Lutzomyia Sand Fly Diversity and Rates of Infection by Wolbachia and an Exotic Leishmania Species on Barro Colorado Island, Panama

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Abstract

Background: Sand flies (Diptera, Psychodidae, Phlebotominae) in the genus Lutzomyia are the predominant vectors of the protozoan disease leishmaniasis in the New World. Within the watershed of the Panama Canal, the cutaneous form of leishmaniasis is a continuous health threat for residents, tourists and members of an international research community. Here we report the results of screening a tropical forest assemblage of sand fly species for infection by both Leishmania and a microbe that can potentially serve in vector population control, the cytoplasmically transmitted rickettsia, Wolbachia pipientis. Knowing accurately which Lutzomyia species are present, what their evolutionary relationships are, and how they are infected by strains of both Leishmania and Wolbachia is of critical value for building strategies to mitigate the impact of this disease in humans.

Methodology and Findings: We collected, sorted and then used DNA sequences to determine the diversity and probable phylogenetic relationships of the Phlebotominae occurring in the understory of Barro Colorado Island in the Republic of Panama. Sequence from CO1, the DNA barcoding gene, supported 18 morphology-based species determinations while revealing the presence of two possible “cryptic” species, one (Lu. sp. nr vespertilionis) within the Vespertilionis group, the other (Lu. gomezi) within the Lutzomyia-cruciata series. Using ITS-1 and “minicircle” primers we detected Leishmania DNA in 43.3% of Lu. trapidoi, 26.3% of Lu. gomezi individuals and in 0% of the other 18 sand fly species. Identical ITS-1 sequence was obtained from the Leishmania infecting Lu. trapidoi and Lu. gomezi, sequence which was 93% similar to Leishmania (viania) naiffi in GenBank, a species previously unknown in Panama, but recognized as a type of cutaneous leishmaniasis vectored broadly across northern and central South America. Distinct strains of the intracellular bacterium Wolbachia were detected in three of 20 sand fly species, including Lu. trapidoi, in which it frequently co-occurred with Leishmania.

Conclusions: Both morphological and molecular methods were used to examine an assemblage of 20 sand fly species occurring in the forests of the Panama Canal area. Two of these species, members of separate clades, were found to carry Leishmania at high frequency and hence are likely vectors of leishmaniasis to humans or other mammal species. A single Leishmania species, identified with high confidence as Le. naiffi, was carried by both species. That Le. naiffi is known to cause cutaneous lesions in South America but has hitherto not been reported or implicated in Panama opens the possibility that its range has recently expanded to include the Isthmus or that it occurs as a recent introduction. The occurrence of Leishmania and Wolbachia in Lu. trapidoi identifies one important vector of the disease as a potential target for gene introductions using Wolbachia population sweeps.

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Introduction

New World sand flies of the genus Lutzomyia are exclusively responsible for transmitting cutaneous and visceral leishmaniasis (CL and VL) to humans, diseases caused by kinetoplast protozoans of the genus Leishmania [1]. While the Isthmus of Panama is a region crucial to world commerce, growing tourism and increasing human population, it also remains an area where leishmaniasis continues to be endemic. Below, we report the results of a study using molecular techniques to clarify species relationships and Leishmania infection status of common sand fly species occurring in the understory of the mature tropical forest on Barro Colorado Island, located in the central watershed of the Panama Canal. The study additionally tests sand fly species for infection by the rickettsia, Wolbachia pipientis, a microbe which could prove useful in future efforts to control species which vector the disease to man.

Leishmania infection is characterized by a species-specific pathology, varying from cutaneous lesions to the potentially fatal visceral form. Found primarily in tropical latitudes, 12 species regularly infect humans in the Neotropics, whereas only 6 species are reported to do so in the Paleotropics [2]. The global burden of
Author Summary

Certain sand fly species living inside or on the edge of tropical forests are well known to transmit a protozoan to humans, which in lowland Panama develops into a cutaneous form of leishmaniasis; open, itching sores on the face and extremities requiring aggressive treatment with antimonial compounds. Morphological characters and DNA sequence from mitochondrial and nuclear gene fragments permitted us to identify and then establish historical relationships among 20 common sand fly species occurring in the understory of Barro Colorado Island, a forested preserve in the middle of the Panama Canal. Individuals in three of these sand fly species were found to be 26–43% infected by *Leishmania naiffi*, a species hitherto known only from the Amazonian region and the Caribbean. We then screened the same 20 sand fly species for the cytoplasmically transmitted bacteria *Wolbachia pipientis*, finding three infected at high rates, each by a distinct strain. *Lutzomyia trapidoi*, the most likely transmitter of *Leishmania* to humans in Panama, was among the *Wolbachia*-infected species, thus marking it as a possible high-value target for future biocontrol studies using the bacteria to either induce mating incompatibilities or to drive selected genes into the population.

