Molecular epidemiological study of *Leishmania* and sand fly vectors

リーシュマニアおよび媒介昆虫サシチョウバエに関する分子疫学的研究

Chukwunonso Onyemaechi Nzelu

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Laboratory of Parasitology

Department of Disease Control

Graduate School of Veterinary Medicine

Hokkaido University

CONTENTS

Abbreviations

AT	adenosine and thymine
BOLD	barcode of life data system
bp	base pair
CDC	Centers for Disease Control and Prevention
CL	cutaneous leishmaniasis
COI	cytochrome c oxidase subunit 1
cyt b	cytochrome *b*
DAT	direct agglutination test
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
EDTA	ethylene diamine tetra-acetate
FDR	fluorescent detection reagent
FTA	Flinders Technology Associates
ITS1	internal transcribed spacer 1
L.	*Leishmania*
LAMP	loop-mediated isothermal amplification
LPG	lipophosphoglycan
Lu.	*Lutzomyia*
M	DNA marker (gene ruler)
MCL	mucocutaneous leishmaniasis
MEGA	molecular evolutionary genetics analysis
MG	malachite green
mtDNA	mitochondrial deoxyribonucleic acid

NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NJ	Neighbor-joining
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	hydrogen-ion exponent
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
S.	*Sergentomyia*
SDS	sodium dodecyl sulfate
ShT	Shannon trap
SSU	small subunit
VL	visceral leishmaniasis
UV	ultra-violet illuminator
Wa	*Warileya*
WHO	World Health Organization

Unit measures abbreviations

%	percentage
µg	microgram
µl	microliter
µM	micromolar
°C	degree Celsius
g	gram

h	hour
km	kilometer
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
sec	second
U	unit

General Introduction

Leishmania is an obligate intracellular protozoan parasite that causes a group of diseases collectively known as the leishmaniasis. The parasite is transmitted by the bite of an infected female phlebotomine sand fly. Leishmaniasis is endemic in 98 countries worldwide, affecting mostly the poorest populations in developing countries, with 350 million people at risk and some 2 million new cases occur yearly (WHO, 2010; Alvar et al., 2012) (Fig. 1). Approximately 20 species of *Leishmania* are reported to be pathogenic to humans. The parasite has a digenetic life-cycle and exists in two main morphological forms; either as amastigotes inside the phagocytes (typically macrophages) of mammalian hosts or as flagellated promasitgotes within the guts of their phlebotomine sand fly vectors. In human, *Leishmania* infection can present diverse clinical manifestations ranging from chronic but often self-healing ulcerative skin lesions at the site of the sand fly bite, cutanous leishmaniasis (CL), to erosive partial or complete destruction of mucous membrane of the nose and mouth, mucocutaneous leishmaniasis (MCL) and a life-threatening systemic infection with hepato-splenomegaly known as visceral leishmaniasis (VL) (Reithinger and Durjardin, 2007; Alvar et al., 2012). The outcome of infection depends on the *Leishmania* species involved (Reithinger and Durjardin, 2007).

The spread of leishmaniasis largely depends on the distribution of phlebotomine sand flies, a proven natural vector of *Leishmania* parasites worldwide. Sand flies are dipteran insects within the family Psychodidae. They are generally no more than 3.5 mm in length and covered with dense hair, holding their wings in a characteristic V-shaped position. The flies are mainly active during the night and early morning, both male and female adults survive on sugary secretions from plants. Like most hematophagous dipterans, female sand flies require vertebrate blood for egg production, and hop across the skin of its vertebrate host in search for a blood-meal.

Although some 800 species of sand flies are known, only few have been incriminated as vectors of leishmaniasis (Munstermann, 2004; Bates, 2007; Kato et al., 2010b).

Control of leishmaniasis relies on early diagnosis and treatment of infected patients, and proper vector surveillance in endemic areas. Therefore, studies aimed at establishment of a simple and field-applicable test for routine screening and diagnosis of patients, as well as efficient tools for accurate identification of sand flies and detection of *Leishmania* DNA within sand fly vectors may contribute to proper understanding of the epidemiology of leishmaniasis and vector control in endemic areas.

In Chapter I, the potential usefulness of mitochondrial gene, cytochrome c oxidase subunit I (COI) as a valid molecular tool for identification of sand fly species was assessed using specimens from leishmaniasis-endemic areas of Peru. Chapter II focused on the identification of potential sand fly vectors and their associated *Leishmania* species for a better understanding of the epidemiology of cutaneous leishmaniasis outbreak in Ghana. In Chapter III, a novel entomological monitoring tool for mass-screening of sand fly for *Leishmania* infection by loop-mediated isothermal amplification was developed and evaluated with field-caught sand fly samples from Ecuador as reliable method for surveillance of leishmaniasis in endemic areas. In Chapter IV, colorimetric malachite green based loop-mediated isothermal amplification test combined with an FTA card as a direct sampling tool for rapid diagnosis of cutaneous leishmaniasis was established and validated with clinical samples from endemic areas of Peru.

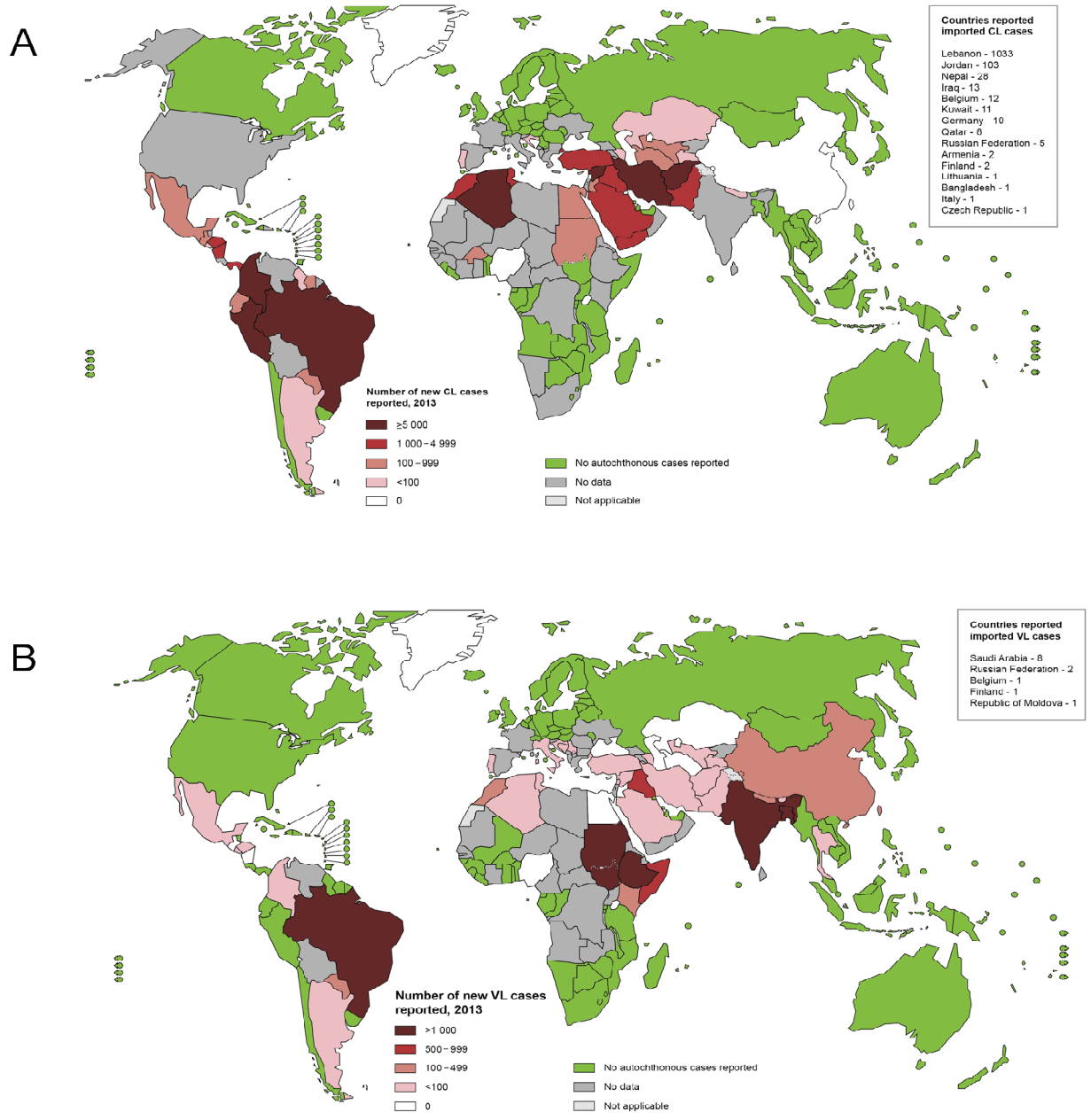

Fig. 1. Geographical distribution of leishmaniasis.

(A) Status of endemicity of cutaneous leishmaniasis, worldwide, 2013. (B) Status of endemicity of visceral leishmaniasis, worldwide, 2013. (Source: http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_2013_CL/VL.png).

Chapter I

DNA barcoding for identification of sand fly species (Diptera: Psychodidae) from leishmaniasis-endemic areas of Peru

1. Introduction

Phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) are of significant public health importance in many parts of the world as vectors of the causative agents of leishmaniasis, bartonellosis and sand fly fever (Maroli et al., 2013). Leishmaniasis remains one of the world's most neglected diseases, caused by several species of cell-infecting parasites of the genus *Leishmania*. Sand flies are the only proven vectors of leishmaniasis and approximately 800 species have been recorded in five major genera; *Phlebotomus* and *Sergentomyia* in the Old World, and *Lutzomyia*, *Brumptomyia* and *Warileya* in the New World (Munstermann, 2004). However, only species belonging to the genera *Phlebotomus* and *Lutzomyia* are the putative vectors of *Leishmania* (Munstermann, 2004; Bates, 2007; Kato et al., 2010b). Therefore, correct sand fly species identification is very important to design strategies for surveillance and control of leishmaniasis in given endemic areas.

In Peru, three sand fly species, *Lutzomyia* (*Lu*.) *peruensis*, *Lu*. *ayacuchensis* and *Lu*. *verrucarum* have been incriminated as the vectors of *Leishmania* (*Viannia*) *peruviana*, the aetiological agent of Andean-type cutaneous leishmaniasis in the endemic areas (Perez et al., 1991, 1994, 2007; Davies et al., 1993; Caceres et al., 2004; Kato et al., 2008, 2011a). Although these sand fly species role in disease transmission has been well studied, detailed molecular taxonomic knowledge of each species is far from complete. Presently, sand flies are typically identified based on morphological features; mainly by internal structures such as the cibarium, pharynx, spermatheca of females and terminal genitalia of males (Young and Duncan, 1994). However, this morphological classification is laborious, time-consuming and often complicated by phenotypic plasticity and cryptic species complexes, as well as demanding considerable skill and taxonomic expertise. To overcome these technical limitations, molecular approaches have

been increasingly employed in the last three decades to explore the taxonomy, population structure and phylogeny in insect vectors including sand flies (Mukhopadhyay et al., 2000; Uribe Soto et al., 2001; Depaquit et al., 2002; Beati et al., 2004; Khalid et al., 2010; Fujita et al., 2012; Yamamoto et al., 2013; Gomez et al., 2014; Kato et al., 2015).

