



Article

Detection of *Leishmania* and *Trypanosoma* DNA in Field-Caught Sand Flies from Endemic and Non-Endemic Areas of Leishmaniasis in Southern Thailand

Pimpilad Srisuton ¹, Atchara Phumee ^{2,3} , Sakone Sunantaraporn ⁴, Rungfar Boonserm ², Sriwatapron Sor-suwan ², Narisa Brownell ², Theerakamol Pengsakul ⁵ and Padet Siriyasatien ^{2,*} 

¹ Medical Parasitology Program, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

² Vector Biology and Vector Borne Disease Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

³ Thai Red Cross Emerging Infectious Diseases-Health Science Centre, World Health Organization Collaborating Centre for Research and Training on Viral Zoonoses, Chulalongkorn Hospital, Bangkok 10330, Thailand

⁴ Medical Science Program, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

⁵ Faculty of Medical Technology, Prince of Songkla University, Songkhla 90110, Thailand

* Correspondence: padet.s@chula.ac.th; Tel.: +66-2256-4387

Received: 8 June 2019; Accepted: 31 July 2019; Published: 2 August 2019



Abstract: Phlebotomine sand flies are tiny, hairy, blood-sucking nematoceran insects that feed on a wide range of hosts. They are known as a principal vector of parasites, responsible for human and animal leishmaniasis worldwide. In Thailand, human autochthonous leishmaniasis and trypanosomiasis have been reported. However, information on the vectors for *Leishmania* and *Trypanosoma* in the country is still limited. Therefore, this study aims to detect *Leishmania* and *Trypanosoma* DNA in field-caught sand flies from endemic areas (Songkhla and Phatthalung Provinces) and non-endemic area (Chumphon Province) of leishmaniasis. A total of 439 sand flies (220 females and 219 males) were collected. Head and genitalia dissection of female sandflies were done for morphology identification, and the remaining parts of those sand flies were then used for the detection of *Leishmania* and *Trypanosoma* parasites. The DNA was extracted from individual female sand flies. Polymerase chain reaction (PCR) anneal, specific to the *ITS1* and *SSU rRNA* gene regions, was used to detect *Leishmania* and *Trypanosoma* DNA, respectively. The positive PCR products were cloned and sequenced. The results showed that the female sand fly species in this study consisted of *Sergentomyia khawi* (35.9%); *Se. anodontis* (23.6%); *Phlebotomus betisi* (18.6%); *Ph. kiangsuensis* (9.5%); *Ph. asperulus* (6.4%); *Se. barraudi* (2.3%); 0.9% of each *Se. indica*, *Ph. stantoni*, and *Ph. major major*; and 0.5% of each *Se. sylvatica* and *Ph. mascomai*. The PCR and sequence analysis were able to detect *Leishmania* and *Trypanosoma* DNA in sand fly samples, which were identified as *L. martiniquensis*, 1/220 (0.45%) in *Se. khawi*, 3/220 (1.36%) of *T. noyesi* in *Se. anodontis*, and *Ph. asperulus*. Fourteen (6.36%) of the unidentified trypanosome species in *Se. khawi*, *Se. indica*, *Se. anodontis*, *Ph. asperulus*, and *Ph. betisi* were found in all of the areas of this study. Interestingly, we found a 1/220 (0.45%) co-infection sample of *L. martiniquensis* and *Trypanosoma* in *Se. khawi* from Songkhla Province. These data indicate that several species of sand flies might be potential vectors of *Leishmania* and *Trypanosoma* parasites in southern Thailand. However, more extensive study for potential vectors using a larger number of sand flies should be conducted to prove whether these sand flies can be natural vectors of leishmaniasis and trypanosomiasis in both humans and animals. In addition, our study could be useful for the future study of infection prevention, including effective vector control for leishmaniasis and trypanosomiasis in Thailand.

Keywords: *Leishmania* spp.; *Trypanosoma* sp.; sand flies; vector; Thailand

1. Introduction

Phlebotomine sand flies are small dipteran, hairy, and blood-sucking insects belonging to the order Diptera and family Psychodidae [1], which are known as important medical vectors of leishmaniasis worldwide. Leishmaniasis is a neglected tropical disease caused by the flagellate protozoa of the genus *Leishmania* [2]. Moreover, Phlebotomine sand flies have been reported to be the potential vector of the *Trypanosoma* species, which can cause trypanosomiasis in mammals, birds, fish, rats, sheep, cows, rabbits, lizards, frogs, and snakes [3–7]. Both parasite genera have been reported in many areas of the world. In Thailand, *Leishmania orientalis* [8], *L. martiniquensis* [9], and *L. donovani* complex [10,11] have been identified as the causative agents of indigenous visceral and cutaneous leishmaniasis. There have been approximately 20 autochthonous leishmaniasis reported in the country from 1996 to the present [12]. The disease is endemic mostly in southern Thailand. Trypanosomiasis is commonly found in animals, for example, *T. lewisi* and *T. evansi*. These animals are the major pathogenic trypanosome of domestic animals, such as rodents, cattle, wild animals, elephants, and tigers [13]. Furthermore, *T. lewisi* [14] and *T. lewisi*-like infection [15] have been reported in an infant in Thailand. It has been almost 20 years since the first case of autochthonous leishmaniasis was reported in Thailand [10], but data of the sand fly vector for the infectious diseases are still limited. In Thailand, several reports of sand flies in the western, central, northern, southern, and northeastern regions have identified four genera of sand flies, as follows: *Sergentomyia*, *Phlebotomus*, *Idiophlebotomus*, and *Chinius* [16–20]. The genus *Sergentomyia* was mostly demonstrated in the Phangnga, Suratthani, and Nakonsitammarat Provinces of southern Thailand, which are considered to be the affected areas of leishmaniasis. Sand fly species, such as *Sergentomyia gemmea* (81.4%), *Se. iyengari*, *Se. barraudi*, *Se. indica*, *Se. sylvatica*, and *Se. perturbans*, as well as another genus, *Phlebotomus* (*Ph. argentipes*), are found in the aforementioned affected areas in the country [21]. Moreover, *Se. (Neophlebotomus) gemmea* collected from an endemic area of leishmaniasis in Thailand were detected for *L. martiniquensis* DNA; therefore, *Se. gemmea* was claimed to be a potential vector of *L. martiniquensis* [22]. Recently, Phumee et al. (2016) reported an unknown *Trypanosoma* sp. DNA detected in a *Ph. stantoni* from Thailand [23]. However, the information on other sand fly species, which may transmit leishmaniasis and trypanosomiasis, is also limited. In this study, we focused on the detection of *Leishmania* and *Trypanosoma* parasites in sand flies collected from endemic and non-endemic areas of leishmaniasis in southern Thailand. The information from this study might help us to gain a better understanding about the prevalence of parasites in the sand fly population, and about the association among the sand fly species as a potential vector in endemic areas of leishmaniasis in Thailand.