Identification of *Lutzomyia* sand flies has traditionally been based on morphological characters found in the terminal segments of the abdomen. Morphology-based classification is sometimes weakened by intra-species variation, the occurrence of cryptic species [30], and the lack of distinguishing morphological characters in the females of some species (i.e. the *Vesiculatoria* Group) [31]. Thus, for phlebotomine sand flies, DNA sequencing provides a useful independent assessment of species relationships. In particular, the cytochrome oxidase 1 mitochondrial gene (CO1) has been used for this purpose in many arthropods [32] and is employed extensively by the Barcode of Life Initiative. This gene has been successfully used to distinguish species [33], and to discover cryptic species in multiple insect orders [34,35], however, its utility as a tool in phylogenetic reconstruction remains controversial [36] unless complemented by sequence from the nuclear genome. Accurate identification of *Lutzomyia* species and species groups will be important in establishing baselines for understanding the spread of the disease and eventually in targeting pest control measures to mitigate *Leishmania* transmission.

Materials and Methods

Phlebotomine Survey

Sand flies were collected on thirteen different dates at one to two week intervals between May 12, 2007 and September 12, 2007 at sampling sites distributed across Barro Colorado Island (Fig. 1). At each site, four “CDC” light traps [38], were placed at 25m intervals in the understory for 12 hours (6PM to 6AM). Each trap was modified to accommodate a small LED light to increase the catch and a 50ml plastic cup half filled with 95% ethanol to rapidly kill and fix small insects. The catch was removed from the traps at the end of each trapping period and stored at −20°C. Four additional specimens were removed from CDC traps run at km 1 of the “Pipeline Road” N of Gamboa, and from malaise trap samples taken near the bases of the canopy cranes at San Lorenzo and Parque Nacional Metropolitano during the same time period (2). Male and female sand flies were identified by a highly experienced *Lutzomyia* taxonomist (R. Rojas) using the keys to species groups [39].

DNA Extraction

DNA extractions were carried out using QIAGEN Puregene Gentra alcohol and salt precipitation. Sand flies were frozen in liquid nitrogen, crushed and run through the kit’s default protocol. As the DNA pellet was very small, the final ethanol wash was removed via micropipette.

Single *Lutzomyia* and *Brumptomyia* extracts were used as templates for PCR amplification. We targeted a conserved region of the *Leishmania* minicircle DNA (a non-nuclear sequence in the kinetoplast organelle present only in protozoans of the class Kinetoplastida) that is copied many-fold and contains a conserved region suitable for PCR with primers 13A and 13B [40].

the disease has been estimated to be about 500,000 cases of visceral leishmaniasis and 1.1–1.5 million for cutaneous leishmaniasis (CL) per year [3,4]. The parasites are transmitted as intracellular amastigotes that spread through binary fission causing symptoms through cell lysis [1]. Humans and a considerable number of other mammals can act as reservoirs for the parasite. There has been a noted world-wide increase in the resistance of *Leishmania* to traditional pentavalent antimonial treatments and the response rate for alternate treatments is species-specific [1,5] raising the importance of accurate species diagnosis. Some strains of *Leishmania* are spreading northward, with cases reported in previously non-endemic areas of North Texas [6].

Some *Lutzomyia* sand fly species are infected with the alpha-proteobacterium (*Rickettsia*) *Wolbachia pipientis* [7], a reproductive parasite of insects [8–11]. *Wolbachia* infects a wide variety of arthropod phyla (including insects, crustaceans and arachnids) and is an endosymbiont within filarial nematodes [12–14]. *Wolbachia* cells concentrate in the reproductive tissues and are transmitted vertically to offspring through egg cytoplasm and horizontally between species through unknown mechanisms. *Wolbachia* is known to infect at least 15-20% of insects worldwide [15,16], and in most filarial nematode species infecting humans [17]. *Wolbachia* manipulates host reproduction in diverse ways, such as male-killing, feminization of genetic males [18], parthenogenesis induction [19], and by causing cytoplasmic incompatibility (CI) of host gametes [20–22]. These adaptations benefit the bacterium by allowing it and the mitochondrial genome of its host to spread quickly through uninfected populations [23,24]. Because of the sweep, *Wolbachia* has been explored as a potential driver for introducing transgenes into natural populations [25–27]. Low levels of maternal transmission in some phlebotomine species may hamper attempts to use it in transgenic strategies [28] but to date this has not been investigated in New World sand flies. However, if *Wolbachia* could be used as a population control mechanism analogous to sterile male release, then less than perfect transmission actually may be desirable [29].
approach has been shown to be sensitive and reliable [41]. Confirmation of *Leishmania* infection was attempted with other primer sets such as 13Y+13Z [40] and LSUC+LSUL [42] but spurious amplification was seen, possibly from non-specific annealing to sand fly or sand fly meal DNA [43] (for discussion on non-specific amplification). Confirmation of infection was easily achieved using primers L5.8S+LITSRn [41] to amplify the ITS-1 region on the *Leishmania* nuclear genome.