Recently, DNA barcoding has been widely shown to be an effective molecular tool for species identification of many insects (Hebert et al., 2003a, b), including dipterans; mosquitoes (Cywinska et al., 2006), black flies (Pramual et al., 2011) and sand flies (Azpurua et al., 2010; Kumar et al., 2012; Contreras Gutiérrez et al., 2014), and revealing sand fly cryptic species (Cohnstaedt et al., 2011; Scarpassa and Alencar, 2012; Kumar et al., 2012). Construction of DNA barcodes for each species of sand fly would provide an efficient tool for their identification. The aim of the present study was to explore the utility of the DNA barcode approach for identification of sand fly species in Peru, where leishmaniasis is endemic, by analyzing morphologically identified adult specimens.

2. Materials and methods

2.1. Sand fly collection

Sand flies were caught using CDC light traps and Shannon traps peridomiciliary, intradomiciliary and extradomiciliary at 12 Departments; Piura, Lambayeque, Cajamarca, La Libertad, San Martin, Huánuco, Lima, Huancavelica, Ayacucho, Apurimac, Cusco and Puno in Peru between 2001 and 2013 (Fig. 2, Table 1). All the flies were captured throughout the night between 18:00 p.m. and 6:00 a.m. with CDC light traps and between 18:00 p.m. and 22:00 p.m. with Shannon traps. The sand flies were morphologically identified mainly based on measurements of wing veins, the ratio of palpus length to length of antenna and the color of the thorax (Young and Duncan, 1994), and then fixed in absolute ethanol and stored at room temperature.

2.2. DNA extraction

Individual ethanol-fixed sand flies were lysed in 50 µl of DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH8.0), 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS)] with 100 µg/ml proteinase K. The samples were incubated at 37 °C overnight. Afterwards, 25 µl of distilled water was added, heated at 95 °C for 5 min, and then, 0.5 µl portions were directly used as templates for PCR amplification.

2.3. Polymerase chain reaction and sequence analysis

The mitochondrial COI was amplified using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). The reaction was carried

out in a volume of 15 µl using a pair of primers (0.4 µM each), 7.5 µl of 2x Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan), and 1 U BIOTaq HS DNA polymerase (Bioline, London, UK). After an initial denaturation at 95 °C for 5 min, amplification was performed with 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 49 °C for 30 sec, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. PCR products were purified using a FastGene Gel/PCR Extraction kit (NIPPON Genetics, Tokyo, Japan) and sequenced with a forward primer by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). To confirm the authenticity of the COI sequences obtained, some individuals from each sand fly species (in total 40 specimens) were subjected to PCR using a high fidelity DNA polymerase (KOD-Plus-ver.2; TOYOBO, Tokyo, Japan), and the sequences were determined as described above.

2.4. Data analysis

The COI sequences were aligned by CLUSTAL W software (Thompson et al., 1997) incorporated into MEGA (Molecular Evolutionary Genetics Analysis) version 5.2 (Tamura et al., 2011). The nucleotide compositions and sequence divergences within and between species were calculated using the distance model Kimura 2-Parameter (K2P) (Kimura, 1980). This model provides the best estimate of divergence when genetic distances are low, as in recently diverged taxa (Nei and Kumar, 2000). The 'best match' method in the SpeciesIdentifier module of TaxonDNA software v1.7.8 (Meier et al., 2006) was used to test the frequency of successful identification. A Neighbor-joining (NJ) tree of K2P distances was created to provide a graphic representation of the clustering pattern among different species (Saitou and Nei, 1987). Branch support for NJ was calculated using the bootstrapping method with 1,000 replicates in MEGA

5.2 (Tamura et al., 2011). All sequences and other specimen information are available in the dataset project ‘DBSFP’ (Process IDs: DBSFP001-15 to DBSFP159-15) on the Barcode of Life Data System (BOLD) at http://www.boldsystems.org. Sequence data are also available in the DDBJ/EMBL/GenBank databases (http://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers AB984357–AB984520 (Table 1).

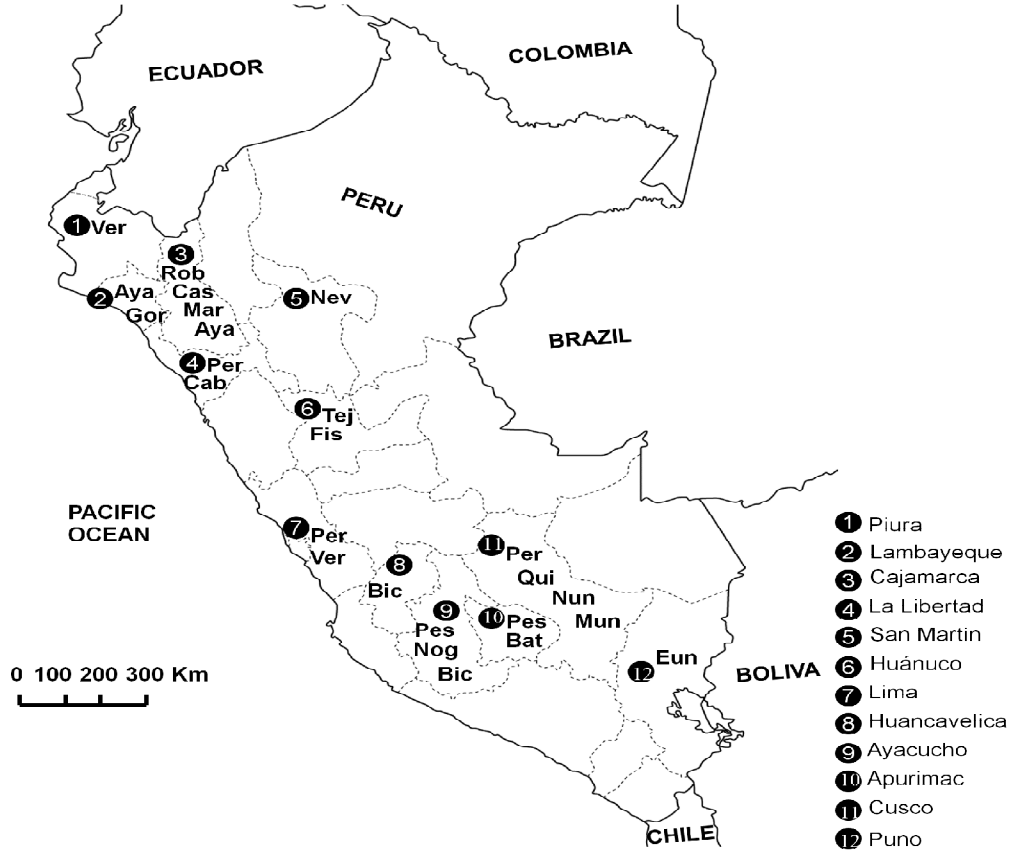

Fig. 2. Map of Peru showing the geographic locations where sand flies were collected along with the respective species analyzed in this study.

Bat: *Lu. battistinii*; Bic: *Lu. bicornuta*; Ver: *Lu. verrucarum*; Nun: *Lu. nuneztovari*; Mar: *Lu. maranonensis*; Nev: *Lu. nevesi*; Rob: *Lu. robusta*; Tej: *Lu. tejadai*; Qui: *Lu. quillabamba*; Cab: *Lu. caballeroi*; Cas: *Lu. castanea*; Pes: *Lu. pescei*; Aya: *Lu. ayacuchensis*; Per: *Lu. peruensis*; Mun: *Lu. munaypata*; Nog: *Lu. noguchii*; Fis: *Lu. fischeri*; Gor: *Lu. gorbitzi*; Eun: *Warileya euniceae*.

3. Results

3.1. Sequence analysis

A total of 159 COI sequences from 19 morphologically identified species of phlebotomine sand flies belonging to 6 subgenera/species groups were obtained. Each species was analyzed using 1 - 17 specimens (Table 1 and 2). The COI sequences obtained varied from 650 to 672 bp by direct sequencing. No deletions, insertions or stop codons were found indicating that all sequences constituted functional mitochondrial products (Funk and Omland, 2003). As these 159 COI sequences contained no indels, alignments were straight forward. The COI sequences from 19 species of sand flies had high adenosine and thymine (AT)-rich content bias (65.7%), similar to that observed for other insects (Simon et al., 1994), with an average nucleotide composition of A=28.0%, T=37.7% , C=17.9%, and G=16.4%.

Intraspecific genetic distances based on Kimura 2-Parameter values for the sand fly species ranged from 0 to 5.96% (Table 2). Maximum intraspecific genetic diversity was observed in 7 of the 19 species, especially within the three vector species of *Leishmania* (*Viannia*) *peruviana*; *Lutzomyia* (*Lu*.) *peruensis* (5.96%), *Lu. verrucarum* (3.48%) and *Lu. ayacuchensis* (2.76%) (Table 2). The high level of intraspecific divergence was related to geographical distribution in some species. The mean intraspecific K2P distance ranged from 0.05% (*Lu. caballeroi*) to 3.36% (*Lu. peruensis*) (Table 2). Interspecific K2P genetic distances for the sand fly species included in the study ranged from 8.39 to 19.08%. The overall nucleotide diversity recorded among the 19 species under the Subfamily Phlebotominae included in the study was found to be 13.9%. Low levels of minimum interspecific divergence typically occurred in closely related species, such as *Lu. tejadai* and *Lu. quillabamba* (8.39%), *Lu. bicornuta* and *Lu. battistinii* (8.88%), and *Lu. castanea* and *Lu. caballeroi* (10.15%). Collectively, the distances between species belonging to

the subgenus *Lutzomyia* (*Lu. bicornuta* and *Lu. battistinii*), and the *Verrucarum* group (*Lu. verrucarum* and *Lu. nuneztovari*) were 8.88 and 12.27%, respectively, whereas the distances between species belonging to the subgenus *Pifanomyia* (*Lu. maranonensis*, *Lu. robusta* and *Lu. nevesi*) ranged from 11.46 to 15.43%. On the other hand, the diversity within the subgenus *Helcocyrtomyia* (*Lu. munaypata*, *Lu. quillabamba*, *Lu. pescei*, *Lu. noguchii*, *Lu. castanea*, *Lu. caballeroi*, *Lu. tejadai*, *Lu. ayacuchensis* and *Lu. peruensis*) ranged from 8.39 to 16.5%. Using SpeciesIdentifier, the overall percentage of correct species identification was 100% as recognized by traditional taxonomy based on morphological features. Generally, the COI divergence within species did not exceed the genetic distance between species. As a result, there was a distinction between intraspecfic and interspecfic genetic divergences, which is critical for DNA barcoding success in taxa discrimination (Fig. 3).

3.2. Neighbor-joining tree

The Neighbor-joining tree showed shallow intraspecific and deep interspecific divergences (Fig. 4). Specimens of the same species were grouped closely together, regardless of the collection site. However, in exceptional cases, obvious geographic differences in sequences within the same species were found in three species, namely, *Lu. peruensis*, *Lu. verrucarum* and *Lu. maranonensis*. Each of these species was monophyletic, but the individuals were divided into two clades corresponding to geographic origin (Fig. 4), namely, *Lu. peruensis*: Cusco (southeast) clade, and Lima (central) and La Libertad (northwest) clade; *Lu. verrucarum*: Lima (central) clade, and Piura (northwest) clade; *Lu. maranonensis*: Querocotillo (Cajamarca-northwest) clade, and San José de Lourdes (Cajamarca-northwest) clade. Additionally, the *Lu. robusta* cluster was

monophyletic, but the individuals were divided into two clades: Cajamarca I (northwest) clade, and Cajamarca II (northwest) clade (Fig. 4).