2. Materials and Methods

2.1. Ethics Statement

The study was approved by the animal research ethics committee of Chulalongkorn University Animal Care and Use Protocol (CU-ACUP), Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA No. 019/2561).

2.2. Sand Fly Collection, Sample Preparation, and Identification

Sand flies were collected from endemic areas, around human settlements (Songkhla and Phatthalung Provinces), and non-endemic areas, in caves (Chumphon Provinces), of leishmaniasis in southern Thailand (Figure 1). The sand fly specimens were collected by Centers for Disease Control and Prevention (CDC) miniature light traps (25 W bulb) without CO₂, during September–October 2017. The 10 traps operated for over 12 h, from 18:00 to 06:00, for one night. The collecting bags were

kept at $-20\text{ }^{\circ}\text{C}$ for 30 min in order to anesthetize the arthropods and separate the sand flies from the other arthropods by morphological features using a stereomicroscope (Olympus, Tokyo, Japan). Each female sand fly was dissected under a stereomicroscope on a sterilized slide using sterile needles. The head and genitalia were mounted on slides with Hoyer's medium for morphological character identification in all female sand flies, under a light microscope (Olympus, Tokyo, Japan), following the taxonomic keys described by Lewis (1978) [24], Phumee et al. (2016) [23], and Depaquit et al. (2019) [25]. The abdomen and the thorax of each female specimen were transferred to sterile 1.5 mL Eppendorf tubes with a lysis buffer for the detection of *Leishmania* and *Trypanosoma* species by the polymerase chain reaction (PCR).

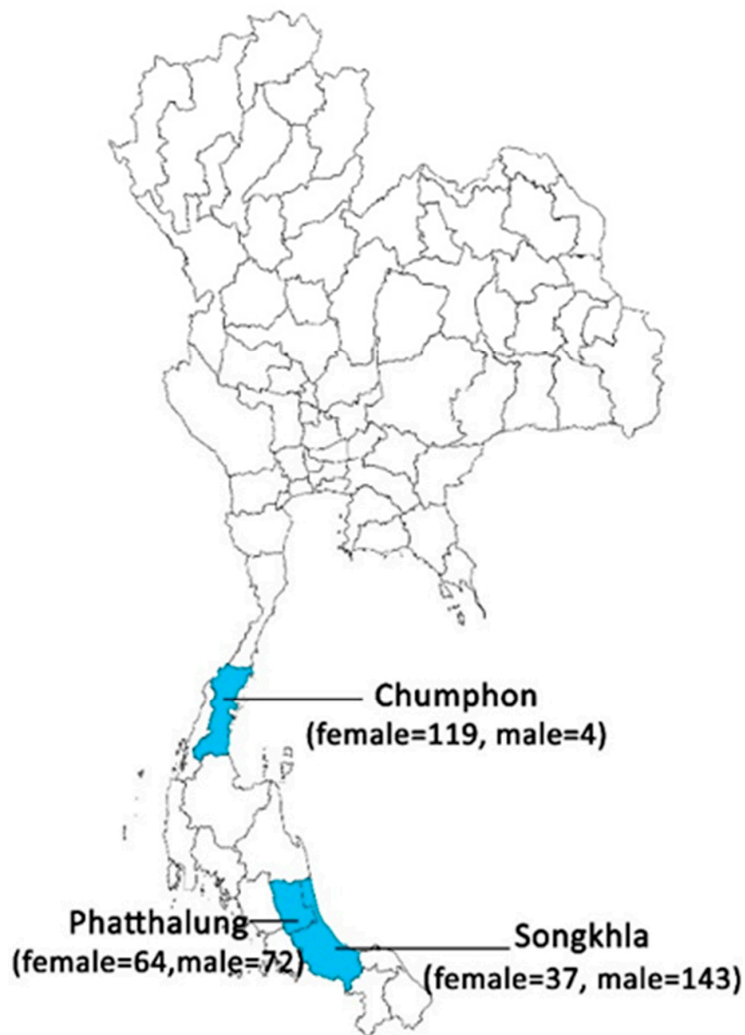


Figure 1. Map of Thailand showing locations of the sand fly sample-collection sites in the three provinces. Blue denotes the collection locations of sand flies.

2.3. DNA Extraction

DNA was extracted from individual sand fly samples using an Invisorb Spin Tissue Mini Kit (STRATEC Molecular, Berlin, Germany), following the manufacturer's instructions. The sand flies were lysed in a 200 μL lysis buffer containing 20 μL of proteinase K. At the final step, DNA was eluted in a 40 μL of elution buffer. The extracted DNA samples were kept at $-80\text{ }^{\circ}\text{C}$ for long-term storage.

2.4. Detection of *Leishmania* and *Trypanosoma* DNA in the Sand Fly Samples

The extracted DNA samples were used to detect the *Leishmania* DNA using polymerase chain reaction (PCR) amplification annealed specifically to the *ITS1* region. The reactions were performed using primers LeR: 5'-CCA-AGT-CAT-CCA-TCG-CGA-CAC-G-3' and LeF: 5'-TCC-GCC-CGA-AAG-TTC-ACC-GAT-A-3', under the conditions previously described by Spanakos et al. (2008) [26]. The reaction was carried out in a total volume of 25 μ L, containing 2.5 μ L of 10 \times PCR Buffer (NH₄SO₄), 1.25 μ L of 25 mM MgCl₂, 2.5 μ L of 2.5 mM of each dNTP, 10 mM of each primer, 1 unit of *Taq* DNA polymerase, and 50 ng of DNA template. After initial denaturation at 95 °C for 5 min, PCR was performed, with 40 cycles consisting of denaturation (95 °C for 1 min), annealing (50 °C for 1 min), extension (72 °C for 1 min), and a final extension at 72 °C for 7 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel for 40 min at 100 volts, stained with ethidium bromide, and visualized with Quantity One Quantification Analysis Software Version 4.5.2 (Gel DocEQ System; Bio-Rad, Hercules, CA, USA). For the identification of the *Trypanosoma* species, the small subunit ribosomal RNA (*SSU rRNA*) region was amplified from the sand fly using primers designed specifically for trypanosomatids. The sequences primers were TRY927F: 5'-GAA-ACA-AGA-AAC-ACG-GGA-G-3' and TRY927R: 5'-CTA-CTG-GGC-AGC-TTG-GA-3' [27]. The reaction and conditions followed the amplification of the *ITS1* region described previously.

