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Short Communication

New Microsatellite Markers for Genetic Studies on *Sergentomyia schwetzi* (Diptera: Psychodidae): A Suspected Vector of *Leishmania infantum* (Trypanosomatida: Trypanosomatidae) in the Canine Leishmaniasis Focus of Mont-Rolland, Senegal

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Abstract

Visceral leishmaniasis is not endemic in West Africa, but prevalence of canine leishmaniasis and seroprevalence of *Leishmania infantum* infection in humans are high in the Mont Rolland community (Thiès region, Senegal). Previous studies in this area showed that *Sergentomyia schwetzi* could be the potential vector of *Le. infantum*. To precisely describe the biology and population structure of this potential vector, we identified eight novel microsatellite loci to characterize *Se. schwetzi* populations. We tested these loci in *Se. schwetzi* populations from five locations at Mont Rolland (Thiès, Senegal). All the loci were polymorphic, with a mean of 17.25 alleles (observed heterozygosity: 0.455). We did not detect any evidence of scoring errors due to stuttering and large allele dropout. Moreover, several of these loci were also amplified in six other sand fly species (*Sergentomyia magna*, *Sergentomyia dubia*, *Sergentomyia minuta*, *Phlebotomus duboscqi*, *Phlebotomus perniciosus*, and *Phlebotomus ariasi*). These preliminary results demonstrate the utility of these microsatellite markers for *Se. schwetzi* (and for the other sand fly species) population genetic studies.

Key words: sand fly, *Sergentomyia*, microsatellite, population genetics

Leishmaniasis is a neglected disease that affects 0.9–1.6 million people worldwide, and causes 26,000–65,000 deaths each year. Over a billion people live in endemic areas at risk of infection (World Health Organization 2016). This disease can present different clinical forms, mostly in function of the infecting *Leishmania* species (Banuls et al. 2011). Only the visceral form is lethal if untreated, and represents 0.2–0.4 million cases each year. Since 2014, an increase of visceral leishmaniasis cases and deaths and of its geographical expansion has been observed (Ibarra-Meneses et al. 2020).

Leishmaniasis is caused by a protozoan parasite belonging to the *Leishmania* genus, transmitted through the bite of infected female sand flies (Abonnenc 1972). Sand flies are relatively small (2–3 mm) insects belonging to the Phlebotominae subfamily within the Psychodidae family (Tesh 1988). Both sexes feed on sweet juices, but only females take bloodmeals that are needed for egg maturation (Abonnenc 1972). Sand flies are distributed in tropical and subtropical areas, arid and semiarid areas, and temperate zones worldwide (Killick-Kendrick 1999). On the approximately 800 sand fly species recorded, two main genera are present in the Old World: *Phlebotomus* (Diptera: Psychodidae) (94 species) and *Sergentomyia* (258 species) (Kato et al. 2010). Species belonging to *Phlebotomus* genus are described as proven vectors of *Leishmania* in the Old World, although several studies also suggest the involvement of *Sergentomyia* species as *Leishmania* vectors (Kanjanopas et al. 2013, Jaouadi et al. 2015, Senghor et al. 2016).