Table 1. Details of sand fly specimens used for DNA barcoding and analysis in the study along with the BOLD Process IDs and GenBank accession numbers

Sand fly species	No. of samples	Location		Trapping Method	Year of capture	BOLD Process IDs	GenBank accession nos.
		District	Department				
Lu. battistinii	10	Abancay	Apurimac	CDC	2012	DBSFP001-15- DBSFP010-15	AB984357-AB984366
Lu. bicornuta	3	Luricocha	Ayacucho	CDC	2009	DBSFP011-15- DBSFP013-15	AB984367-AB984369
Lu. bicornuta	5	San Marcos de Rocchac	Huancavelica	CDC	2011	DBSFP014-15- DBSFP018-15	AB984370-AB984374
Lu. munaypata	10	Maranura	Cusco	CDC	2007	DBSFP019-15- DBSFP028-15	AB984375-AB984384
Lu. quillabamba	2	Calca	Cusco	CDC	2011	DBSFP029-15- DBSFP030-15	AB984385-AB984386
Lu. tejadai	11	Huánuco	Huánuco	CDC	2007, 2012	DBSFP031-15- DBSFP041-15	AB984387-AB984397
Lu. pescei	9	Luricocha	Ayacucho	CDC	2009	DBSFP042-15- DBSFP050-15	AB984398-AB984406
Lu. pescei	1	Chacoche	Apurimac	CDC	2012	DBSFP051-15	AB984407
Lu. caballeroi	10	La Cuesta	La Libertad	CDC	2007	DBSFP052-15- DBSFP061-15	AB984408-AB984417
Lu. castanea	5	San José de Lourdes	Cajamarca	ShT	2001	DBSFP062-15- DBSFP066-15	AB984418-AB984422
Lu. castanea	4	San Ignacio	Cajamarca	CDC	2007	DBSFP067-15- DBSFP070-15	AB984423-AB984426
Lu. fischeri	12	Ambo	Huánuco	CDC	2013	DBSFP071-15- DBSFP082-15	AB984428-AB984439
Lu. gorbitzi	2	Motupe	Lambayeque	CDC	2013	DBSFP083-15- DBSFP084-15	AB984440-AB984441
Lu. ayacuchensis	7	Motupe	Lambayeque	CDC	2013	DBSFP085-15- DBSFP091-15	AB984442-AB984448
Lu. ayacuchensis	1	San José de Lourdes	Cajamarca	CDC	2013	DBSFP092-15	AB984449
Lu. peruensis	2	Ambar	Lima	CDC	2007	DBSFP093-15- DBSFP094-15	AB984450-AB984451
Lu. peruensis	3	La Cuesta	La Libertad	CDC	2007	DBSFP095-15- DBSFP097-15	AB984452-AB984454
Lu. peruensis	1	Cusipata	Cusco	CDC	2011	DBSFP098-15	AB984455
Lu. peruensis	1	Urubamba	Cusco	CDC	2012	DBSFP099-15	AB984456
Lu. peruensis	3	Ollantaytambo	Cusco	CDC	2012	DBSFP100-15- DBSFP102-15	AB984457-AB984459
Lu. verrucarum	3	Huancabamba	Piura	CDC	2004	DBSFP103-15- DBSFP105-15	AB984460-AB984462
Lu. verrucarum	2	San Antonio de Chaclla	Lima	CDC	2013	DBSFP106-15- DBSFP107-15	AB984463-AB984464
Lu. verrucarum	6	Santa Eulalia	Lima	CDC	2013	DBSFP108-15- DBSFP113-15	AB984465-AB984470
Lu. nuneztovari	3	Maranura	Cusco	CDC	2007	DBSFP114-15- DBSFP116-15	AB984471-AB984473
Lu. noguchii	9	Pullo	Ayacucho	CDC	2007	DBSFP117-15- DBSFP125-15	AB984474-AB984482
Lu. nevesi	2	Tarapoto	San Martin	CDC	2007	DBSFP126-15- DBSFP127-15	AB984483-AB984484
Lu. maranonensis	2	San José de Lourdes	Cajamarca	CDC	2001	DBSFP128-15- DBSFP129-15	AB984485-AB984486
Lu. maranonensis	12	Querocotillo	Cajamarca	CDC	2006	DBSFP130-15- DBSFP141-15	AB984487-AB984498
Lu. robusta	2	San José de Lourdes	Cajamarca	ShT	2001	DBSFP142-15- DBSFP143-15	AB984501-AB984502
Lu. robusta	10	San José de Lourdes	Cajamarca	CDC	2001	DBSFP144-15- DBSFP153-15	AB984503-AB984512
Lu. robusta	5	San Juan de Cutervo	Cajamarca	CDC	2007	DBSFP154-15- DBSFP158-15	AB984513-AB984517
Wa. euniceae	1	Alto Inambari	Puno	ShT	2013	DBSFP159-15	AB984520

Lu: *Lutzomyia*; *Wa*: *Warileya*; CDC: CDC light trap; ShT: Shannon trap; BOLD: Barcode of Life Data system.

Table 2. Intraspecific Kimura 2-Parameter (K2P) pairwise distances of sand fly species from Peru

Subgenera/Species group	Sand fly species	No. of specimens	Minimum (%)	Mean (%)	Maximum (%)
Lutzomyia	*Lu. battistinii*	10	0	0.27	0.46
	Lu. bicornuta	8	0	0.76	1.25
Verrucarum	*Lu. verrucarum*	11	0	1.59	3.48
	Lu. nuneztovari	3	0	0.30	0.46
Pifanomyia	*Lu. maranonensis*	14	0	0.81	1.87
	Lu. nevesi	2	0.93	0.93	0.93
	Lu. robusta	17	0	0.37	1.55
Helcocyrtomyia	*Lu. tejadai*	11	0	0.16	0.46
	Lu. quillabamba	2	0.15	0.15	0.15
	Lu. caballeroi	10	0	0.05	0.15
	Lu. castanea	9	0	0.49	1.24
	Lu. pescei	10	0	0.58	1.69
	Lu. ayacuchensis	8	0.30	1.85	2.76
	Lu. peruensis	10	0	3.36	5.96
	Lu. munaypata	10	0	0.28	0.46
	Lu. noguchii	9	0.15	0.92	1.72
Pintomyia	*Lu. fischeri*	12	0	0.19	0.75
Blancasmyia	*Lu. gorbitzi*	2	0.15	0.15	0.15
–	*Wa. euniceae*	1		N/A	

N/A: not applicable.

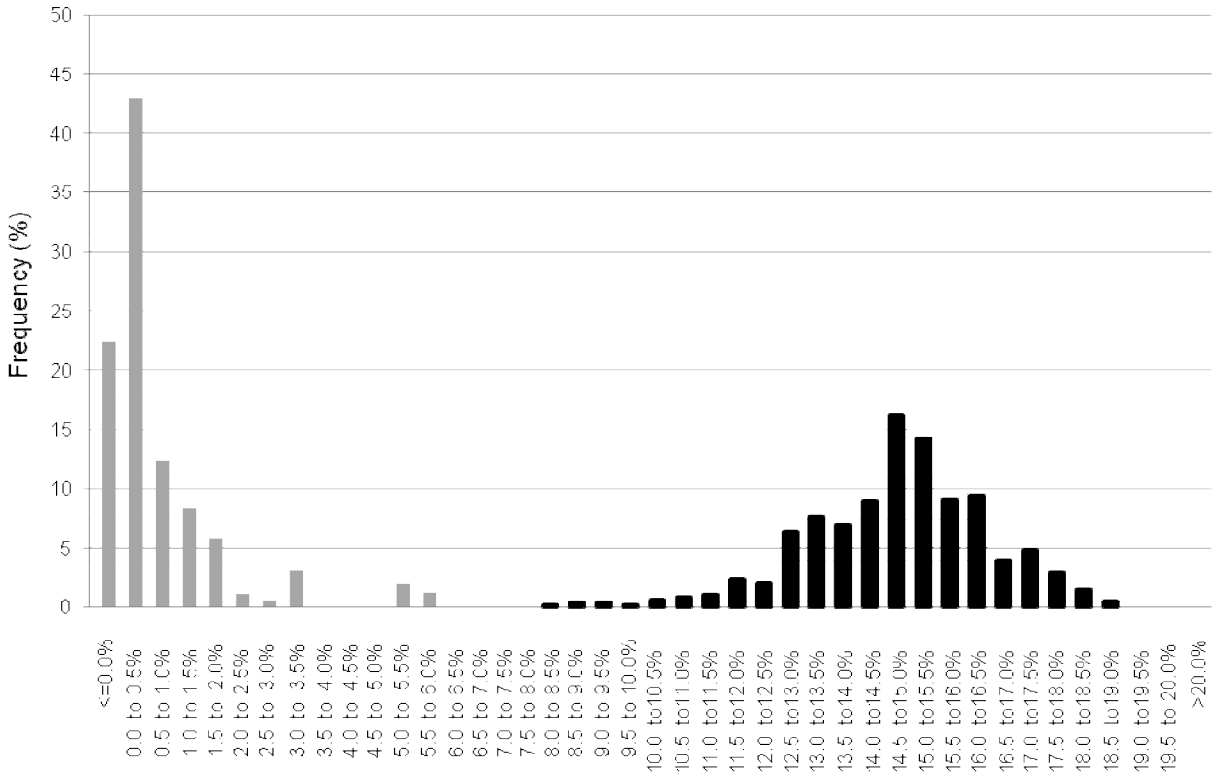

Fig. 3. Distribution of intraspecific (grey) and interspecific (black) genetic distances for the cytochrome c oxidase subunit I (COI) sequences of sand fly species from Peru.