2.5. Molecular Cloning and Nucleotide Sequencing

The amplified PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The ligated vectors were transformed into *E. coli* DH5 α competent cells. The recombinant plasmids were screened using the blue-white screening systems. Suspected chimeric colonies were cultured and subjected to plasmid DNA extraction by the Invisorb Spin Plasmid Mini Kit (STRATEC Molecular, Berlin, Germany), following the manufacturer's instructions. The purified plasmids were sequenced by the sequencing service of Macrogen Inc., Seoul, Korea, using a universal forward T7 primer. The nucleotide sequences were analyzed by comparison with the GenBank database using a BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences were aligned using BioEdit Sequence Alignment Editor Version 7.0.5.3 [28]. The phylogenetic tree was constructed using the maximum likelihood method with Kimura's two-parameter and bootstrap analysis with 1000 replications in MEGAX version 10.0.1 [29].

3. Results

The number of sand flies collected from all of the sites (Songkhla, Phatthalung, and Chumphon Provinces) using a CDC light trap was 439 (220 females and 219 males). For the morphological characteristics, the female sand flies (n = 220) were classified into two genera and 11 species, which were from the Songkhla (*Se. anodontis*, *Se. khawi*, *Se. barraudi*, and *Ph. stantoni*), Phatthalung (*Se. khawi*, *Se. barraudi*, *Se. indica*, *Ph. betisi*, and *Ph. kiangsuisensis*), and Chumphon (*Se. anodontis*, *Se. sylvatica*, *Ph. asperulus*, *Ph. betisi*, *Ph. kiangsuisensis*, *Ph. major major*, and *Ph. mascomai*) provinces. The most abundant species in our samples was *Se. khawi* (35.9%; Table 1). All of the samples of female sand flies were screened for *Leishmania* and *Trypanosoma* infection using *ITS1*-PCR and *SSU rRNA*-PCR, respectively.

In the endemic area of leishmaniasis, Songkhla province, one out of thirty-seven female *Se. khawi* DNA extracts was positive, by PCR, for *L. martiniquensis* DNA, using *ITS1*-PCR (Figure S1), and one sample showed co-infection between *L. martiniquensis* and *Trypanosoma* parasites (Table 2). An analysis of the *L. martiniquensis* amplified sequences showed 99% compatibility with *L. martiniquensis* (accession no. JQ001751; Figure S2). Moreover, PCR targeting the *SSU rRNA* region of the four positive samples showed 99% identity with *Trypanosoma* sp. (accession no. AB520638; Figure S3). The female sand flies collected from Phatthalung were 50 sand flies of *Se. khawi* and two sand flies of *Se. indica*. One positive

sample showed a 99% similarity with *Trypanosoma* sp. (accession no. AB520638) in each species, whereas *Leishmania* DNA was not detected in this area (Figure S4).

The female sand flies were collected from the non-endemic area of leishmaniasis, Chumphon Province. The results showed that 2/119 of *Se. anodontis* and 1/119 of *Ph. asperulus* were detected for *T. noyesi* DNA, with 98% identity (accession no. KX008320); furthermore, we found positive *Trypanosoma* sp. in four samples of *Se. anodontis* and two samples of each *Ph. asperulus* and *Ph. betisi*, which showed 96%–99% similarity with *Trypanosoma* sp. (accession no. AB520638). However, *Leishmania* DNA was not detected in the non-endemic area of leishmaniasis.

Table 1. Morphological identification of female sand flies from southern Thailand.

| Species | Provinces | | | Total |
|---------------------------|-----------|-------------|------------|------------|
| | Songkhla | Phatthalung | Chumphon | |
| <i>Se. anodontis</i> | 4 | 0 | 48 | 52 |
| <i>Se. khawi</i> | 29 | 50 | 0 | 79 |
| <i>Se. sylvatica</i> | 0 | 0 | 1 | 1 |
| <i>Se. barraudi</i> | 2 | 3 | 0 | 5 |
| <i>Se. indica</i> | 0 | 2 | 0 | 2 |
| <i>Ph. stantoni</i> | 2 | 0 | 0 | 2 |
| <i>Ph. asperulus</i> | 0 | 0 | 14 | 14 |
| <i>Ph. betisi</i> | 0 | 3 | 38 | 41 |
| <i>Ph. kiangsuenensis</i> | 0 | 6 | 15 | 21 |
| <i>Ph. major major</i> | 0 | 0 | 2 | 2 |
| <i>Ph. mascomai</i> | 0 | 0 | 1 | 1 |
| Total | 37 | 64 | 119 | 220 |

Table 2. Molecular detection of *Leishmania* DNA of *ITS1* and *Trypanosoma* DNA of *SSU rRNA* from sand fly samples collected in southern Thailand.

| Province | Positive Sand Fly Species | Detection of <i>Leishmania</i> spp. and <i>Trypanosoma</i> sp. | | |
|--------------------------------|---------------------------|--|------------------|------------------------|
| | | <i>L. martiniquensis</i> | <i>T. noyesi</i> | <i>Trypanosoma</i> sp. |
| Songkhla (n = 37) | <i>Se. khawi</i> | 2 * | ND | 4 |
| Phatthalung (n = 64) | <i>Se. khawi</i> | ND | ND | 1 |
| | <i>Se. indica</i> | ND | ND | 1 |
| Chumphon (n = 119) | <i>Ph. asperulus</i> | ND | 1 | 2 |
| | <i>Se. anodontis</i> | ND | 2 | 4 |
| | <i>Ph. betisi</i> | ND | ND | 2 |
| Total (n = 220) | | 1 | 3 | 14 |

* One specimen was co-infected by *L. martiniquensis* and *Trypanosoma* sp.; ND—not detected.