**Polymerase Chain Reaction (PCR)**

A 20 µL PCR mix consisted of a final concentration of 1× buffer, 1.25mM MgCl₂, 0.2 mM dNTP, 1 µM of each of the two primers, and 0.5 units of Taq polymerase (Applied Biosystems AmpliTaq). The thermal cycler protocol depended on the gene being amplified.

**Lutzomyia cytochrome oxydase 1.** We used universal CO1 primers [32]. Thermal cycle protocol: initial melting 94°C for 2 minutes; 37 cycles of melting at 94°C for 30 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1 minute 30 seconds. A final extension time of 10 minutes at 72°C was followed by holding at 4°C.

**Lutzomyia 18S.** Primers were 18SA F&R and 18SB F&R [44]. Thermal cycler protocol included a ‘touch-up’ step: initial melting 94°C for 2 minutes; 10 cycles of melting at 94°C for 35 seconds, annealing at 51.4°C for 30 seconds, extension at 72°C for 30 seconds; 24 cycles of melting at 94°C for 30 seconds, annealing at 54.8°C for 30 seconds, and extension at 72°C for 40 seconds. A final extension time of 10 minutes at 72°C was followed by holding at 4°C.

**Leishmania kinetoplast.** Primers were 13A and 13B [40]. Thermal cycler protocol as CO1, except annealing was 60°C.

**Leishmania ITS-1.** Primers were ITS-1 primers L5.8s and LITSRn, general to all *Leishmania* [45]. Thermal cycle protocol: initial melting 95°C for 4 minutes; then 36 cycles of melting at 95°C for 40 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute. A final extension time of 6 minutes at 72°C was followed by holding at 4°C.

**Wolbachia 16S.** Primers were WSPEC specific to *Wolbachia* 16S [16]. Thermal cycler protocols were as in CO1, including annealing temperature.

**Wolbachia MLST genes.** Primers are degenerate MLST primers [46]. Thermal cycler protocols were as in CO1 except annealing temperatures were 54°C for coxA, ftsZ, gatB, hcpA and 59°C for wsp and hbpA.

**DNA Sequencing**

Leftover primers and dNTPs were removed from PCR products via simultaneous incubation with exonuclease and shrimp alkaline phosphatase (ExoSAP, USB Corporation). For some PCR reactions, non-specific products were visible, so the desired product band was cut followed by Gelase (Epigenent Biotechnologies) digestion. Purified PCR products were then sequenced by BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems). Sequencing reactions were cleaned using Sephadex G-50 columns on Millipore Multiscreen 96-well filtration plates or via BigDye Terminator kit (Applied Biosystems). The clean sequencing reaction was run through an ABI 3130× sequencer.

**Phylogenetetic Tree Construction**

Sequences were examined using Sequencer 4.7 (Gene Codes Corporation, 2006). Ends were trimmed to clearly defined peaks. Forward and Reverse sequences for each sample were compared to correct dye blots and to ensure non-contamination. Sequences were aligned with Clustal W [47] online implementation [48], and trimmed to equal length before analysis in PAUP 4.0 [49], ModelTest [50], and MrBayes [51,52].