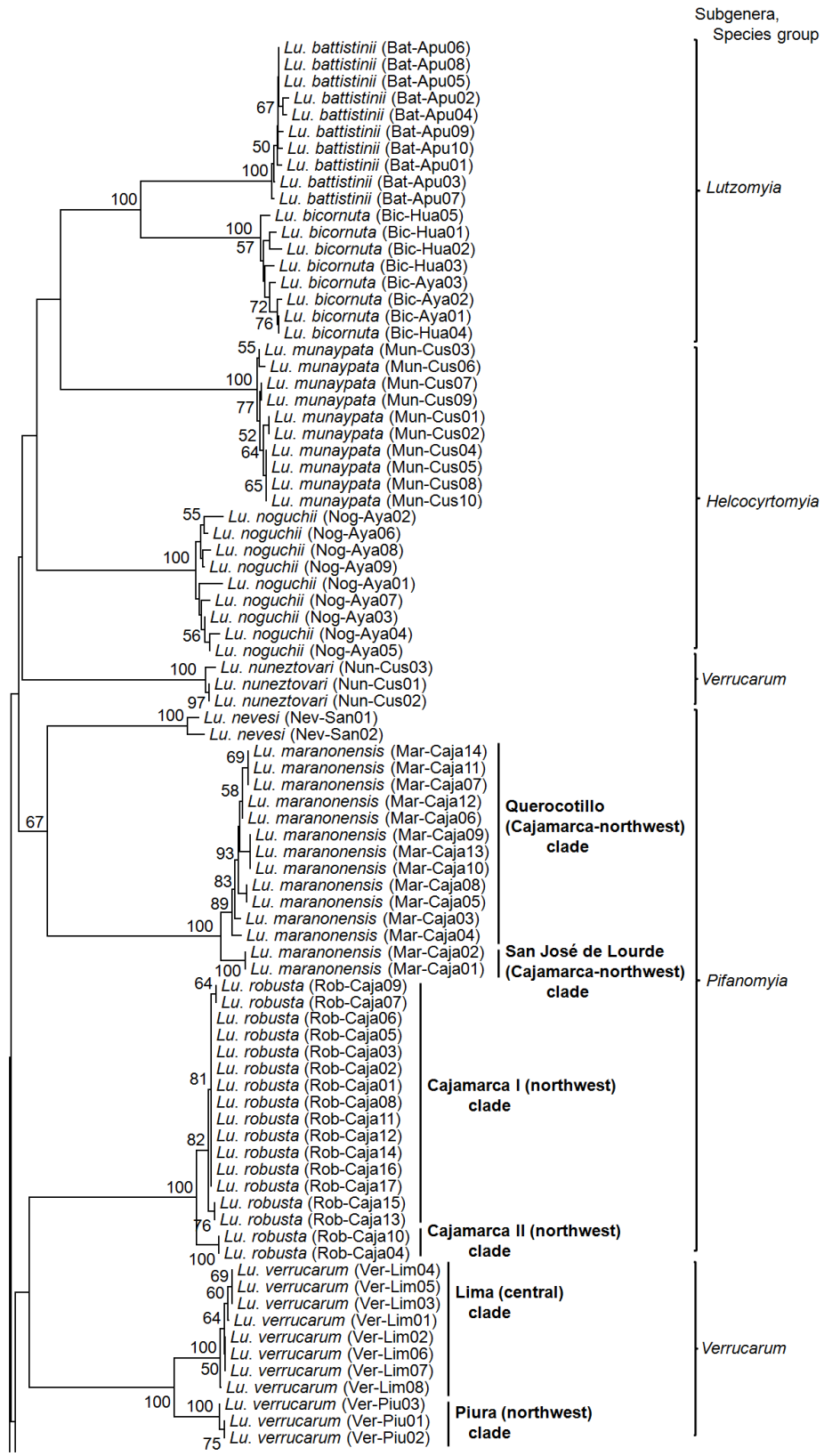

Fig. 4. (Continued)

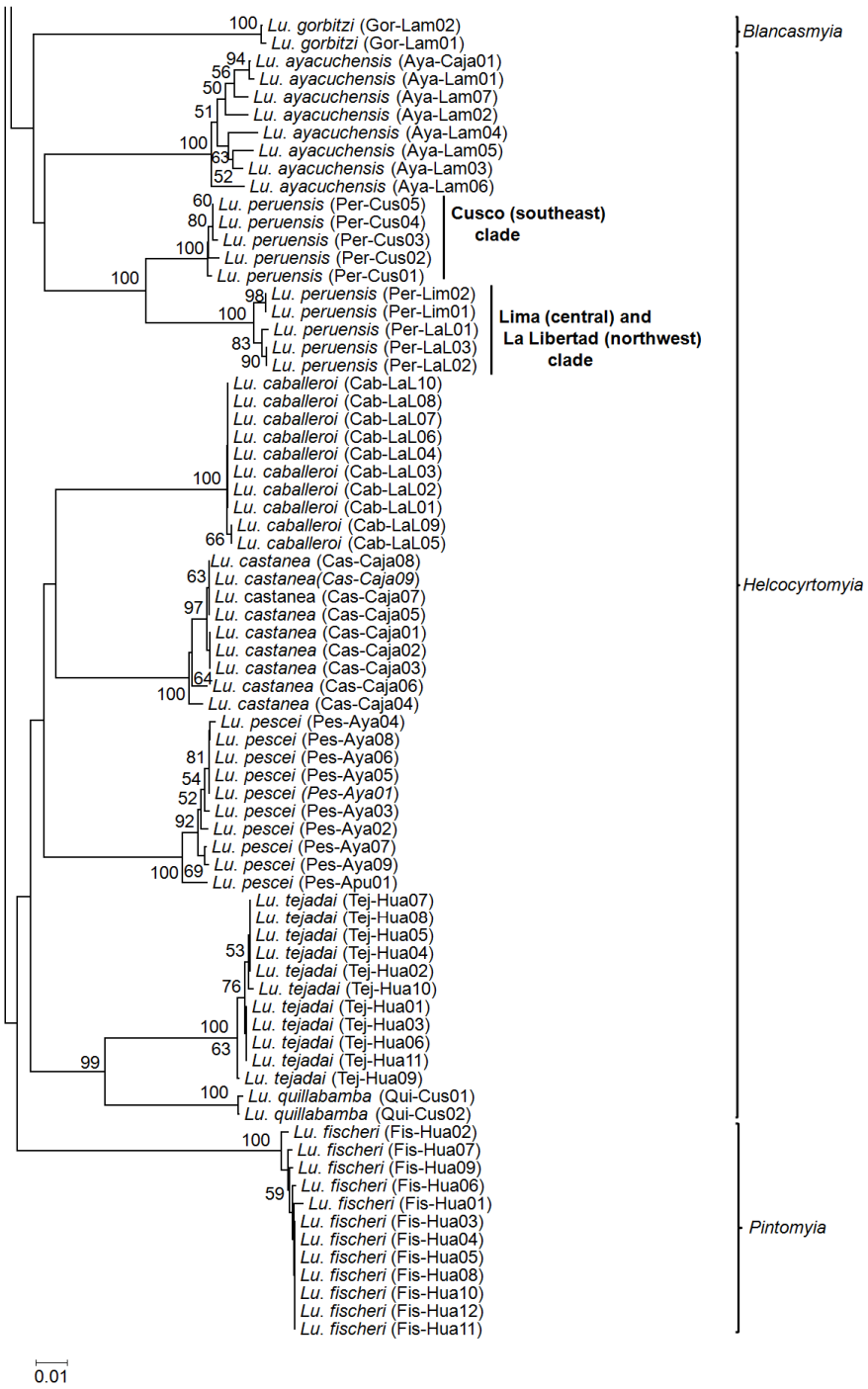

Fig. 4. Neighbor-joining tree based on Kimura 2-parameter genetic distances of mitochondrial COI sequences of *Lutzomyia* sand fly species from Peru.

The bar scale represents 0.01% divergences. Bootstrap values are shown above or below branches. Specimen IDs (in parentheses).

4. Discussion

Adequate knowledge of the ecological and medical importance of sand flies relies on correct identification of these minute and fragile insects. The gold standard for any robust taxonomic system is its ability to deliver reliable and accurate species identifications (Hebert et al., 2003a). In the present study, the targeted COI fragments were recovered and aligned from all sand fly species examined. The strongly biased AT-richness noted within the DNA barcode sequences was consistent with results from other insects including different sand fly genera (Cywinska et al., 2006; Pramual et al., 2011; Kumar et al., 2012; Contreras Gutiérrez et al., 2014). The range of intraspecific variation was between 0 and 5.96%, while the interspecific variability for species differentiation ranged between 8.39 and 19.08%. The range of intraspecific genetic divergence in this study, 0 to 5.96%, was very similar to values reported for New World sand flies in Colombia (0 to 6%; Contreras Gutiérrez et al., 2014), whereas the 5.96% maximum intraspecific divergence was higher than that earlier reported for Old World sand flies in India (2%; Kumar et al., 2012). High intraspecific sequence divergence was found among the three vector species of *Leishmania* (*Viannia*) *peruviana* in Peru (Perez et al., 1991, 1994, 2007; Davies et al., 1993; Caceres et al., 2004; Kato et al., 2008, 2011a); *Lu*. (*Helcocyrtomyia*) *peruensis* (5.96% maximum intraspecifc divergence), *Lu*. (*Verrucarum*) *verrucarum* (3.48%), and *Lu.* (*Helcocyrtomyia*) *ayacuchensis* (2.76%) (Table 2). Overall, there were no overlaps between the intra- and inter-specific divergence levels, despite the high values of intraspecific divergences observed in some species, supporting the utility of mtDNA COI in discriminating species (Hebert et al., 2003b).

Inclusion of DNA sequence data in phylogenetic analysis of individuals allows for quick determination of monophyletic groups and recognition of hidden or potential species. In addition to delineation of species, DNA barcoding can reveal cryptic diversity (Hebert et al., 2004;

Pramual et al., 2011; Kumar et al., 2012). The Neighbor-joining (NJ) tree analysis distinctively clustered the COI sequences obtained from individual *Lutzomyia* sand flies as previously determined by morphological features; specimens of single species formed barcode clusters with tight cohesion that were clearly distinct from those of allied species. However, in this study four taxa, *Lu. peruensis*, *Lu. verrucarum*, *Lu. maranonensis* and *Lu. robusta* were found to be complexes of at least 2 genetically distinct groups. The NJ tree revealed two deep-divergence reciprocally monophyletic clades in *Lu. peruensis*, the main vector of *L.* (*V.*) *peruviana* (Perez et al., 1991, 2007; Kato et al., 2008, 2011a) that clustered by geographic collection area (Fig. 4), and indicated that the *Lu. peruensis* group from Cusco (southeast) was genetically distinct from those from Lima (central) and La Libertad (northwest). A similar scenario was previously observed in *Lu. peruensis* based on the analysis of *cyt* b gene, among specimens from two regions, Ancash and Lima (central), and La Libertad (northwest) (Yamamoto et al., 2013). Additionally, high genetic distance between the Lima (central) and Piura (northwest) groups of *Lu. verrucarum* was observed. Therefore, these observed genetic distances among *Lu. peruensis* and *Lu. verrucarum* groups indicated that the groups in each taxa were highly isolated, with little or no genetic exchange between the groups.

Phlebotomine sand flies are known to have limited sustained flight and tend to remain associated with their emergence sites, typically not dispersing more than a half kilometer (Alexander, 1987; Killick-Kendrick, 1990; Morrison et al., 1993). Such putatively low flight capacity and dispersal range of sand flies coupled with the heterogeneous terrain of the Andes may cause the isolation of these populations throughout their distributions, thereby producing genetically distinct but morphologically similar individuals. Consequently, genetic differentiation observed within some species, may be ascribable to local adaptation or genetic

drift. Moreover, the observation of genetic differentiation among the vector species (*Lu. peruensis*, *Lu. verrucarum*, and *Lu. ayacuchensis*) may have significant implications for epidemiological studies and vector control strategies. Indeed it has been shown that insecticide susceptibility and vector capacity can vary within evolutionary lineages, even within the same species (Lanzaro et al., 1993; O' Loughlin et al., 2008; Hassan et al., 2012). Collectively, the fact that some species showed higher conspecific divergence does not compromise the use of COI sequences for their identification; rather, it allows delineation of the regional lineages that constitute them (Hebert et al., 2003b). Nonetheless, further investigations with more specimens from various localities will be required to provide additional information on the levels of divergence within these vector species and other prevalent sand fly species in Peru. Such studies may also contribute to more effective leishmaniasis control in endemic areas.