The phylogenetic tree of *L. martiniquensis* of the *ITS1* region was clearly shown in a clade of the *L. enriettii* complex (Figure 2). The phylogenetic tree of all of the positive *Trypanosoma* parasites with the *SSU rRNA* regions was divided into two groups. The first group of 15 samples contained *Trypanosoma* sp. in the Anura clade described in Pakistan. The second group of three samples was grouped together with *T. noyesi* with small branch lengths in the *T. cruzi* clade (Figure 3). The partial nucleotide sequence of the *ITS1* and *SSU rRNA* obtained in this study was deposited in GenBank under the following accession numbers: MK603807–MK603827.

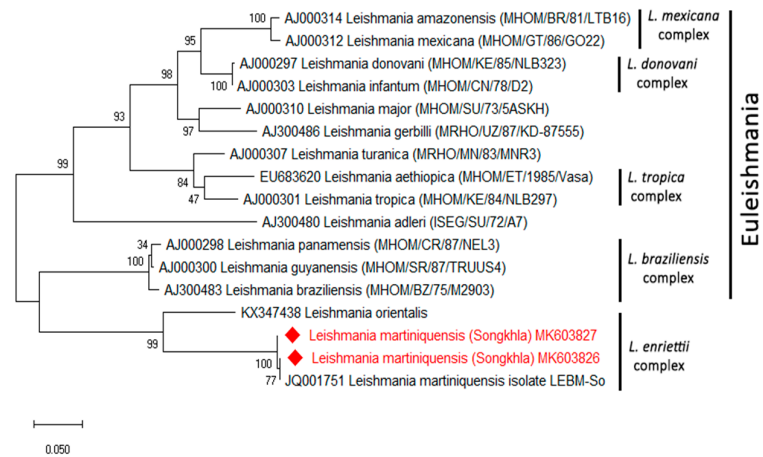


Figure 2. A maximum likelihood tree was constructed from the partial *ITS1* sequences of *Leishmania* spp. and was compared with the reference sequences using the Kimura two-parameter model with the maximum likelihood method, by testing with 1000 bootstrap values. The sequences from this study are indicated with a red color.

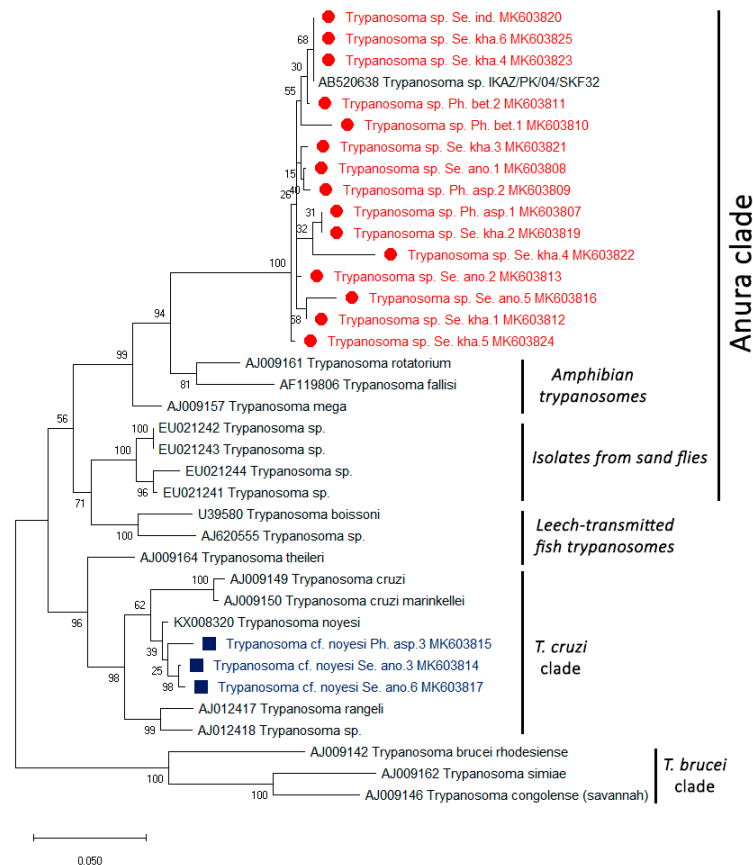


Figure 3. Phylogenetic tree of the *Trypanosoma* species in the sand flies constructed from the partial *SSU rRNA* sequences from all of the regions. The sequences from this study, indicated in red and blue, were compared with the reference sequences obtained from GenBank. The tree was derived using the maximum likelihood method based on the Kimura two-parameter model (bootstrapped 1000 times).

4. Discussion

Autochthonous leishmaniasis in Thailand is caused by two major *Leishmania* species, namely, *L. orientalis* and *L. martiniquensis* (Kinetoplastida: Trypanosomatidae) [8,9,12,30–40]. Studies on the vector of the disease are essential for controlling the disease and for gaining a better understanding

of the disease transmission dynamics; however, there are limited data on this disease in Thailand. Ready (2013) has demonstrated that female sand flies are able to transmit several parasites, including *Leishmania* spp., *Trypanosoma* spp., and other viruses [41]. In the current study, we described a method for the detection of *Leishmania* and *Trypanosoma* protozoans by PCR using primers specific for *Leishmania* and *Trypanosoma* DNA in a natural population of sand flies collected from endemic and non-endemic areas of leishmaniasis in southern Thailand. We showed that the only *L. martiniquensis*-positive sand flies were *Se. khawi*, by analysing the *ITS1* regions. Moreover, one sample showed co-infection between *L. martiniquensis* and *Trypanosoma* sp. DNA in *Se. khawi* (*Trypanosoma* sp. *Se. kha*) from the Songkhla Province, an endemic area of leishmaniasis. However, we did not conduct a dissection to demonstrate live parasites in the midgut and did not observe any blood meal in the female sand fly samples; however, we also detected parasite DNA in the samples. These results could infer that there may be some live parasites in the midgut, even after the female sand flies have been defecated. In the next survey study, we will dissect the sand fly midguts for the live parasites. There have been reports of the potential role of *Sergentomyia* species as a vector of leishmaniasis [42]. *L. donovani* DNA has been detected in *Se. babu* from India [43]. *L. major* DNA has been detected in *Se. sintoni* from Iran [44], *Se. minuta* from Portugal [45], and *Se. clydei* [46] and *Se. minuta* [47] from Tunisia. In addition, *L. infantum* DNA has been detected in *Se. dubia*, *Se. magna*, and *Se. schwetzi* from Senegal [48]. In Thailand, *L. martiniquensis* DNA was detected in both *Se. gemmea* [12,22] and *Se. barraudi* [49]. Recently, Siripattanapipong et al. (2018) suggested that *L. orientalis* was found in *Se. iyengari* (Diptera: Psychodidae) from southern Thailand [50]. However, some reports have described that the species of *Se. gemmea* and *Se. iyengari* sand flies in Thailand may be misidentified based on morphology and molecular techniques [23,25]; moreover, all of the sequences of *Se. iyengari* deposited in GenBank (accession numbers MG770900, MG770901, MG770913, MG770914, MG770916–MG770918, MG770924, MG770926, and MG770927) and *Se. gemmea* deposited in GenBank (accession numbers MG770900, MG770901, MG770913, MG770914, MG770916–MG770918, MG770924, MG770926, and MG770927) are grouped as *Se. hivernus* (Raynal and Gaschen) and *Se. khawi* (Raynal) by using *CytB*-PCR [23,25]. In this study, the most abundant species in our samples was *Se. khawi*, which could be a potential vector of *L. martiniquensis* infection in both humans and animals in Thailand. However, in order to prove that *Se. khawi* is a competent vector of leishmaniasis, future studies are needed in order to reveal exact sand fly species in both the morphological and molecular characterization. Additionally, the isolation and cultivation of *Leishmania* parasites are needed to support the complete life cycle of leishmaniasis in Thailand.