The phylogenetic relationships among *Lutzomyia* species were investigated by sequencing two fragments of the 18S gene (18SA 402bp, 18SB 482bp) from single *Lutzomyia* extracts. The two sequences were aligned separately using Clustal W2 [47,46]; then the concatenated sequence was analyzed using ModelTest for AIC and hLTR best-fit models of evolution [50]. The hLTR recommendation by ModelTest was a General Time Reversible model with invariant site (I) and gamma shape (G) parameter adjustments. The AIC recommendation was the Hasegawa-Kishino-Yano 1985 (HK85) model, with I and G adjustments. These models were implemented in the Maximum Likelihood analysis in PAUP for tree construction, and gave identical topologies. The ML tree was bootstrapped using the HK85 model. The concatenated sequences were also used for Bayesian Inference (BI) analysis with MrBayes [51,52]. The HK85 model was implemented in MrBayes so that BI posterior probabilities could be directly compared to bootstrap values in ML from PAUP. All trees were generated using *Brumptomyia galindoi* as an outgroup.

Highly variable loop regions in 18S where alignment was difficult or non-existent were excised after alignment. No attempt was made to code gaps as binary or multi-state characters.

CO1 barcode sequence was obtained from 49 individuals identified by morphology in 18 species. More than one specimen from each species was sequenced when available (Table 1). We aligned sequences manually as no insertions or deletions were present in the protein-coding fragment. Sequence reliability was determined by comparing the forward and reverse reads, as well as in silico translation using the invertebrate mitochondria genetic code to ensure nonsense codons were not present in the fragment. Aligned sequences were exported to PAUP 4.0 [49] where a neighbor-joining tree was generated using a Kimura 2-Parameter correction for DNA differences.

**Results**

**Phlebotomine Collection**

More than 437 sand fly individuals collected from seven shoreline and five interior forest sites on Barro Colorado Island, Panama contained 17 *Lutzomyia* and 2 *Brumptomyia* species determined by morphology (Table 1). Abundance of *Lu. panamensis*, the numerically dominant species was not accurately determined due to its extremely high numbers (thousands) in the combined sample. Less abundant species were counted precisely and ranged from one to a total of 63 individuals per species. Five man-biting species (*Lu. panamensis*, *Lu. tropidio*, *Lu. olmeca*, *Lu. gomezi* and *Lu. carrerai thula*) accounted for more than 61% of individuals collected on BCI. The relative abundances of the species collected in this study were significantly correlated to those found in a previous intensive study of sand fly community composition [33] at the “Río Limbo” site on the mainland, 7 km NW of Gamboa and 10 km east from BCI.
with peaks in activity at dawn and dusk, while different levels within the forest. Five species are nocturnal feeders
samples, three principally are canopy species but all can occur at three others in the dry season. Of the six man-biting species in our
peak densities in the early wet season, three in the late wet and the present study (including three man-biting species) reach their
''Rio Limbo'' study [54,55], suggest 11 of the species collected in man-biting species, Lu. panamensis
CO1 Barcoding Gene and Sand Fly Species
(rs = 0.64, n = 18, p<0.01) (Fig. 1). That 16-month study collected over 30,000 Lutzomyia individuals in 35 species, with Lu. triannulata and Lu. pananensis comprising the two most abundant species coming to understory light traps during the May to August period. A seventh man-biting species, Lu. yephiletrix (Fairchild and Hertig), collected in the Rio Limbo study was not present in BCI samples.

The seasonal trends in adult population density recorded by the “Rio Limbo” study [54,55], suggest 11 of the species collected in the present study (including three man-biting species) reach their peak densities in the early wet season, three in the late wet and three others in the dry season. Of the six man-biting species in our samples, three principally are canopy species but all can occur at different levels within the forest. Five species are nocturnal feeders with peaks in activity at dawn and dusk, while Lu. carrerai thula actively feeds at all hours.

CO1 Barcoding Gene and Sand Fly Species
Neighbor-joining analysis of CO1 sequence extracted from 49 sand fly specimens separated the genera, Brumptomyia and Lutzomyia, and unambiguously assigned 43 individuals to 13 morphological species (Fig. 2). The status of the three species represented by single specimens remained unresolved within the Lutzomyia clade but occurred separate from the 13 other morphological species. Single specimens within two morphological species groups diverged sufficiently from conspecifics to possibly indicate species differences. The separation of specimen 394 within Lu. vespertilionis, received 100% bootstrap support as did specimen 54 within Lu. gomezi. Thus, the presence of two cryptic or misdiagnosed species within our group of 16 morphologically determined Lutzomyia species is suggested.