In conclusion, this study provided the COI barcodes for several Peruvian sand flies and showed their effectiveness in discriminating species recognized through prior conventional taxonomic work. Aside from its success in discriminating known species, DNA barcoding was found to be useful in revealing population differentiation. A better understanding of the implication of the genetic differentiation among the vector species will require further molecular (genetic information from multiple loci), ecological and morphological studies.

Summary

Phlebotomine sand flies are the only proven vectors of leishmaniases, a group of human and animal diseases. Accurate knowledge of sand fly species identification is essential in understanding the epidemiology of leishmaniasis and vector control in endemic areas. Classical identification of sand fly species based on morphological characteristics often remains difficult and requires taxonomic expertise. Here, DNA barcodes of the cytochrome c oxidase subunit 1 (COI) gene using 159 adult specimens morphologically identified to be 19 species of sand flies, belonging to 6 subgenera/species groups circulating in Peru, including the vector species were generated. Neighbor-joining analysis based on Kimura 2-Parameter genetic distances formed non-overlapping clusters for all species. The levels of intraspecific genetic divergence ranged from 0 to 5.96%, whereas interspecific genetic divergence among different species ranged from 8.39 to 19.08%. The generated COI barcodes could discriminate between all the sand fly taxa. Besides its success in separating known species, DNA barcoding was found to be useful in revealing population differentiation and cryptic diversity, and thus promises to be a valuable tool for epidemiological studies of leishmaniasis.

Chapter II

First detection of *Leishmania tropica* DNA and *Trypanosoma* species in *Sergentomyia* sand flies (Diptera: Psychodidae) from an outbreak area of cutaneous leishmaniasis in Ghana

1. Introduction

Cutaneous leishmaniasis (CL) is a public health problem caused by cell-infecting flagellate protozoa of the genus *Leishmania*, transmitted by female phlebotomine sand flies. The disease occurs in 98 tropical, subtropical and temperate countries worldwide and it is estimated that 1.2 million new cases of CL occur per year (Alvar et al., 2012). CL of the Old World is caused by five species of *Leishmania*: *Leishmania* (*Leishmania*) *tropica*, *L.* (*L.*) *major*, *L.* (*L.*) *aethiopica*, *L.* (*L.*) *infantum* and *L.* (*L.*) *donovani* (WHO, 2010). Clinically, cutaneous lesions due to *L.* (*L.*) *tropica* last much longer and are more difficult to treat than those due to *L.* (*L.*) *major* (Klaus and Frankenburg, 1999). To date, there is no information available on the distribution of *L.* (*L.*) *tropica* in West Africa.

Sand fly species of the genus *Phlebotomus* are the putative vectors of *Leishmania* in the Old World (Munstermann, 2004); however, few studies have recently suggested the possible involvement of some species of the genus *Sergentomyia* in the transmission of *Leishmania* in the Old World. Studies conducted in cutaneous leishmaniasis foci in Iran, Mali and Portugal have shown the detection of *L.* (*L.*) *major* DNA in *S. sintoni* (Parvizi and Amirkhani, 2008), *S. darlingi* (Berdjane-Brouk et al., 2012) and *S. minuta* (Campino et al., 2013), respectively. Earlier, *L.* (*L.*) *major* was also isolated from *S. garnhami* in Kenya (Mutinga et al., 1994). Other reports have also detected *L.* (*L.*) *donovani* DNA in *S. babu* in India (Mukherjee et al., 1997) and more recently, *L. siamensis* DNA in *S. gemmea* in Thailand (Kanjanopas et al., 2013).

In Ghana, *L.* (*L.*) *major* (Fryauff et al., 2006) and uncharacterized species (Villinski et al., 2008) have been identified as the causative agents of human cutaneous leishmaniasis in an area of CL outbreak. In order to control the transmission of *Leishmania* spp. in an endemic area, information on potential sand fly vectors as well as their associated *Leishmania* species is of

paramount importance since vector dispersion is one of the major factors that determine the potential rate of pathogen dissemination. Thus, this study was designed to determine and detect *Leishmania* DNA within sand flies collected in the outbreak area of CL in Ghana by molecular analysis in order to ascertain if they were infected by either *L*. (*L*.) *major* (Fryauff et al., 2006) or the yet to be characterized species (Villinski et al., 2008). This information is necessary to implicate the potential vectors in the area for a better understanding of the epidemiology of leishmaniasis in Ghana. Here, the confirmation of *L*. (*L*.) *major* DNA and first detection of *L*. (*L*.) *tropica* DNA in *Sergentomyia* species using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing analyses, as well as *Trypanosoma* species associated with sand fly infections in Ghana were reported.

2. Materials and methods

2.1. Sand fly capture and taxonomic identification

Previously collected indoor resting sand flies from three CL outbreak communities; Hlefi, Klefe and Taviefe in Ho District of Ghana were used in this study (Fig. 5). Verbal informed consent was obtained from residents in each of the community. The collections were done in August, September and November 2007 from human habitats using manual aspirator. Each unfed and blood-fed female fly was dissected under sterile conditions by cutting off the head and last three abdominal segments with a pair of sterilized entomological needles and forceps. The head and last three abdominal segments of each fly was mounted on microscope slides in Puri's medium and identified using taxonomic keys (Abonnenc, 1972; Niang et al., 2000). For each blood-fed fly, the remainder of the body was processed under sterile condition and subjected to blood-meal analysis (unpublished results; Kweku et al., 2011). Afterwards, the dissected thorax and attached anterior abdomen of unfed and the processed specimens known to have fed on human-blood (unpublished results; Kweku et al., 2011) were separately pooled according to species and locality (Table 3) for DNA extraction and infection molecular analysis.

2.2. DNA extraction, Leishmania ITS1 PCR-RFLP and trypanosomatid SSU rRNA gene amplification

Ten (10) unfed and blood-fed flies belonging to the same species were pooled and homogenized in animal tissue lysis buffer (Qiagen) containing proteinase K. DNA was extracted using the Qiagen DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer instructions and 0.5 µl of the extract was used as PCR templates.

The leishmanial ribosomal internal transcribed spacer 1 (ITS1) region was amplified using primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3') (El Tai et al., 2000). The reactions were carried out in volumes of 25 µl containing 200 µM of each dNTP, 1.5 mM $MgCl_2$, 2 U Taq polymerase and 500 nM of each primer. Each PCR reaction included a positive control (DNA from a reference strain: *L.* (*L.*) *major* - IPAP/EG/1989/S1-177 and *L.* (*L.*) *tropica* - MHOM/SU/1974/K27) and a negative control (water). After initial denaturation at 95 °C for 2 min, PCR amplification were performed with 34 cycles consisting of denaturation (95 °C for 20 sec), annealing (53 °C for 30 sec), and extension (72 °C for 1 min) followed by a final extension cycle at 72 °C for 6 min. An aliquot of 5 µl of each PCR reaction was separated by electrophoresis on 2% agarose gel and visualized. Each ITS1 amplicon was digested with the restriction endonuclease *Hae*III (Invitrogen) for the species identification. The restriction fragments were run and visualised on 2% agarose gel and compared with those of reference strains of *L.* (*L.*) *major* (IPAP/EG/1989/S1-177) and *L.* (*L.*) *tropica* (MHOM/SU/1974/K27).

For the identification of *Trypanosoma* species, the small subunit ribosomal RNA (SSU rRNA) gene was amplified from the sand fly using primer specific for SSU rRNA gene of trypanosomatids (TRY927F: 5'-GAAACAAGAAACACGGGAG-3' and TRY927R: 5'-CTACTGGGCAGCTTGGA-3') (Noyes et al., 1999; Hamilton et al., 2004; Kato et al., 2010a).

2.3. PCR amplification of sand fly COI and18S rRNA genes

Validation of morphologically identified sand fly specimens was done using mitochondrial cytochrome c oxidase gene subunit I (COI) and 18S rRNA genes. Amplification of the COI gene was accomplished using the primers LCO I490 (5'-GGTCAACAAATCATAAAGATATTGG-3')

and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994; Hebert et al., 2003a). The reaction was carried out in a volume of 15 µl using a pair of primers (1 µM each) and 2 x AmpliTaq Gold PCR Master Mix (Applied Biosystems, NJ, USA). After initial denaturation at 95 °C for 5 min, amplification was performed with 37 cycles consisting of denaturation at 94 °C for 30 sec, annealing at 55 °C for 45 sec, extension at 72 °C for 1 min 30 sec, followed by a final extension at 72 °C for 10 min. For the 18S rRNA locus, the sequence of interest was amplified using the primers Lu. 18S rRNA-1S (5'-TGCCAGTAGTTATATGCTTG-3') and Lu. 18S rRNA-1R (5'-TTACGCGCCTGCTGCCTTCC-3') (Kato et al., 2005). The reaction was carried out in a volume of 15 µl using a pair of primers (0.4 µM each) and 2 x AmpliTaq Gold PCR Master Mix (Applied Biosystems, NJ, USA). An initial denaturation was done for 5 min at 95 °C, followed by PCR amplification for 30 cycles of denaturation (95 °C for 1 min), annealing (55 °C for 1min), and polymerization (72 °C for 1 min), with a final extension of 10 min at 72 °C. Amplified products were resolved on 2% agarose gels.

2.4. Molecular cloning and nucleotide sequencing

Infection and sand fly PCR products were directly cloned into plasmid using a pGEM-T Easy Vector System (Promega, Madison, WI). *Escherichia coli* DH5 α cells was transformed with a ligated product and plated onto Luria Bertani agar plates containing ampicillin (50 µg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactoside (36 µg/ml), and isopropyl β-D-thiogalactoside (40 µg/ml). Plasmid DNAs were extracted with QIAprep Spin Miniprep Kit (Qiagen) and the insert of the plasmids were sequenced by the dideoxy chain termination method using BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

2.5. Phylogenetic analysis

Sequences from both strands were aligned using Clustal W software (Thompson et al., 1997) and imported into MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 (Tamura et al., 2007). Phylogenetic trees were constructed by the neighbor-joining (NJ) method with the algorithm of MEGA program. Bootstrap values were determined with 1,000 replicates of the datasets. ITS1 gene sequences used for the analysis were *L.* (*L.*) *major* (IPAP/EG/1989/SI-177, DQ295824; MHOM/GH/2004/HO-004, DQ295825; MHOM/EG/2006/RTC-64, FJ460456; MHOM/TN/1997/LPN162, FN677342; MHOM/IL/1967/JERICHO II, EU326229), *L.* (*L.*) *tropica* (MHOM/EG/2006/RTC-66, FJ460457; IROS/NA/1976/ROSSI-II, AJ000302; MHOM/KE/1984/NLB297, AJ000301; MHOM/TN/1988/TAT3, AJ300485) and *Leishmania* sp. (MHOM/GH/2006/TVE, EF524071). The trypanosomatid SSU rRNA gene sequence was analyzed with those of *T. avium* (AB566384, AF416559), *T. brucei rhodesiense* (AJ009142), *T. corvi* (AY461665), *T. microti* (AJ009158), *T. kuseli* (AB175626), *T. cruzi* (AJ009149, AJ009150), *T. rangeli* (AJ012417), *T. congolense* (AJ009145, AJ009146), *T. mega* (AJ009157), *T. rotatorium* (AJ009161), *T. fallisi* (AF119806) and *Trypanosoma* species isolated from toads (EU021231, EU021232), *Lutzomyia* sand flies (EU021241, EU021242, EU021243, EU021244, EU021245, EU021237), *Phlebotomus* sand fly (AB520638), mosquitoes (AF416561), and hippoboscid (AF416562).