For *Trypanosoma* DNA detection, we found 1.81% of *T. noyesi* in *Se. anodontis* (*Trypanosoma* cf. *noyesi* *Se. ano*) and *Ph. asperulus* (*Trypanosoma* cf. *noyesi* *Se. asp*) and 6.36% of *Trypanosoma* sp. *Se. kha*, *Se. indica* (*Trypanosoma* sp. *Se. ind*), *Se. anodontis* (*Trypanosoma* sp. *Se. ano*), *Ph. asperulus* (*Trypanosoma* sp. *Ph. asp*), and *Ph. betisi* (*Trypanosoma* sp. *Ph. bet*) from both endemic and non-endemic regions of leishmaniasis. Three positive samples were clustered together with *T. noyesi* (accession no. KX008320) with small branch lengths and in the *T. cruzi* clade. Botero et al. (2016) determined that their study provided novel information on the morphological, behavioral observations in vitro, and genetic variability of an Australian trypanosome of *T. noyesi* within the *T. cruzi* clade isolated from the critically endangered woylie (*Bettongia penicillata*) [51]. A previous report in Thailand was the first study on *Trypanosoma* sp. DNA detection in a *Ph. stantoni* collected from southern Thailand. It was suspected that the detected sequences belonged to a novel species of the genus *Trypanosoma* [23]. In this study, we found that 15 samples were grouped to *Trypanosoma* sp. from *Ph. Kazeruni*, and Theodor and Mesghali described this in Pakistan (accession no. AB520638), in an Anura clade [52]. In contrast, we have not found unknown *Trypanosoma* sp. in sand flies, as described by Phumee et al., (2016) in Thailand. This might be because of the low number of samples, and the vector for unknown *Trypanosoma* sp. may be other sand fly species. Many reports also described the detection of *Trypanosome* DNA in sand flies. For example, Nzelu et al. (2014) revealed that *L. tropica* and *L. major* DNA, and *Trypanosoma* DNA can be detected in *Sergentomyia* sand flies in Ghana [53]. However, the information of *Leishmania* and *Trypanosoma* parasites in sand flies associated with their hosts, geographic distribution, and human or

animal diseases are quite limited. Therefore, more extensive and systematic epidemiological surveys on potential vectors by using a larger number of sand flies should be conducted for proving whether these sand flies can be natural vectors of leishmaniasis and trypanosomiasis in both humans and animals. In addition, a better understanding of the epidemiological and ecological relationships of the disease could be used for studying the prevention, as well as effective control for both *Leishmania* and *Trypanosoma* vectors in Thailand.

5. Conclusions

The natural infection of sand flies by *Leishmania* or *Trypanosoma* alone and the co-infection of both parasites were detected in both the *Sergentomyia* and *Phlebotomus* genera of sand flies. The detection and identification of natural infections in sand flies by these parasites in endemic and non-endemic areas of Thailand are key factors in assessing the disease transmission risk and potential vectors, designing prevention and control measures, and predicting disease epidemics in leishmaniasis and trypanosomiasis in Thailand.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2075-4450/10/8/238/s1>. Figure S1: *ITS1*-PCR amplification for *Leishmania* parasites. Lane M: molecular mass marker (100 base pairs [bp]); lane P: *L. martiniquensis* culture, lane N: negative control, lanes 1–10 female sand fly samples. Figure S2: Sequence alignment of *Leishmania* parasites based on the partial *ITS1* gene. Figure S3: *SSU rRNA*-PCRs for *Trypanosoma* parasites. Lane M: molecular mass marker (100 base pairs [bp]); lane P: *Trypanosoma* sp. culture, lane N: negative control, lanes 1–10 female sand fly samples. Figure S4: Sequence alignment of *Trypanosoma* parasites base on the partial *SSU rRNA* gene

Author Contributions: Conceptualization, P.S. (Padet Siriyasatien); methodology, P.S. (Pimpilad Srisuton); software, P.S. (Pimpilad Srisuton), S.S. and A.P.; validation, P.S. (Pimpilad Srisuton), P.S. (Padet Siriyasatien), and A.P.; formal analysis, P.S. (Padet Siriyasatien); investigation, R.B., S.S.-s. and T.P.; resources, P.S. (Padet Siriyasatien); data curation, P.S. (Padet Siriyasatien); writing—original draft preparation, P.S. (Pimpilad Srisuton); writing—review and editing, P.S. (Padet Siriyasatien), A.P. and N.B.; visualization, P.S. (Padet Siriyasatien); supervision, P.S. (Padet Siriyasatien); project administration, P.S. (Pimpilad Srisuton); funding acquisition, P.S. (Padet Siriyasatien).

Funding: This study was supported by Rachadapisaek Sompote Fund (Grant No. RA 62/016), Faculty of Medicine, Chulalongkorn University and Thailand research fund (Grant No. RSA 5780024).