Phylogenetic Analysis using Nuclear Sequence
Both Maximum Likelihood (ML) and Bayesian Inference (BI) trees apportioned the 16 Lutzomyia species identically among two apparently deep clades within the genus Lutzomyia (Fig. 3). Both trees recovered the three species within the Psychodopygus group as a monophyletic group within Clade “A”. Further, the two species within the Aragaoi group were recovered as a separate subgroup within Clade “A”. Within clade “B” only the Pressatia subgenus was recovered with high support in both trees. The two Leishmania infected species, Lu. trapidoi and Lu. gomezi, occur isolated within separate, well-supported clades in both BI and ML trees. Similarly, the two Wolbachia infected species, Lu. trapidoi and Lu. vespertilionis, occur in separate, well-supported clades.

Leishmania Infections
Only Lu. trapidoi and Lu. gomezi of 20 species tested gave replicable amplification of the k inetoplast gene fragment. The prevalence of Leishmania ranged from 0% in 18 species to 26.3% in Lu. gomezi and 43.3% in Lu. trapidoi (Table 1). We collected one or both of these species at six of our 12 collecting sites on BCI, including traps deep within the forest and near the edge of the island.

When the amplified products from the ITS-1 region were sequenced and BLASTed against the nucleotide collection in

| Species                     | Subgenus (Group) - Series          | Individuals Collected | % (N) Individuals Infected | Genbank Accession Numbers                   |
|-----------------------------|------------------------------------|-----------------------|----------------------------|---------------------------------------------|
| Lutzomyia carpenteri        | (Fairchild and Hertig)             | 22                    | 0 (20) 0 (20)              | GU001729-31 FJ977587 GU048921              |
| Lutzomyia rufipes           | (Fairchild and Hertig)             | 1                     | 0 (1) 0 (1)                | GU001754 FJ977598 GU048922                |
| Lutzomyia aculeifera        | (Fairchild and Hertig)             | 33                    | 0 (20) 0 (20)              | GU001724, 25 FJ977588 GU048918             |
| Lutzomyia triannulata       | (Fairchild and Hertig)             | 32                    | 0 (22) 0 (20)              | GU001766, 67 FJ977589 GU048911             |
| Lutzomyia trinidadensis     | (Neustead)                         | 4                     | 0 (2) 0 (4)                | GU001765 FJ977593 GU048926                |
| Lutzomyia abonnenci         | (Floh and Chassignet)              | 1                     | 0 (1) 0 (1)                | — — —                                     |
| Lutzomyia ovallesi          | (Ortiz)                            | 13                    | 0 (13) 0 (13)              | GU001744-46 FJ977594 GU048916             |
| Lutzomyia vespertilionis    | (Fairchild and Hertig)             | 15                    | 0 (15) 73.3 (15)           | GU001768, 69, 71,72 FJ977590 GU048910     |
| Lutzomyia ssp. nr vespertilionis |                           | 1                     | 0 (1) 100 (1)              | GU001770 FJ977591 GU048909                |
| Lutzomyia sanguinaria       | (Fairchild and Hertig)             | 3                     | 0 (3) 0 (3)                | GU001755-57 FJ977592 GU048923             |
| Lutzomyia gomezi            | (Nitzulescu)                       | 24                    | 26.3 (19) 0 (20)           | GU001737-39 FJ977599 GU048914             |
| Lutzomyia olmeca            | (Vargas and Diaz-Najera)           | 15                    | 0 (15) 0 (15)              | GU001741-43 FJ977585 GU048919             |
| Lutzomyia trapidoi          | (Fairchild and Hertig)             | 30                    | 43.3 (30) 53.3 (30)        | GU001758-64 FJ977595 GU048920             |
| Lutzomyia camposi           | (Rodriguez)                        | 18                    | 0 (18) 0 (18)              | GU001726-28 FJ977582 GU048912             |
| Lutzomyia dysponeta         | (Fairchild and Hertig)             | 20                    | 0 (18) 0 (19)              | GU001732, 33 FJ977583 GU048913             |
| Lutzomyia carrerai thula    | (Young)                            | 63                    | 0 (18) 0 (16)              | GU001751-53 FJ977584 GU048924             |
| Lutzomyia guyanensis        | (= gencilatula) (Floh and Abonnenc) | 5                     | 0 (5) 0 (5)                | GU001736 FJ977586 GU048925                |
| Lutzomyia pananensis        | (Shannon)                          | 133+                  | 0 (29) 0 (29)              | GU001747-50 FJ977596 GU048917             |
| Brumptomyia galindo         | (Fairchild and Hertig)             | 3                     | 0 (3) 0 (3)                | GU001734, 35 FJ977581 GU048908            |
| Brumptomyia hamata          | (Fairchild and Hertig)             | 1                     | 0 (1) 0 (1)                | GU001740 — — —                            |

The sand fly species collected (man-biting species in bold), their higher classification, the numbers of field-collected individuals, the percent and number which tested positive for Leishmania and Wolbachia using PCR and the Genbank accession numbers for the COI bar-code gene and two fragments of the nuclear-ribosomal 18S gene.