Sand fly COI gene sequences were analyzed with those of *Sergentomyia babu babu* (HQ585351, HQ585357), *S. vadhanurensis* (HQ585345, HQ585348), *S. bailyi* (HQ585381 and HQ585384), *S. punjabenensis* (HQ585375, HQ585379), *Phlebotomus papatasi* (JN172077) and the New World sand fly species *Lutzomyia longiflocosa* (FJ437273). The sand fly 18S rRNA gene sequences were analyzed with those of *S. barraudi* (JQ790518), *S. queenslandi* (HM775498), *S.*

magna (AJ391741), *S. babu* (JN581685), *S. minuta* (AJ244419), *S. schwetzi* (AJ391739), *S. buxtoni* (AJ391737), *S. dentata* (AJ244423), *S. dubia* (AJ391738), *S. clydei* (AJ391742), *S. ghesquierei* (AJ391743), *P. papatasi* (AJ391726) and the New World sand fly species *Lu. longipalpis* (AJ244429).

2.6. Statistical analysis

The infection rate in sand flies was determined using the poolScreen2 program generously provided by Dr. Charles Katholi (Department of Biostatistics and Division of Geographic Medicine, University of Alabama at Birmingham, USA) (Katholi et al., 1995). The algorithm was used to calculate the maximum likelihood estimate (MLE) of infection in sand fly species at 95% confidence intervals.

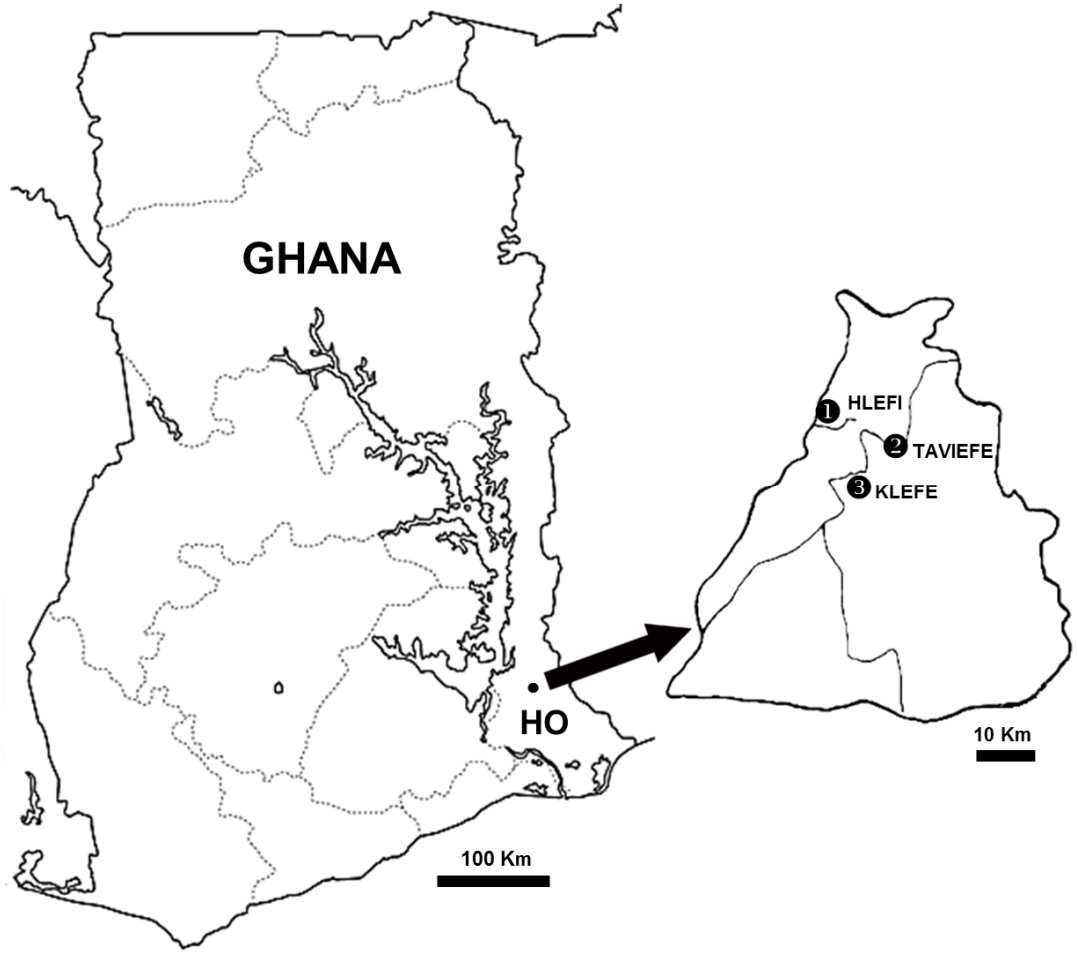

Fig. 5. Map of Ghana showing the location of Ho District and outset showing the areas where sand flies were collected.

(Adapted from a map available at http://english.freemap.jp/; Fryauff et al., 2006)

3. **Results**

3.1. Detection of trypanosomatids within sand flies

Sergentomyia sand flies of 102 pools were analyzed (Table 3), each containing ten females (in total 1,020 insects): *S. africana africana* (2 blood-fed, 28 unfed pools), *S. ingrami* (4 blood-fed, 21 unfed pools), *S. simillima* (4 blood-fed, 19 unfed pools), *S. dissimillima* (19 unfed pools), and *S. hamoni* (5 unfed pools). ITS1-PCR showed 6 positives for trypanosomatids, with a unique band of approximately 340 bp in 5 pools, namely, 4 *S. ingrami* and 1 *S. hamoni* as expected from the results obtained from the reference strains and the other yielding a fragment of approximately 500 bp in 1 *S. africana africana* pool (Fig. 6A). No *Leishmania* DNA was detected in *S. simillima* and *S. dismillima* pools from the three CL foci. The six positive samples were subjected to RFLP for species identification and their subsequent digestion with the endonuclease *Hae*III produced fragments characteristic of *L.* (*L.*) *major* in samples 3-5 and the *L.* (*L.*) *major* reference DNA, and *L.* (*L.*) *tropica* in samples 6-7 and the *L.* (*L.*) *tropica* reference DNA, whereas sample 8 displayed an uncharacteristic RFLP pattern (Fig. 6B). To confirm authenticity of the RFLP analysis, both the 340 bp and 500 bp ITS1 PCR products obtained in the sand fly positive samples were subjected to sequencing analysis. The ITS1 DNA sequences obtained from the 3 (1 blood-fed from Klefe, 2 unfed from Hlefi) *S. ingrami* pools [IING/GH/2007/HLE-9 (GeneBank accession number: AB759711), IING/GH/2007/KLE-2 (AB759712), and IING/GH/2007/HLE-22 (AB759713)] were identical to each other and showed 99% identity to several *L.* (*L.*) *major* ITS1 sequences in GenBank, including isolates from Ghana, Egypt, Kenya, Sudan and Tunisia. The ITS1 sequences obtained from 1 unfed *S. ingrami* pool from Taviefe [IING/GH/2007/TAV-51 (AB787189)] and 1 unfed *S. hamoni* pool from Klefe [IHAM/GH/2007/KLE-18 (AB787190)] were different at 4 positions of the nucleotides, which include the deletion of 2 nucleotides in the former. These

samples showed high similarity with several *L.* (*L.*) *tropica* ITS1 sequences in GenBank at 99% identity. In the phylogenetic tree, the 3 *L.* (*L.*) *major* sequences determined in this study were grouped into *L.* (*L.*) *major* cluster with 100% bootstrap support value, and the 2 *L.* (*L.*) *tropica* sequences determined were located in *L. tropica* cluster (Fig. 7).

The ITS1 gene sequence of the 500 bp amplicon was determined and analyzed with the NCBI BLAST. Unexpectedly, the sequence did not match with those of *Leishmania* species but rather showed similarity to *Trypanosoma* spp., suggesting that the DNA sequence obtained belongs to the genus *Trypanosoma.* For further identification, the sample was analyzed with the SSU rRNA gene because this gene has been well studied in trypanosomatids (Noyes et al., 1999; Hamilton et al., 2004; Kato et al., 2010a). The SSU rRNA gene sequence obtained [IAFR/GH/2007/HLE-82 (AB787191)] had 93% identity with those of trypanosomes isolated from toads. A phylogenetic tree constructed with this sequence and SSU rRNA sequences of *Trypanosoma* species showed that the *Trypanosoma* DNA sequence determined from *S. africana africana* pool collected from Hlefi was located in the clade comprising trypanosomes isolated from Brazilian toads (*Trypanosoma* sp. 362 and 364) (Ferreira et al., 2008), but the sequence did not completely match those from any reported species (Fig. 8). In addition, the *Trypanosoma* SSU rRNA sequence determined was separated from *Trypanosoma* species isolated from Amazon sand flies (*T.* sp. 101, 120, 887, 103, 888, and 1155) (Ferreira et al., 2008) and in a desert area of Pakistan (*T.* sp. SKF32) (Kato et al., 2010a), which have closer relationships with anuran trypanosomes (Fig. 8). This result suggests that the *Trypanosoma* DNA detected within *S. africana africana* in this study is a novel or genetically uncharacterized *Trypanosoma* species possibly of amphibians.

The minimum infection rate, assuming that one infected insect was present in each positive pool was 0.49% for *Leishmania* spp. and 0.10% for *Trypanosoma* infection among the total

population of pooled female sand flies. Using poolScreen2 algorithm, the maximum *Leishmania* DNA infection rate was estimated as follows: *L*. (*L*.) *tropica* infection rate in *S. ingrami* (1 out of 25 pools) and *S. hamoni* (1 out of 5 pools) were 4.07% (95% CI 1.259−2.080) and 2.21% (95% CI 6.864−1.094), respectively. *Trypanosoma* DNA infection in *S. africana africana* (1 out of 30 pools) was estimated at 3.38% (95% CI 1.045−1.731).

3.2. Validation of sand fly species by molecular biological method

The COI gene sequences from infected flies (accession numbers: AB759971−AB759973, AB787192−AB787194) showed greater homology with those of *Sergentomyia* species registered in GeneBank than any other genus. A phylogenetic analysis was performed to observe the phylogenetic relationships of COI among *Sergentomyia* species. Among the three different infected *Sergentomyia* species included in phylogenetic analysis, only *S. africana africana* of the subgenus *Parrotomyia* was monophyletic (Fig. 9). All the four *S. ingrami* and one *S. hamoni* samples of the subgenera *Neophlebotomus* and *Sergentomyia*, respectively, were clustered in the same clade (Fig. 9). Similarly, 18S rRNA gene sequences from infected flies (accession numbers: AB759714−AB759716, AB787195–AB787197) had a greater degree of homology with those of *Sergentomyia* species (98 − 99%) than any *Phlebotomus* and *Lutzomyia* species (92 − 97%). In the phylogenetic tree, all species characterized in this study and those of other *Sergentomyia* species were located in their respective subgenus cluster (Fig. 10). These results confirmed that sand flies infected by *Leishmania* DNA belong to the genus *Sergentomyia*.