Acknowledgments: We would like to express my sincere thanks to the staff of the staff of Vector Biology and Vector Borne Disease Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Thailand.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Killick-Kendrick, R. The biology and control of phlebotomine sand flies. *Clin. Dermatol.* **1999**, *17*, 279–289. [[CrossRef](#)]
2. Akhoundi, M.; Kuhls, K.; Cannet, A.; Votýpka, J.; Marty, P.; Delaunay, P.; Sereno, D. A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. *PLoS Negl. Trop. Dis.* **2016**, *10*, e0004349. [[CrossRef](#)] [[PubMed](#)]
3. Cooper, C.; Clode, P.L.; Peacock, C.; Thompson, R.C.A. Chapter Two—Host–Parasite Relationships and Life Histories of Trypanosomes in Australia. In *Advances in Parasitology*; Rollinson, D., Stothard, J.R., Eds.; Academic Press: Cambridge, MA, USA, 2017; Volume 97, pp. 47–109.
4. Njagu, Z.; Mihok, S.; Kokwaro, E.; Verloo, D. Isolation of *Trypanosoma brucei* from the monitor lizard (*Varanus niloticus*) in an endemic focus of rhodesian sleeping sickness in Kenya. *Acta Trop.* **1999**, *72*, 137–148. [[CrossRef](#)]
5. Ferreira, R.C.; De Souza, A.A.; Freitas, R.A.; Campaner, M.; Takata, C.S.; Barrett, T.V.; Shaw, J.J.; Teixeira, M.M. A phylogenetic lineage of closely related trypanosomes (Trypanosomatidae, Kinetoplastida) of anurans and sand flies (Psychodidae, Diptera) sharing the same ecotopes in Brazilian Amazonia. *J. Eukaryot. Microbiol.* **2008**, *55*, 427–435. [[CrossRef](#)] [[PubMed](#)]
6. Lemos, M.; Morais, D.H.; Carvalho, V.T.; D’Agosto, M. First record of *Trypanosoma chattoni* in Brazil and occurrence of other *Trypanosoma* species in Brazilian frogs (*Anura*, Leptodactylidae). *J. Parasitol.* **2008**, *94*, 148–151. [[CrossRef](#)]

7. Viola, L.B.; Campaner, M.; Takata, C.S.; Ferreira, R.C.; Rodrigues, A.C.; Freitas, R.A.; Duarte, M.R.; Grego, K.F.; Barrett, T.V.; Camargo, E.P.; et al. Phylogeny of snake trypanosomes inferred by SSU rDNA sequences, their possible transmission by phlebotomines, and taxonomic appraisal by molecular, cross-infection and morphological analysis. *Parasitology* **2008**, *135*, 595–605. [[CrossRef](#)]
8. Jariyapan, N.; Daroontum, T.; Jaiwong, K.; Chanmol, W.; Intakhan, N.; Sor-Suwan, S.; Siriyasatien, P.; Somboon, P.; Bates, M.D.; Bates, P.A. *Leishmania (Mundinia) orientalis* n. sp. (Trypanosomatidae), a parasite from Thailand responsible for localised cutaneous leishmaniasis. *Parasites Vectors* **2018**, *11*, 351. [[CrossRef](#)]
9. Chiewchanvit, S.; Tovanabutra, N.; Jariyapan, N.; Bates, M.D.; Mahanupab, P.; Chuamanochan, M.; Tantiworawit, A.; Bates, P.A. Chronic generalized fibrotic skin lesions from disseminated leishmaniasis caused by *Leishmania martiniquensis* in two patients from northern Thailand infected with HIV. *Br. J. Dermatol.* **2015**, *173*, 663–670. [[CrossRef](#)]
10. Thisyakorn, U.; Jongwutiwes, S.; Vanichsetakul, P.; Lertsapcharoen, P. Visceral leishmaniasis: The first indigenous case report in Thailand. *Trans. R. Soc. Trop. Med. Hyg.* **1999**, *93*, 23–24. [[CrossRef](#)]
11. Maharom, P.; Siripattanapipong, S.; Mungthin, M.; Naaglor, T.; Sukkawe, R.; Pudkorn, R.; Wattana, W.; Wanachiwanawin, D.; Areechokchai, D.; Leelayoova, S. Visceral leishmaniasis caused by *Leishmania infantum* in Thailand. *Southeast Asian J. Trop. Med. Public Health* **2008**, *39*, 988–990.
12. Leelayoova, S.; Siripattanapipong, S.; Manomat, J.; Piyaraj, P.; Tan-Ariya, P.; Bualert, L.; Mungthin, M. Leishmaniasis in Thailand: A Review of Causative Agents and Situations. *Am. J. Trop. Med. Hyg.* **2017**, *96*, 534–542. [[CrossRef](#)]
13. Milocco, C.; Kamyngkird, K.; Desquesnes, M.; Jittapalapong, S.; Herbreteau, V.; Chaval, Y.; Douangboupha, B.; Morand, S. Molecular demonstration of *Trypanosoma evansi* and *Trypanosoma lewisi* DNA in wild rodents from Cambodia, Lao PDR and Thailand. *Transbound. Emerg. Dis.* **2013**, *60*, 17–26. [[CrossRef](#)]
14. Sim, S.T.; Wiwanitkit, V. Emerging human trypanosomiasis in Southeast Asia: A coming new threat? *J. Nat. Sci.* **2015**, *1*, e29.
15. Sarataphan, N.; Vongpakorn, M.; Nuansrichay, B.; Autarkool, N.; Keowkarnkah, T.; Rodtian, P.; Stich, R.W.; Jittapalapong, S. Diagnosis of a *Trypanosoma lewisi*-like (Herpetosoma) infection in a sick infant from Thailand. *J. Med. Microbiol.* **2007**, *56*, 1118–1121. [[CrossRef](#)]
16. Apiwathnasorn, C.; Sucharit, S.; Rongsriyam, Y.; Leemingsawat, S.; Kerdpibule, V.; Deesin, T.; Surathin, K.; Vutikes, S.; Punavuthi, N. A brief survey of phlebotomine sandflies in Thailand. *Southeast Asian J. Trop. Med. Public Health* **1989**, *20*, 429–432.
17. Apiwathnasorn, C.; Samung, Y.; Prummongkol, S.; Phayakaphon, A.; Panasopolkul, C. Cavernicolous species of phlebotomine sand flies from Kanchanaburi Province, with an updated species list for Thailand. *Southeast Asian J. Trop. Med. Public Health* **2011**, *42*, 1405–1409.
18. Artemiev, M.A. Classification of the subfamily Phlebotominae. *Parassitologia* **1991**, *33*, 69–77.
19. Marcondes, C.B. A proposal of generic and subgeneric abbreviations for Phlebotomine sandflies (Diptera: Psychodidae: Phlebotominae) of the World. *Entomol. News* **2007**, *118*, 351–356. [[CrossRef](#)]
20. Galati, E.A.B. *Classificação de Phlebotominae, in Flebotomíneos do Brasil*; Rangel, E.F., Lainson, R., Eds.; Fiocruz: Rio de Janeiro, Brazil, 2003; pp. 23–51.
21. Sukra, K.; Kanjanopas, K.; Amsakul, S.; Rittaton, V.; Mungthin, M.; Leelayoova, S. A survey of sandflies in the affected areas of leishmaniasis, southern Thailand. *Parasitol. Res.* **2013**, *112*, 297–302. [[CrossRef](#)]
22. Kanjanopas, K.; Siripattanapipong, S.; Ninsaeng, U.; Hitakarun, A.; Jitkaew, S.; Kaewtaphaya, P.; Tan-ariya, P.; Mungthin, M.; Charoenwong, C.; Leelayoova, S. *Sergentomyia (Neophlebotomus) gemmea*, a potential vector of *Leishmania siamensis* in southern Thailand. *BMC Infect. Dis.* **2013**, *13*, 333. [[CrossRef](#)]
23. Phumee, A.; Tawatsin, A.; Thavara, U.; Pongsakul, T.; Thammapalo, S.; Depaquit, J.; Gay, F.; Siriyasatien, P. Detection of an Unknown *Trypanosoma* DNA in a *Phlebotomus stantoni* (Diptera: Psychodidae) Collected From Southern Thailand and Records of New Sand Flies With Reinstatement of *Sergentomyia hivernus* Raynal & Gaschen, 1935 (Diptera: Psychodidae). *J. Med. Entomol.* **2017**, *54*, 429–434. [[CrossRef](#)]
24. Lewis, D.J. The phlebotomine sandflies (Diptera: Psychodidae) of the oriental region. *Bull. Br. Mus. (Nat. Hist.) B. Entomol.* **1978**, *37*, 217–343. [[CrossRef](#)]
25. Depaquit, J.; Vongphayloth, K.; Siriyasatien, P.; Polseela, R.; Phumee, A.; Loyer, M.; Vol, A.; Varlot, G.; Rahola, N.; Brey, P.T.; et al. On the true identity of *Sergentomyia gemmea* and description of a closely related species: *Se. raynali* n. sp. *Med. Vet. Entomol.* **2019**. [[CrossRef](#)]