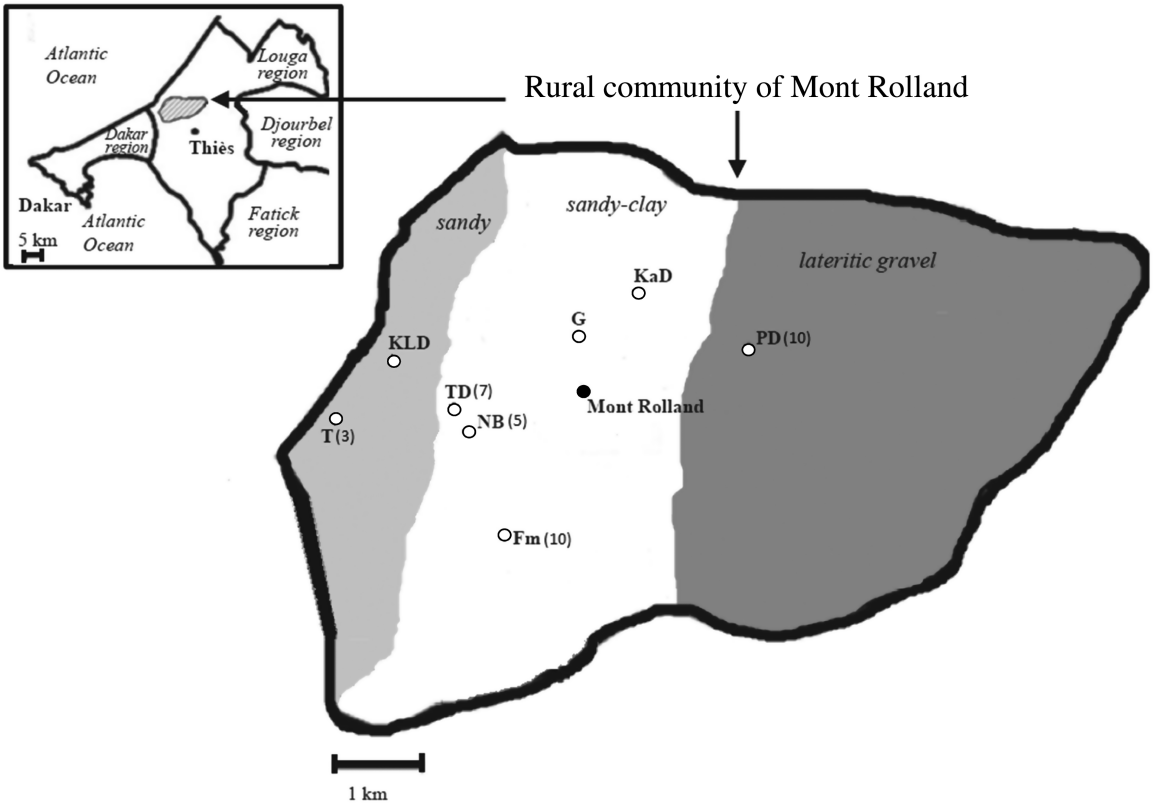

Fig. 1. Map of the study area. Description of the Mont-Rolland community (Thiès region, Senegal). Identification of the villages: Fouloum (Fm), Guidieur (G), Khaye Diagal (KaD), Keur Lat Diop (KLD), Ndiaye Bopp (NB), Pallo Diale (PD), Thiaye (T), and Twin Djassa (TD). The number of *Sergentomyia schwetzi* individuals collected (in brackets) and the soil characteristics are indicated. Adapted from Senghor et al. (2011) and Cassan et al. (2016).

This work is specifically focused on *Se. schwetzi* Adler, Theodor et Parrot 1929. Even if identification keys were established (Abonnenc 1972, Ba 1999), few articles were published on the systematic classification of *Sergentomyia* genus (Davidson 1990, Randrianambinintsoa et al. 2014) since until a few years ago they were not considered as potential vectors.

Visceral leishmaniasis is not endemic in West Africa. However, the prevalence of infections by *Leishmania infantum*, a species that causes visceral leishmaniasis in dogs, and its seroprevalence in humans are high in the Mont Rolland community (Thiès region, Senegal) (Faye et al. 2010, Faye et al. 2011). Entomological studies in this area showed that unsuspected sand fly species could be the *Le. infantum* vectors: *Sergentomyia dubia* and *Sergentomyia schwetzi* (Senghor et al. 2011, Senghor et al. 2016). However, no information is available on the population structure of these potential vectors. Yet, a better knowledge of these vectors is needed to precisely understand *Leishmania* transmission. Although few full sand fly genome sequences are available, the microsatellite marker-based method still remains one of the best approaches to study their population structure (Prudhomme et al. 2020).

Therefore, the aim of this work was to identify microsatellite markers to study *Se. schwetzi* population structure. We describe preliminary results based on 35 *Se. schwetzi* samples collected in the Mont Rolland community and using eight new polymorphic microsatellite loci. Moreover, we tested the transferability of these microsatellite markers to six other sand fly species (*Sergentomyia magna*, *Se. dubia*, *Sergentomyia minuta*, *Phlebotomus duboscqi*, *Phlebotomus perniciosus*, and *Phlebotomus ariasi*).

Material and Methods

Sand Fly Collection and Identification

In December 2012, sand flies were captured in the rural community of Mont Rolland, in the Thiès region (West Senegal), using sticky traps (20 × 20 cm white paper strips covered with castor oil), and CDC miniature light traps (John W. Hock Co., FL) (Alten et al. 2015, Ayhan et al. 2017). *Se. schwetzi* specimens were collected at Pallo Diale, Fouloum, Twin Djassa, Ndiaye Bopp and Thiaye. *Se. magna*, *Se. dubia*, and *Ph. duboscqi* specimens were collected at Fouloum, Guidieur, Keur Lat Diop, Khaye Diagal and Ndiaye Bopp (Fig. 1; Table 1, and Supp Table 1 [online only]). *Se. minuta*, *Ph. Perniciosus*, and *Ph. ariasi* specimens were captured at Roquedur (Gard, France) between July 2011 and 2013 (Table 1, and Supp Table 1 [online only]).