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GenBank, the closest match between *Leishmania* found in *Lu. trapidoi* and *Lu. gomezi* was found with *Le. naiffi* (100% coverage, 94% identity; GenBank Accession Number 76577722). The next closest match was *Le. lainsoni* (73% coverage, 96% identity). As a control, we sequenced a serum sample from blood infected with *Le. panamensis*. The amplification band and sequence were different from that obtained from infected BCI *Lutzomyia* spp. and corresponded (100% coverage, 99% identity) to *Le. panamensis* (GenBank Accession Number 2764481).

*Wolbachia* Infections. Single *Lutzomyia* and *Brumptomyia* extracts were used as templates for a PCR reaction using the WSPEC *Wolbachia* specific primer [16]. Gel electrophoresis showed two species, *Lu. trapidoi* and *Lu. vespertilionis*, were infected. The prevalence of *Wolbachia* ranged from 0% in 17 species to 53.3% in *Lu. trapidoi*, and 68.9% in *Lu. vespertilionis*. The species, *Lu. sp. nr vespertilionis*, also tested positive for Wolbachia (Table 1).

*Wolbachia* multi-locus sequence typing (MLST) is preferred over the surface-protein (WSP) for characterizing Wolbachia strains [46]. We amplified fragments of five MLST genes and the WSP gene [56] for four individuals from each of the sand fly species groups testing positive for *Wolbachia*. We found that *Lu. trapidoi* individuals were uniformly infected with a single *Wolbachia* strain. Within the *Vespertilionis* species group three specimens were infected by a single *Wolbachia* strain, while the fourth specimen with the divergent CO1 sequence (no. 394, *Lu. sp. nr vespertilionis*) carried a second *Wolbachia* strain (Table 2).

From the 20 species tested for both *Wolbachia* and *Leishmania* infections, two species (*Lu. vespertilionis* and *Lu. sp. nr vespertilionis*) were solely infected by *Wolbachia*, one species (*Lu. gomezi*) was solely infected by *Leishmania*, and one species (*Lu. trapidoi*) was infected by both *Wolbachia* and *Leishmania*. Among the 30 *Lu. trapidoi* individuals tested for both types of infection, 13 individuals (43.3%) were positive for *Leishmania* and 16 individuals (53.3%) were positive for *Wolbachia*. The seven double infected individuals were no more common than would be expected by chance (Fisher’s exact test, p = 0.99, n = 30).

**Discussion**

Neighbor-joining analysis clustered the CO1 sequences obtained from 46 individuals into 13 taxa previously determined by spermalethal morphology. However, one specimen was discovered within each of two species groups (the subgenus and series, *Lutzomyia-crucata* and the group, *Vespertilionis*) containing sequence sufficiently divergent from conspecifics to identify them as likely cryptic or misdiagnosed species. Sequencing larger numbers of individuals might increase the numbers of individuals with divergent mitochondrial haplotypes. The utility of CO1 sequencing as a tool for detecting species level genetic variation within morphologically determined species of New World Phlebotominae seems substantial. However, while analyses of CO1 sequence give high levels of support for species groups, more basal nodes were unsupported regardless of method of tree construction. Homoplasy, mitochondrial sweeps caused by *Wolbachia* [57] or genetic drift likely limit the usefulness of CO1 for phylogeny reconstruction in *Lutzomyia*, a conclusion reached in other studies [31].

Individual no. 394 in the *Vespertilionis* group with aberrant CO1 was sequenced only after we discovered it contained a different *Wolbachia* strain MLST (see below). The females of both *Lu. vespertilionis* and *Lu. isovespertilionis* (Fairchild & Hertig), are practically indistinguishable. Further they are the only two species in this group known to occur in Panama. Only subtle difference in the male paramere allow the two species to be determined [39]. Alternatively, individual no. 394 may not be *Lu. isovespertilionis* but
an undescribed, cryptic species. This ambiguity will likely remain until additional collecting and sequencing of the *Lu. vespertilionis* species group is carried out. Below we provisionally refer to this individual as *Lu. sp. nr vespertilionis*.

