query coverage, 97% similarity, and an E-value of $\leq 10^{-2}$, which was the case for all *Cannabis* classifications. On the other hand, if two or more hits passed the above thresholds, the taxonomic information was attributed to the family that had more local species. In summary, BLAST "hits" were used to assign reads to plant species, genera, or families, and statistical significance was measured by the E-value. Reads that did not fulfill these conditions were considered potential chimeric sequences and discarded. Finally, the plant meal(s) of each sand fly were determined by calculating the proportion of reads matching each plant species, genus, or family.

Circular graphs (circos) (Figs. 3–5). Circular plots (Figs. 3*B*, 4, and 5) were created using the plant source-specific, generic, or familial *rbcL* frequencies in the various groups (e.g., location, sand fly sex, *Leishmania* infection status) with the chord diagram function of the circlize package in R 3.4.3. The inner-superior semicircles represent the cumulative number of sand flies that had fed on a specific plant (family/genus/species). The outer-superior semicircles represent the numbers of sand flies from each group, and the origin is represented by colored links that match the group of origin [inferior semicircles (labeled)]. Categories of sand flies within study sites (e.g., males vs. females in Kfar Adumim; *Leishmania*-infected vs. uninfected in Tubas, Brazil, and Kazakhstan) were compared using *t* tests. Comparisons between *C. sativa* feeding rates in Tubas and other field sites were performed using χ^2 tests.

Haplotype network based on* C. sativa rbcL *(Fig. 3A). Multiple alignment of the *C. sativa rbcL* sequences was performed with the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program using the iterative algorithm with default settings (48). Alignments were double checked and, where necessary, edited using BioEdit 5.0.9 software (49). The haplotype network analysis was performed using the median joining network method with the Population Analysis with Reticulate Trees (PopART) software package (50).

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