Samples were prepared, dissected, mounted, and identified as previously described in Prudhomme et al. (2020).

Selected Specimens

Fifty *Se. schwetzi* individuals were pooled and used to designed and synthesized the microsatellite markers. Then, 66 sand fly specimens were used to test these markers: 35 *Se. schwetzi* (Senegal), 5 *Se. magna* (Senegal), 5 *Se. dubia* (Senegal), 5 *Se. minuta* (Senegal), 5 *Ph. duboscqi* (Senegal), 6 *Ph. perniciosus* (France), and 5 *Ph. ariasi* (France) (Table 1 and Supp Table 1 [online only]).

DNA Extraction, Isolation of Microsatellite Loci, and Identification

DNA was extracted from the thorax, wings, legs, and abdomen of each sand fly specimen using the Cetyl Trimethyl Ammonium

Bromide (CTAB) method, as previously described in Prudhomme et al. (2015). *Se. schwetzi* specimens were used, by a private company (StarSEQ, Mainz, Germany), to isolate, clone, and sequence microsatellite markers using an Illumina MiSeq system (Prudhomme et al. 2015). Among the 84 locus pairs designed and synthesized, 8 were successfully amplified (Table 2).

DNA Amplification and Genotyping

All sand fly DNA samples were individually amplified. Each 25 µL reaction mix included 10 pmol of forward (labeled with a fluorochrome, FAM, ATT0565, or HEX) and reverse primers (Table 2), 2 µL of DNA template, 5 pmol of dNTP mix, 5 µL of 5X buffer, 2 µL of $MgCl_2$, and 2 units of Taq polymerase (Promega). DNA was amplified using the following program: initial denaturation step at 95°C for 3 min, then 35 cycles at 95°C for 30 s, the annealing temperature of each locus (Table 1) for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 5 min.

Genotyping was performed on a 3500xL Genetic Analyzer (Applied Biosystems) automated sequencer with 1 µL of PCR product added to a standard loading mix: 0.2 µL of internal standard-size GENESCAN 500LIZ and 15 µL of formamide (HiDi) (both Applied Biosystems). The GENEMAPPER 4.0 (Applied Biosystems) software was used to read and analyze the profiles. As the PCR products (loci) were of different sizes and labeled with different fluorochromes (Table 2), multiplex amplification was possible.

Table 1. Description of the sand fly specimens used in this work

Country	Locality	Species	Number
Senegal (Thiès)	Pallo Diale	*Sergentomyia schwetzi*	10
Senegal (Thiès)	Fouloum	*Se. schwetzi*	10
		Se. magna	5
		Se. dubia	3
Senegal (Thiès)	Twin Djassa	*Se. schwetzi*	7
Senegal (Thiès)	Thiaye	*Se. schwetzi*	3
Senegal (Thiès)	Ndiaye Bopp	*Se. schwetzi*	5
		Phlebotomus duboscqi	2
Senegal (Thiès)	Guidieur	*Se. dubia*	2
Senegal (Thiès)	Keur Lat Diop	*Ph. duboscqi*	2
Senegal (Thiès)	Khaye Diagal	*Ph. duboscqi*	1
France (Gard)	Roquedur	*Ph. ariasi*	5
		Ph. perniciosus	6
		Se. minuta	5

Data Analysis

Raw data were formatted for CREATE (Coombs et al. 2008) that allows their conversion for the different software programs used. Linkage disequilibrium (LD) between each locus pair was measured with the *G*-based test (10,000 permutations) (Goudet et al. 1996, Prudhomme et al. 2020).

F_{STAT}, version 2.9.4 (Goudet 1995) was used to compute the observed heterozygosity (H_o), expected heterozygosity (H_e), mean genetic diversity (H_s), allelic richness (AR), and inbreeding coefficient (F_{IS}). Deviations from the Hardy–Weinberg equilibrium were measured using GENEPOP, version 4.2.2 (Rousset 2008). The individual estimations of H_o, H_s, H_e, and fixation index (F_{ST}) in the populations from the different locations, as well as the Cavalli-Sforza and Edwards genetic distances (Cavalli-Sforza and Edwards 1967) between populations were calculated using GENETIX 4.05 (Belkhir et al. 2009). These distances were used to build a Neighbor Joining (NJ) tree using Mega V6 (Tamura et al. 2013). Scoring errors due to stuttering, large allele dropout, and homozygote excess were computed with MicroChecker 2.2.3 (Van Oosterhout et al. 2004).