Table 3. Summary of *Sergentomyia* sand flies screened by molecular biological method in this study

Location	Sand fly species	Number of unfed pools* (positive pools)	Number of blood-fed pools*¶ (positive pools)	Total
Klefe	*S. africana africana*	5	1	6
	S. ingrami	10	2 (1)[c]	12
	S. simillima	12	2	14
	S. dissimillima	13	0	13
	S. hamoni	5 (1)[a]	0	5
	Total	45	5	50
Hlefi	*S. africana africana*	19 (1)[b]	1	20
	S. ingrami	7 (2)[c]	1	8
	S. simillima	4	2	6
	S. dissimillima	5	0	5
	Total	35	4	39
Taviefe	*S. africana africana*	4	0	4
	S. ingrami	4 (1)[a]	1	5
	S. simillima	3	0	3
	S. dissimillima	1	0	1
	Total	12	1	13

*Ten female specimens in each pool.

¶ Identified human-blood fed specimens (unpublished results).

[a]*L.* (*L.*) *tropica* DNA detection according to sequence analysis.

[b]*Trypanosoma* sp. DNA detection according to sequence analysis.

[c]*L.* (*L.*) *major* DNA detection according to sequence analysis.

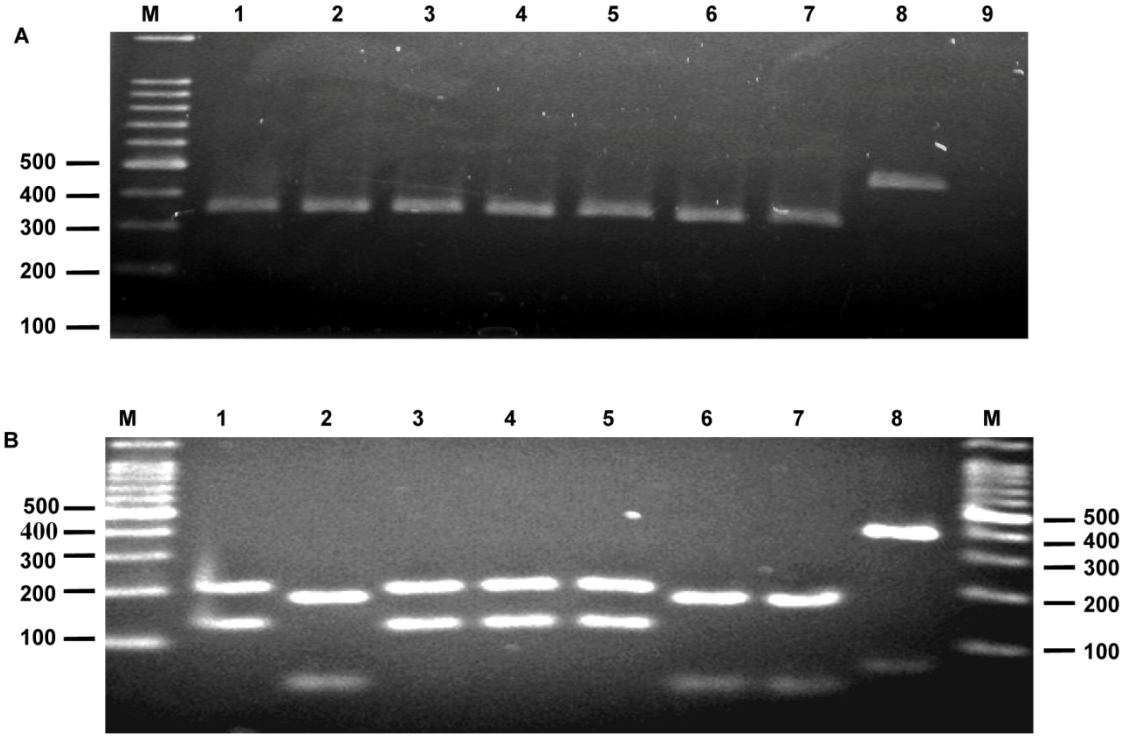

Fig. 6. Detection of *Leishmania* DNA in sand flies.

(A) PCR of *Leishmania* internal transcribed spacer 1 (ITS1) region amplified within pools of female *Sergentomyia* sand flies captured indoors and *Leishmania* spp. controls. M: 100 bp size marker; Lanes 1 and 2 (*L.* (*L.*) *major* and *L.* (*L.*) *tropica* reference strains, respectively); Lanes 3 to 6, *S. ingrami* pools (KLE-2, HLE-9, HLE-22 and TAV-51, respectively), Lane 7, *S. hamoni* pool (KLE-18); Lane 8, *S. africana africana* pool (HLE-82) (~500 bp) and Lane 9, negative control. (B) *Hae*III digestion of restriction fragment length polymorphisms of ITS1 PCR products shown in A. M: 100 bp size marker; Lane 1, *L.* (*L.*) *major* (IPAP/EG/1989/S1-177); Lane 2, *L.* (*L.*) *tropica* (MHOM/SU/1974/K27); Lanes 3 to 6, *S. ingrami* pools; Lane 7, *S. hamoni* pool and Lane 8, *S. africana africana* pool. HLE: Hlefi community; KLE: Klefe community; TAV: Taviefe community.

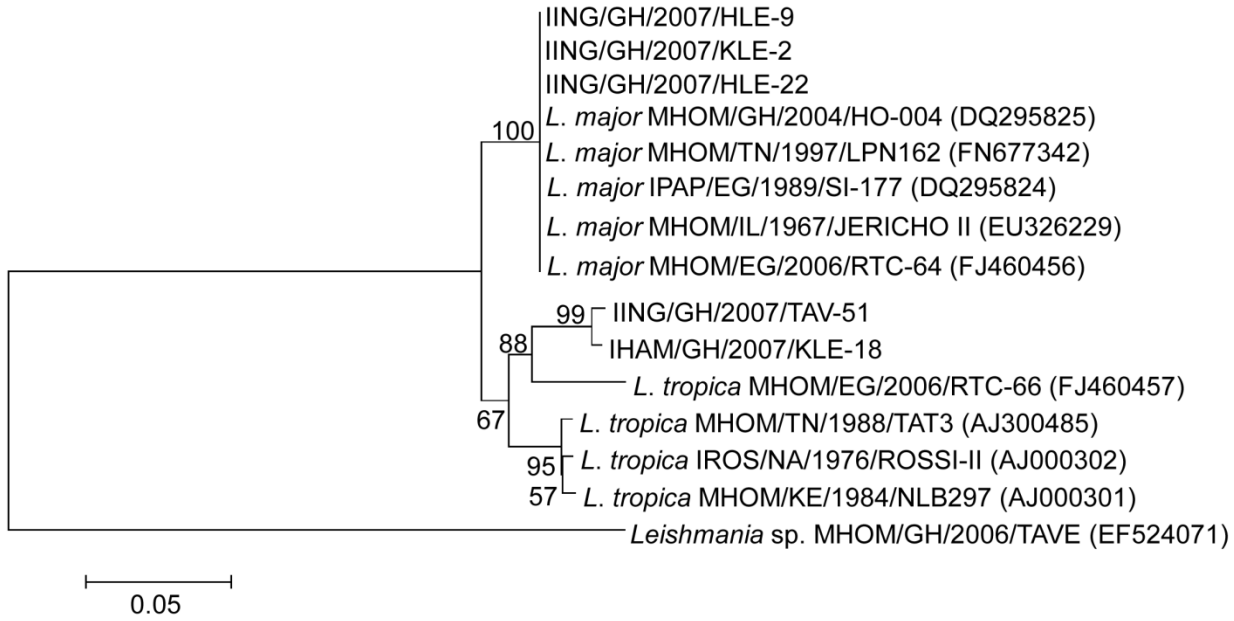

Fig. 7. Phylogenetic tree of ITS1 gene sequences among species.

Leishmania ITS1 gene was amplified within *Leishmania* DNA positive *S. ingrami* pools (IING/GH/2007/HLE-9, IING/GH/2007/KLE-2, IING/GH/2007/HLE-22 and IING/GH/2007/TAV-51) and *S. hamoni* pool (IHAM/GH/2007/KLE-18) and sequenced. Analysis was performed by the neighbor-joining method on the sequences together with those from 10 *Leishmania* species including the 2 human isolates from Ghana, *L. major* (MHOM/GH/2004/HO-004) and an uncharacterized *Leishmania* sp. (MHOM/GH/2006/TAVE). The scale bar represents 0.05% divergence. Bootstrap values are reported at nodes.

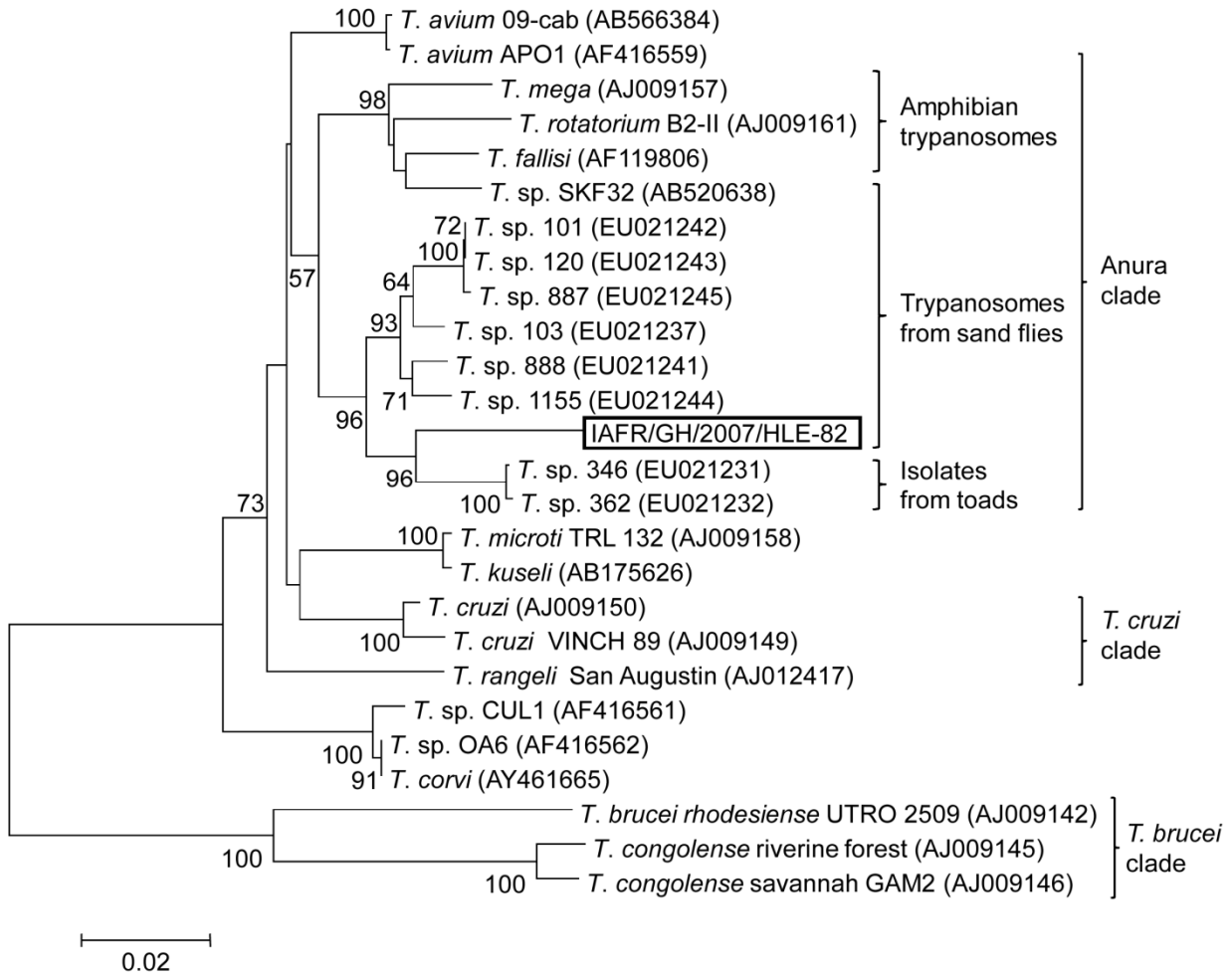

Fig. 8. Phylogenetic tree of SSU rRNA gene sequences among species.