26. Spanakos, G.; Piperaki, E.T.; Menounos, P.G.; Tegos, N.; Fliemetakis, A.; Vakalis, N.C. Detection and species identification of old world *Leishmania* in clinical samples using a PCR-based method. *Trans. R. Soc. Trop. Med. Hyg.* **2008**, *102*, 46–53. [[CrossRef](#)]
27. Noyes, H.A.; Stevens, J.R.; Teixeira, M.; Phelan, J.; Holz, P. A nested PCR for the *ssrRNA* gene detects *Trypanosoma binneyi* in the platypus and *Trypanosoma* sp. in wombats and kangaroos in Australia. *Int. J. Parasitol.* **1999**, *29*, 331–339. [[CrossRef](#)]
28. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* **1999**, *41*, 95–98.
29. Kumar, S.; Stecher, G.; Li, M.; Niyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [[CrossRef](#)]
30. Bualert, L.; Charungkiattikul, W.; Thongsuksai, P.; Mungthin, M.; Siripattanapipong, S.; Khositnithikul, R.; Naaglor, T.; Ravel, C.; El Baidouri, F.; Leelayoova, S. Case Report: Autochthonous disseminated dermal and visceral leishmaniasis in an AIDS patient, southern Thailand, caused by *Leishmania siamensis*. *Am. J. Trop. Med. Hyg.* **2012**, *86*, 821–824. [[CrossRef](#)]
31. Chusri, S.; Hortiwakul, T.; Silpapojakul, K.; Siriyasatien, P. Case Report: Consecutive cutaneous and visceral leishmaniasis manifestations involving a novel *Leishmania* species in two HIV patients in Thailand. *Am. J. Trop. Med. Hyg.* **2012**, *87*, 76–80. [[CrossRef](#)]
32. Jungudomjaroen, S.; Phumee, A.; Chusri, S.; Kraivichian, K.; Jariyapan, N.; Payungporn, S.; Siriyasatien, P. Detection of *Leishmania martiniquensis* DNA in various clinical samples by quantitative PCR. *Trop. Biomed.* **2015**, *32*, 736–744.
33. Noppakun, N.; Kraivichian, K.; Siriyasatien, P. Disseminated dermal leishmaniasis caused by *Leishmania siamensis* in a systemic steroid therapy patient. *Am. J. Trop. Med. Hyg.* **2014**, *91*, 869–870. [[CrossRef](#)]
34. Osatakul, S.; Mungthin, M.; Siripattanapipong, S.; Hitakarun, A.; Kositnitikul, R.; Naaglor, T.; Leelayoova, S. Recurrences of visceral leishmaniasis caused by *Leishmania siamensis* after treatment with amphotericin B in a seronegative child. *Am. J. Trop. Med. Hyg.* **2014**, *90*, 40–42. [[CrossRef](#)]
35. Phumee, A.; Kraivichian, K.; Chusri, S.; Noppakun, N.; Vibhagool, A.; Sanprasert, V.; Tampanya, V.; Wilde, H.; Siriyasatien, P. Detection of *Leishmania siamensis* DNA in saliva by polymerase chain reaction. *Am. J. Trop. Med. Hyg.* **2013**, *89*, 899–905. [[CrossRef](#)]
36. Phumee, A.; Chusri, S.; Kraivichian, K.; Wititsuwannakul, J.; Hortiwakul, T.; Thavara, U.; Silpapojakul, K.; Siriyasatien, P. Multiple Cutaneous Nodules in an HIV-Infected Patient. *PLoS Negl. Trop. Dis.* **2014**, *8*, e3291. [[CrossRef](#)]
37. Pothirat, T.; Tantiworawit, A.; Chaiwarith, R.; Jariyapan, N.; Wannasan, A.; Siriyasatien, P.; Supparatpinyo, K.; Bates, M.D.; Kwakye-Nuako, G.; Bates, P.A. First isolation of *Leishmania* from Northern Thailand: Case report, identification as *Leishmania martiniquensis* and phylogenetic position within the *Leishmania enriettii* complex. *PLoS Negl. Trop. Dis.* **2014**, *8*, e3339. [[CrossRef](#)]
38. Siriyasatien, P.; Chusri, S.; Kraivichian, K.; Jariyapan, N.; Hortiwakul, T.; Silpapojakul, K.; Pym, A.M.; Phumee, A. Early detection of novel *Leishmania* species DNA in the saliva of two HIV-infected patients. *BMC Infect. Dis.* **2016**, *16*, 89. [[CrossRef](#)]
39. Suankratay, C.; Suwanpimolkul, G.; Wilde, H.; Siriyasatien, P. Case Report: Autochthonous visceral leishmaniasis in a human immunodeficiency virus (HIV)-infected patient: The first in Thailand and review of the literature. *Am. J. Trop. Med. Hyg.* **2010**, *82*, 4–8. [[CrossRef](#)]
40. Sukmee, T.; Siripattanapipong, S.; Mungthin, M.; Worapong, J.; Rangsin, R.; Samung, Y.; Kongkaew, W.; Bumrungsana, K.; Chanachai, K.; Apiwathanasorn, C.; et al. A suspected new species of *Leishmania*, the causative agent of visceral leishmaniasis in a Thai patient. *Int. J. Parasitol.* **2008**, *38*, 617–622. [[CrossRef](#)]
41. Ready, P. Biology of phlebotomine sand flies as vectors of disease agents. *Annu. Rev. Entomol.* **2013**, *58*, 227–250. [[CrossRef](#)]
42. Maia, C.; Depaquit, J. Can *Sergentomyia* (Diptera, Psychodidae) play a role in the transmission of mammal-infecting *Leishmania*? *Parasite* **2016**, *23*, 55. [[CrossRef](#)]
43. Mukherjee, S.; Hassan, M.Q.; Ghosh, A.; Ghosh, K.N.; Bhattacharya, A.; Adhya, S. *Leishmania* DNA in *Phlebotomus* and *Sergentomyia* species during a kala-azar epidemic. *Am. J. Trop. Med. Hyg.* **1997**, *57*, 423–425. [[CrossRef](#)]