Results and Discussion

Selected Microsatellite Markers

Among the 84 microsatellite loci found (available on request), eight successfully amplified loci were selected on the basis of the criteria described in Prudhomme et al. (2015). These dinucleotide microsatellite loci are described in Table 2 (sequences available in GenBank). Their polymorphism in *Se. schwetzi* was tested using the 35 specimens from Senegal.

Genetics Analysis

In the *Se. schwetzi* samples from the five localities of Mont Rolland (Senegal), all eight loci were polymorphic (mean: 17.25 alleles

Table 2. Details of the eight microsatellite markers selected: name, primer sequences, structure of the repeat array, annealing temperature (Ta), labeling dye, multiplexing group, allele number (*Na*), allele size range, observed heterozygosity (H_o), mean genetic diversity (H_s), expected heterozygosity (H_e), allelic richness (AR) and GenBank accession number

Name	Primer sequence (5'-3')	Repeat type	Ta (°C)	Labeling dye	Groups	Na	Allele size (bp)	H_o	H_s	H_e	AR	GenBank accession no.
SSC1	5′-AATCATGCGTTCTCGAGTCA-3′ 5′-TGCCAGCATAATTCCATCTTC-3′	TC	57	ATT0565	1	22	182–294	0.488	0.913	0.959	1.950	MZ027289
SSC2	5′-CCCAGAGACAGGTGTTGAGA-3′ 5′-CCCTAACCATTCCTGTGCAT-3′	AG	57	FAM	1	13	151–235	0.534	0.855	0.886	1.890	MZ027290
SSC3	5′-CACAAACCATAAATTGGCCC-3′ 5′-TCCCGAGAAGAAAGAAGTGC-3′	AT	57	HEX	1	9	133–161	0.444	0.684	0.717	1.757	MZ027291
SSC4	5′-GCGTGGGAAATTACGAAAGA-3′ 5′-TTGGATTGCACGAGAAGAAA-3′	TA	57	ATT0565	2	21	171–265	0.147	0.869	0.949	1.945	MZ027292
SSC5	5′-CGGAAATTATTCTGCGGTGT-3′ 5′-GTCAGCAATTACTGCCCGAT-3′	GA	57	FAM	2	17	171–245	0.551	0.900	0.917	1.904	MZ027293
SSC6	5′-TCGATTTCTCACTTCGTGACC-3′ 5′-GAGTGCAACTCCGTGTCTTG-3′	TC	57	HEX	2	20	135–177	0.765	0.922	0.939	1.936	MZ027294
SSC7	5′-ATAACCCACCCACAAACCAA-3′ 5′-CGACGTCCTGAATTTGTTCC-3′	TA	58	ATT0565	3	22	190–270	0.261	0.955	0.960	1.949	MZ027295
SSC8	5′-TGACACATGCGAAACGTGAT-3′ 5′-ATTTGCCTCCATTCATCGTC-3′	AT	57	HEX	3	14	119–159	0.447	0.695	0.777	1.804	MZ027296

Table 3. Estimation of the allele number (*Na*), number of dinucleotide repeats (allele size range), observed heterozygosity (H_o), mean genetic diversity (H_s), expected heterozygosity (H_e), inbreeding coefficient (F_{IS}), and allelic richness (AR) for the five *Se. schwetzi* populations (Pop, population; PD, Pallo Diale; Fm, Fouloum; TD, Twin Djassa; T, Thiaye; and NB, Ndiaye Bopp)