Our attempt to recover phylogenetic relationships among Panamanian sand flies is based on two fragments of the nuclear ribosomal 18S gene (Fig. 3). While the information within this relatively short (884 bp) segment limits the support for certain nodes within the tree, the separation of all taxa into either the A or B clades is well supported by both Bayesian Inference and Maximum Likelihood analysis. There is bootstrap support at three nodes within the tree representing *Pressatia* (Mangabeira) and *Psychodopygus* subgenera and the *Aragoi* group (Theodor).

*L. p. infection was confirmed by PCR in two of the 18 *Lutzomyia* species collected on BCI. Both are man-biting species with high rates of infection, 43.3% and 26.3%, in *Lu. trapidoi* and *Lu. gomezi*, respectively. *Leishmania* has been discovered previously in both of these species in Panama as well as in *Lu. ylephiletrix* and *Lu. panamensis* [58]. We found none of 29 individuals of the numerically dominant species, *Lu. panamensis*, infected in our study. However, the rate of infection in *Lu. panamensis* reported by Christensen et al. [58] was extremely low (1.2%; 4 of 306) and accordingly, we may easily have missed infected individuals by chance.

Methods for detection, diagnosis and species identification of *Leishmania* parasites currently in use are microscopy of clinical samples, PCR, and RFLP, each with a different degree of sensitivity, rate of false negatives and positives, and species-specificity. We successfully detected *Leishmania* from single sand fly DNA extracts with a single PCR step using the general kinetoplast primers and general ITS-1 primers. Previous work has also shown this is possible with semi-nested PCR [37] and with genus-specific primers [59]. Other work has shown that PCR amplification is as reliable as visual identification when applied to lab-infected strains [60]. Direct comparisons of the reliability of our approach are not possible as we did not try to detect the parasites any other way, such as by visual inspection of collected *Lutzomyia* flies. However, it is noteworthy that two primer sets developed under different circumstances for different purposes yielded congruent results.

The detection of *Leishmania (Viannia) naiffi* in *Lu. trapidoi* and *Lu. gomezi* is, to our knowledge, the first record of this species in Panama. Both sand fly species are known carriers of *Leishmania panamensis*, with *Lu. trapidoi* considered the main vector of CL in Panama (Panamerican Health Organization Bulletin 44, 1997). *Leishmania naiffi* is known to occur widely in South America and the Caribbean, including Brazil, Ecuador, Peru, French Guiana, and Martinique where it causes CL in humans [61]. The presence of *Le. naiffi* has been confirmed in tissue samples of humans in the Amazon region (Panamerican Health Organization Bulletin 44, 1997). The main reservoir for the parasite is thought to be the armadillo *Dasypus novemcinctus* [62], a mammal distributed widely across the Americas including Barro Colorado Island and mainland Panama.

There are several hypotheses that would account for the presence and late discovery of *Le. naiffi* in Panama: 1) *Leishmania*

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**Table 2.** Multilocus strain-types (MLST) of *Wolbachia* extracted from infected *Lutzomyia* species.

| Species              | Individual ID | WSP | fbpA | coxA | ftsZ | gatB | hcpA |
|----------------------|---------------|-----|------|------|------|------|------|
| *Lu. trapidoi*       | 35            | 5   | 26   | 2    | 21   | 23   | hcpA-a |
|                      | 208           | 5   | 26   | 2    | 21   | 23   | hcpA-a |
|                      | 218           | 5   | 26   | 2    | 21   | 23   | hcpA-a |
|                      | 220           | 5   | 26   | 2    | 21   | 23   | hcpA-a |
| *Lu. vespertilionis* | 183           | wsp-a | fbpA-a | 66 | ftsZ-a | gatB-a | hcpA-b |
|                      | 389           | wsp-a | fbpA-a | 66 | ftsZ-a | gatB-a | hcpA-b |
| *Lu. sp. nr vespertilionis* | 394 | wsp-b | fbpA-a | 66 | ftsZ-a | gatB-a | hcpA-b |

*Numerical responses within cells identify alleles occurring in the general Wolbachia MLST data base [56] whose sequences are identical to those recovered in this study. Non-numeric entries indicate allele sequences unique to this study.*

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naiffi occurs naturally in Panama but early surveys of Leishmania overlooked or misidentified it because of limitations in methodology. 2) the primarily South American range of Le. naiffi has expanded northward to include the Isthmus in the past 40 years, perhaps because of climate change or deforestation (Barro Colorado Island has a clear record of increasing night time temperature, a factor that certainly could affect the nocturnal and circumpupcular activity of sand flies.), 3) Leishmania naiffi was introduced to the Caribbean coast and canal area by workers coming to Panama from the West Indies to construct the Panama Railroad and/or the Panama Canal during the mid to late 1800’s and the first two decades of the 1900’s, or 4) Le. naiffi was accidentally introduced to BCI during the late 1960’s by the import of exotic primate species collected by ethologists in the upper Amazon drainage. Further molecular analysis directed toward Leishmania vectoring sand fly species (especially L. trapidoi and L. gomezi) collected on the adjacent mainland and sites in the Darien Province should provide an easy test of these hypotheses. Indeed, much stands to be gained by a geographic survey of sand flies across the entire Isthmus using molecular methods of detection and sequence comparison.