44. Arvizi, P.; Amirkhani, A. Mitochondrial DNA characterization of *Sergentomyia sintoni* populations and finding mammalian *Leishmania* infections in this sandfly by using ITS-rDNA gene. *Iran. J. Vet. Res.* **2008**, *9*, 9–18.
45. Campino, L.; Cortes, S.; Dionísio, L.; Neto, L.; Afonso, M.O.; Maia, C. The first detection of *Leishmania major* in naturally infected *Sergentomyia minuta* in Portugal. *Mem. Inst. Oswaldo Cruz* **2013**, *108*, 516–518. [[CrossRef](#)]
46. Ayari, C.; Ben Othman, S.; Chemkhi, J.; Tabbabi, A.; Fisa, R.; Ben Salah, A.; BenAbderrazak, S. First detection of *Leishmania major* DNA in *Sergentomyia (Sintonius) clydei* (Sinton, 1928, Psychodidae: Phlebotominae), from an outbreak area of cutaneous leishmaniasis in Tunisia. *Infect. Genet. Evol.* **2016**, *39*, 241–248. [[CrossRef](#)]
47. Aouadi, K.; Ghawar, W.; Salem, S.; Gharbi, M.; Bettaieb, J.; Yazidi, R.; Harrabi, M.; Hamarsheh, O.; Ben Salah, A. First report of naturally infected *Sergentomyia minuta* with *Leishmania major* in Tunisia. *Parasites Vectors* **2015**, *8*, 649. [[CrossRef](#)]
48. Senghor, M.; Niang, A.; Depaquit, J.; Faye, M.; Ferté, H.; Elguero, E.; Gaye, O.; Alten, B.; Perktas, U.; Cassan, C.; et al. Transmission of *Leishmania infantum* in the canine leishmaniasis focus of Mont-Rolland, Senegal: Ecological, parasitological and molecular evidence for a possible role of *Sergentomyia* sand flies. *PLoS Negl. Trop. Dis.* **2016**, *10*, e0004940. [[CrossRef](#)]
49. Chusri, S.; Thammapalo, S.; Chusri, S.; Thammapalo, S.; Silpapojakul, K.; Siriyasatien, P. Animal reservoirs and potential vectors of *Leishmania siamensis* in southern Thailand. *Southeast Asian J. Trop. Med. Public Health* **2014**, *45*, 13–19.
50. Siripattanapipong, S.; Leelayoova, S.; Ninsaeng, U.; Mungthin, M. Detection of DNA of *Leishmania siamensis* in *Sergentomyia (Neophlebotomus) iyengari* (Diptera: Psychodidae) and Molecular Identification of Blood Meals of Sand Flies in an Affected Area, Southern Thailand. *J. Med. Entomol.* **2018**, *55*, 1277–1283. [[CrossRef](#)]
51. Botero, A.; Cooper, C.; Thompson, C.K.; Clode, P.L.; Rose, K.; Thompson, R.C. Morphological and Phylogenetic Description of *Trypanosoma noyesi* sp. nov.: An Australian Wildlife Trypanosome within the *T. cruzi* Clade. *Protist* **2016**, *167*, 425–439. [[CrossRef](#)]
52. Kato, H.; Uezato, H.; Sato, H.; Bhutto, A.M.; Soomro, F.R.; Baloch, J.H.; Iwata, H.; Hashiguchi, Y. Natural infection of the sand fly *Phlebotomus kazeruni* by *Trypanosoma* species in Pakistan. *Parasites Vectors* **2010**, *3*, 10. [[CrossRef](#)]
53. Nzelu, C.O.; Kato, H.; Pupilampu, N.; Desewu, K.; Odoom, S.; Wilson, M.D.; Sakurai, T.; Katakura, K.; Boakye, D.A. First detection of *Leishmania tropica* DNA and *Trypanosoma* species in *Sergentomyia* sand flies (Diptera: Psychodidae) from an outbreak area of cutaneous leishmaniasis in Ghana. *PLoS Negl. Trop. Dis.* **2014**, *8*, e2630. [[CrossRef](#)]