Pop	Locus	SSC1	SSC2	SSC3	SSC4	SSC5	SSC6	SSC7	SSC8
PD	Na	9	8	6	6	7	9	11	9
	Allele size (bp)	182–288	151–221	133–149	171–219	171–207	135–177	198–270	119–157
	H_e	0.8750	0.8611	0.7778	0.8200	0.8200	0.8047	0.8900	0.8438
	H_s	0.9333	0.9394	0.8485	0.9111	0.9111	0.8583	0.9368	0.9000
	H_o	0.3750	0.5000	0.8333	0.200	0.8000	0.6250	0.4000	0.7500
	F_{IS}	0.6150	0.4920	0.0200	0.800	0.1350	0.2860	0.5860	0.1760
	AR	1.933	1.939	1.848	1.911	1.911	1.858	1.937	1.900
Fm	Na	12	8	4	8	6	11	6	7
	Allele size (bp)	192–294	179–221	133–143	199–265	187–203	139–167	192–236	121–159
	H_e	0.8950	0.8600	0.6563	0.8600	0.7734	0.8850	0.7917	0.6900
	H_s	0.9421	0.9053	0.7500	0.9053	0.8250	0.9316	0.8636	0.7263
	H_o	0.6000	0.5000	0.5000	0.0000	0.5000	0.7000	0.3333	0.4000
	F_{IS}	0.3760	0.4610	0.3680	1.0000	0.4110	0.2590	0.6360	0.4630
	AR	1.942	1.905	1.750	1.905	1.825	1.932	1.864	1.726
TD	Na	10	8	4	6	9	9	8	5
	Allele size (bp)	268–292	205–235	133–141	225–247	189–211	139–163	192–234	121–139
	H_e	0.8673	0.8265	0.6633	0.7857	0.8367	0.8776	0.8571	0.7551
	H_s	0.9341	0.8901	0.7143	0.8462	0.9011	0.9451	0.9231	0.8132
	H_o	0.7143	0.5714	0.2857	0.2857	0.8571	1.0000	0.5714	0.2857
	F_{IS}	0.2500	0.3770	0.6190	0.6800	0.0530	−0.0630	0.4000	0.6670
	AR	1.934	1.890	1.714	1.846	1.901	1.945	1.923	1.813
T	Na	1	2	1	1	2	3	3	1
	Allele size (bp)	226	213–219	133	225	193–195	143–161	194–262	121
	H_e	0.0000	0.3750	0.0000	0.0000	0.5000	0.6250	0.6667	0.0000
	H_s	0.0000	0.5000	0.0000	0.0000	0.6667	0.8333	0.8000	0.0000
	H_o	0.0000	0.5000	0.0000	0.0000	0.0000	0.5000	0.0000	0.0000
	F_{IS}	NA	0.0000	NA	NA	1.0000	0.5000	1.0000	NA
	AR	1.000	1.500	1.000	1.000	1.667	1.833	1.800	1.000
NB	Na	7	4	5	3	7	7	4	5
	Allele size (bp)	234–294	211–225	133–161	235–243	191–245	145–171	190–208	121–143
	H_e	0.8438	0.7000	0.7400	0.5313	0.8400	0.8400	0.7500	0.7600
	H_s	0.9643	0.7778	0.8222	0.6071	0.9333	0.9333	0.8571	0.8444
	H_o	0.7500	0.6000	0.6000	0.2500	0.6000	1.0000	0.0000	0.8000
	F_{IS}	0.2500	0.2500	0.2940	0.6250	0.3850	−0.0810	1.0000	0.0590
	AR	1.964	1.778	1.822	1.607	1.933	1.933	1.857	1.844

per loci; range 9–22) (Table 3). The mean H_o, H_s, and H_e were 0.455 ± 0.187, 0.849 ± 0.103, and 0.888 ± 0.092, respectively. No deviation from the Hardy–Weinberg equilibrium was observed, and no LD was detected between loci. No evidence of scoring errors due to stuttering and large allele dropout was found.

The allelic richness per locus and sampling localities varied from 1 to 1.945 (Table 3). The fixation index (F_{ST}) revealed a low genetic differentiation between Pallo Diale and Fouloum (F_{ST} = 0.0091), Pallo Diale and Twin Djassa (F_{ST} = −0.0018), Pallo Diale and Ndiaye Bopp (F_{ST} = 0.02460), Fouloum and Twin Djassa (F_{ST} = −0.00790), Fouloum and Ndiaye Bopp (F_{ST} = 0.00189), and Twin Djassa and Ndiaye Bopp (F_{ST} = −0.01626). The F_{ST} was moderate between Thiaye and Pallo Diale (F_{ST} = 0.10489), Thiaye and Fouloum (F_{ST} = 0.07588), and Thiaye and Twin Djassa (F_{ST} = 0.12828). The F_{ST} was higher between Ndiaye Bopp and Thiaye (F_{ST} = 0.18741). However, none of these F_{ST} values was significant, possibly due to the small number of individuals collected at each locality. In agreement with the low F_{ST} value, no strong structuring was observed between sampling localities on the NJ phenogram (Fig. 2). Additional molecular studies will be performed with more samples to determine *Se. schwetzi* genetic structure at different geographic scales. These analyses will bring genetic information on *Se. schwetzi* vector versus nonvector population. Nevertheless, these preliminary