Wolbachia was initially detected in 2 of 17 sand fly species surveyed (11.7%). This is lower than reported by a previous study in the Paleotropics (27%) [9] and estimates for arthropod species globally (16–20%) [29]. MLST analysis reveals that individuals in the Vespertilionis group are separately infected by two different strains of Wolbachia, with no indication of simultaneous infection. Three Lu. vespertilionis specimens carried one Wolbachia strain, while a divergent strain was carried by a fourth specimen. We confirmed the specimen with the divergent strain was genetically distinct from other Lu. vespertilionis flies (Fig. 1) by comparing CO1 sequences from the four specimens. Effectively, this results in 3 out of 18 (16.6%) Lutzomyia species positive for Wolbachia. We speculate that Wolbachia may be part of a genetic barrier that separates the very similar species, Lu. vespertilionis and Lu. sp. nr vespertilionis, but more research is required to confirm the species identities and the occurrence of intra-specific Wolbachia incompatibilities. The extent to which Wolbachia induced cytoplasmic incompatibility contributes to species divergence in sand flies [63] deserves additional study.

MLST shows that the Wolbachia strains carried by Lu. vespertilionis and Lu. sp. nr vespertilionis are highly divergent and hence may represent independent acquisitions by horizontal transfer. It is important to note that the Wolbachia strain infecting Lu. sp. nr vespertilionis shares three of the six alleles found in the Lu. trapidoi Wolbachia strain, while sharing no alleles with the Wolbachia strain in Lu. vespertilionis. This may indicate that the agent responsible for stochastic horizontal transmission of Wolbachia occurs in or interacts with both Lu. trapidoi and the cryptic species. The considerable phylogenetic distance between the species in the Vespertilionis group and Lu. trapidoi present in the 18S tree (Fig. 3) suggests that the Wolbachia strains carried by these two species are unlikely to have descended from a single transfer event.

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The moderate Wolbachia infection rate we recorded within sand fly species (Table 1) is a feature consistent with other Wolbachia research. This was also seen in studies of Phlebotomus papatasi, and was due to low vertical maternal transmission [29]. If low maternal vertical transmission also occurs in New World phlebotomines, then Wolbachia will probably not be useful as a mechanism for driving transgenes into sand fly populations, as has been studied with mosquitoes [64]. If Wolbachia were to be used in a manner analogous to sterile males, low transmission may actually increase the effectiveness due to the population staying polymorphic with Wolbachia infection [29]. Wolbachia is possibly already limiting Lu. trapidoi numbers naturally. It is also important to note that while Wolbachia produces CI in Old World sand flies, its phenotype in New World Lutzomyia species remains unknown.

Our goal after testing species boundaries within Lutzomyia using the CO1 barcoding gene, was to examine phylogenetic relationships of the sand fly species in our samples. We found that the two 18S fragments we sequenced contain useful information and amplified with minor optimization. However, greater resolution ultimately will be needed, perhaps by combining sequence from 18S and 28S [31]. Nevertheless, the phylogeny we obtained based solely on 18S clearly indicates the two sand fly vectors in this study and a third species known to vector Le. panamensis (Lutzomyia panamensis) is not closely related, indicating their vectoring capacities may have evolved independently. Although no clinical data is available indicating Le. naiffi infects humans in Panama, residents of BCI and surrounding mainland areas have developed Leishmania symptoms and received antimonal treatment. The occurrence of Wolbachia in one of the principal vectors of leishmaniasis may provide an extra tool for future control of this disease on the Isthmus and perhaps elsewhere in the Neotropics.

Supporting Information

Alternative Language Abstract S1 Spanish translation of the abstract by JA. Found at: doi:10.1371/journal.pntd.0000627.s001 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: DDIC AV. Performed the experiments: JA DDIC. Analyzed the data: JA DW. Contributed reagents/materials/analysis tools: DW. Wrote the paper: JA DW.