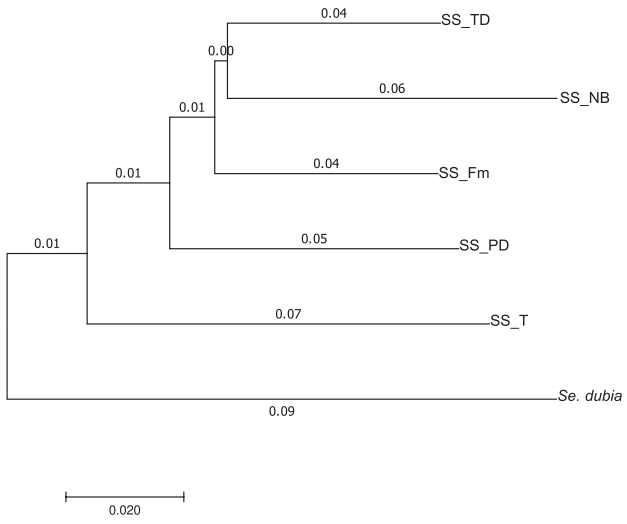

Fig. 2. Neighbor-joining phenogram based on the Cavalli-Sforza chord measure, for the five *Sergentomyia schwetzi* populations collected at Fouloum (SS_Fm), Ndiaye Bopp (SS_NB), Pallo Diale (SS_PD), Thiaye (SS_T), and Twin Djassa (SS_TD). *Sergentomyia dubia* (*Se. dubia*) was used as outgroup.

Table 4. Transferability of the eight microsatellite loci selected to other sand fly species (*n* = number of tested individuals)

Locus	*Sergentomyia magna* (*n* = 5)	*Se. dubia* (*n* = 5)	*Se. minuta* (*n* = 5)	*Phlebotomus duboscqi* (*n* = 5)	*Ph. perniciosus* (*n* = 6)	*Ph. ariasi* (*n* = 5)
SSC1	–	–	1	–	2	–
SSC2	–	–	3	–	4	1
SSC3	–	3	5	4	5	1
SSC4	–	–	4	–	2	–
SSC5	3	4	5	2	–	–
SSC6	2	–	1	–	1	–
SSC7	1	–	3	1	3	–
SSC8	–	3	2	3	–	–

results suggest that these eight microsatellite loci are suitable for *Se. schwetzi* genetic study.

Transferability Test to Other Species

Some loci could be amplified also in *Se. magna* (SSC5, SSC6, SSC7), *Se. dubia* (SSC3, SSC5, and SSC8), *Se. minuta* (all loci), *Ph. duboscqi* (SSC3, SSC5, SSC7, and SSC8), *Ph. perniciosus* (SSC1, SSC2, SSC3, SSC4, SSC6, and SSC7), and *Ph. ariasi* (SSC2 and SSC3) DNA samples. The number of alleles per locus for these loci are listed in Table 4, and Supp Table 1 (online only). Therefore, these microsatellite loci can be used for genetic, phylogeographic, and evolutionary analyses of different species within the *Phlebotomus* and *Sergentomyia* genera.

Conclusions

Microsatellites are useful for assessing genetic diversity, specimen identification, phylogenetic studies, and as a tool to measure inbreeding and differences among populations. As microsatellites have a rapid evolution rate, they are particularly valuable to understand the relationships among closely related species (Okumu et al. 2017). These preliminary results demonstrate the utility of these microsatellite markers for *Se. schwetzi* (and for other sand fly species) population genetic studies.

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Author Contributions

J.P., B.F., M.W.S, A.A.N, and A.L.B. designed the study, T.M. and S.H. performed the molecular characterization. J.P. and T.M. analyzed the data. J.P., T.M., S.H., C.T., C.C., M.W.S, A.A.N, B.F. and A-L.B. contributed to field sampling, sand fly species identification or sample preparation. J.P. and A-L.B. wrote the manuscript with support from T.M., S.H., C.T., M.W.S., A.A.N., B.F. and C.C. All authors have read and approved the actual version of the manuscript.

Data Availability

All resources used in this article are provided in the Supp Table S1 [online only] and all the analyses are detailed allowing the assessment or verification of the manuscript's findings.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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