The SSU rRNA (IAFR/GH/2007/HLE-82) gene was amplified within *S. africana africana* pool and sequenced. Phylogenetic analysis of the SSU rRNA gene sequences was performed by the neighbor-joining method on the sequence together with those from 25 *Trypanosoma* species. The sequences from the database are represented by the name of the species, isolates and GeneBank (accession number). The scale bar represents 0.02% divergence. Bootstrap values are reported at nodes.

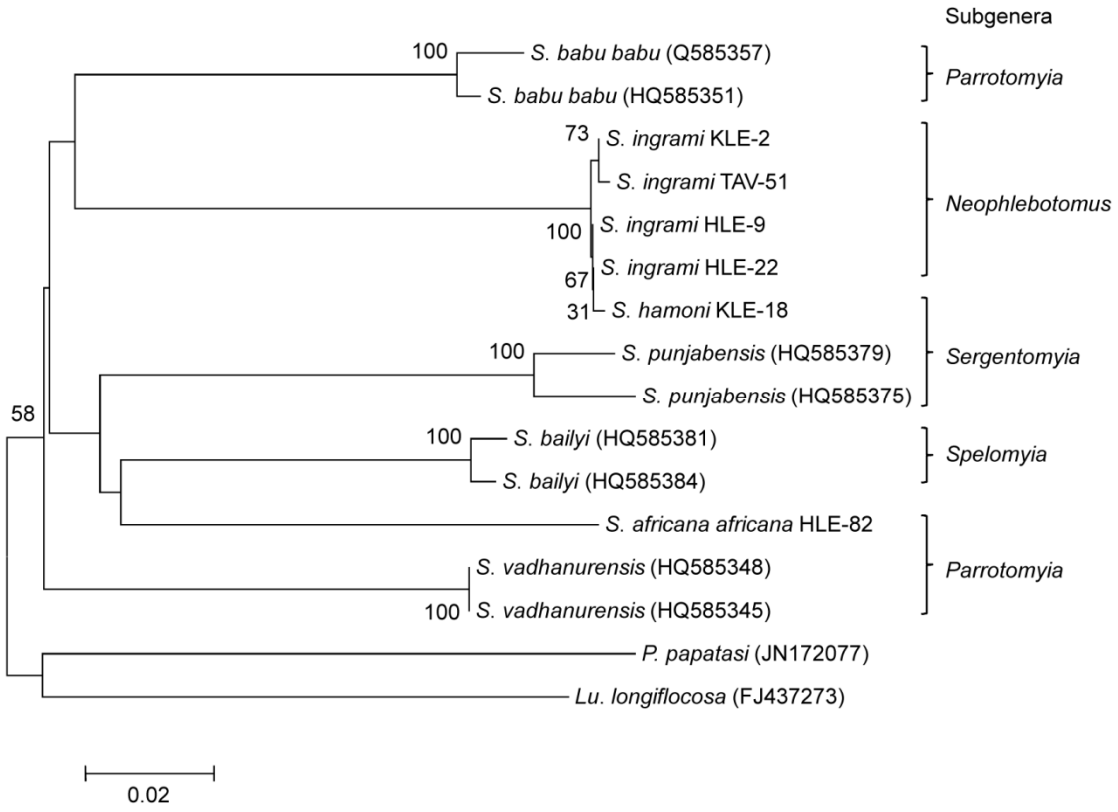

Fig. 9. Phylogenetic tree of COI gene sequences among sand fly species.

The COI (*S. ingrami* HLE-9, *S. ingrami* HLE-22, *S. ingrami* KLE-2, *S. ingrami* TAV-51, *S. hamoni* KLE-18 and S. *africana africana* HLE-82) gene was amplified from infected sand flies and sequenced. Analysis of the sequences together with those from *Sergentomyia* species, *Phlebotomus* species and *Lutzomyia* species registered in GeneBank were performed. The bar scale represents 0.02% divergences. Bootstrap values are shown above or below branches. HLE: Hlefi community; KLE: Klefe community; TAV: Taviefe community.

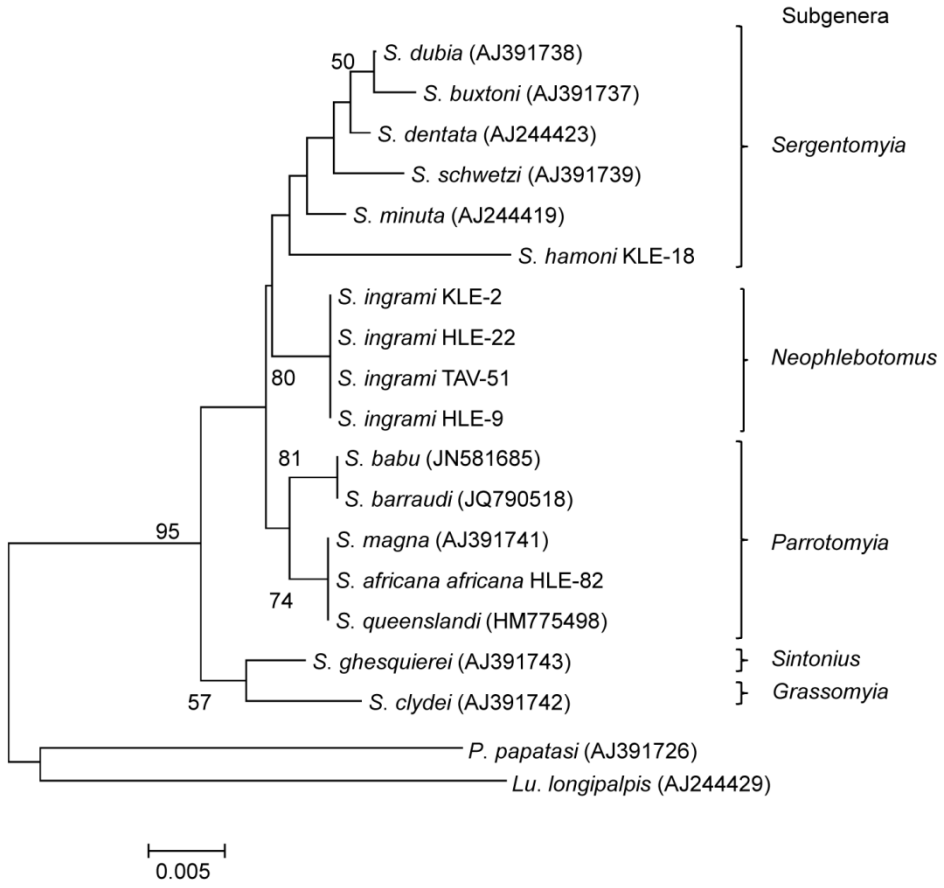

Fig. 10. Phylogenetic tree of 18S rRNA gene sequences among sand fly species.

The 18S rRNA (*S. ingrami* HLE-9, *S. ingrami* HLE-22, *S. ingrami* KLE-2, *S. ingrami* TAV-51, *S. hamoni* KLE-18 and S. *africana africana* HLE-82) gene was amplified from infected sand flies and sequenced. Analysis of the sequences together with those from *Sergentomyia* species, *Phlebotomus* species and *Lutzomyia* species registered in GeneBank were performed. The bar scale represents 0.005% divergences. Bootstrap values are shown above or below branches.

4. Discussion

In this study, the detection of *Leishmania* DNA within *Sergentomyia* species and their comparison with *Leishmania* species previously reported from humans in Ho District were revealed. The correct identification and comparison of *Leishmania* species from sand flies and humans is of epidemiological importance for predictions of the risk and expansion of the disease in endemic and surrounding areas. Different PCR-based techniques are applied either in individual sand flies (Kato et al., 2005; Berdjane-Brouk et al., 2012) or in sand fly pools (Martin-Sánchez et al., 2006).

Three cutaneous leishmaniasis foci Klefe, Hlefi and Taviefe were identified in Ho, where both *L.* (*L.*) *major* (Fryauff et al., 2006) and uncharacterized species (Villinski et al., 2008) were found as the etiologic agents. In the present study, only sand fly species of the genus *Sergentomyia* were identified from the CL focal area, consistent with previous reports from the same area (Boakye et al., 2005; Kweku et al., 2011) and suggests the possible involvement of this genus in the transmission of CL. The present study revealed infection of *L.* (*L.*) *major* DNA in *S. ingrami* pools, and *L.* (*L.*) *tropica* DNA in one *S. ingrami* and one *S. hamoni* pools by ITS1 PCR-RFLP and sequencing analyses. These observations represent the first detection of *L.* (*L.*) *tropica* DNA and confirmation of *L.* (*L.*) *major* DNA in sand flies by gene sequencing in Ghana and support the earlier hypothesis that species of the genus *Sergentomyia* might be the vectors of CL in this area (Boakye et al., 2005; Kweku et al., 2011). The detection of *L.* (*L.*) *tropica* DNA in Ho focus is a novel finding and the first report in West Africa. In addition, DNA of *Trypanosoma* species was detected within one *S. africana africana* pool by the SSU rRNA gene analysis, which also represents the first report on infection of sand fly by trypanosomes other than *Leishmania* in West Africa. Furthermore, the blood-meal analysis of the blood-fed flies used in this study, revealed 3

anthropophilic *Sergentomyia* species; *S. ingrami*, *S. africana africana* and *S. simillima* (unpublished results; Kweku et al., 2011).

Vector incrimination is an important part of any epidemiological studies on leishmaniasis. The vector role of a sand fly species in leishmaniasis focus is epidemiologically suspected when the species is predominant and proved anthropophilic behavior. This suspicion is strengthened when the same sand fly is found infected with the same leishmanial parasite as that found in man in the same place (Killick-Kendrick, 1990). Phylogenetic analysis of the leishmanial ribosomal internal transcribed spacer 1 region indicates that the sequences from *L.* (*L.*) *major* DNA determined from *S. ingrami* align most closely with a human isolate from Ghana (MHOM/GH/2004/HO-004) (Fryauff et al., 2006). Thus, based on the documented anthropophilic nature of *S. ingrami* (unpublished results; Kweku et al., 2011), its abundance and the detection of *L.* (*L.*) *major* DNA within this sand fly as the same *Leishmania* species found in man from the same CL focus, suggests that *S. ingrami* is a possible vector of *L.* (*L.*) *major* transmission in the study area.

The amplification of *Leishmania* DNA in *S. ingrami* and *S. hamoni*, collected in Taviefe and Klefe communities, respectively, which clustered together with other ITS1 sequences of *L.* (*L.*) *tropica*, suggests that *L.* (*L.*) *tropica* might also be associated with the cases of CL in the outbreak area. Although phylogenetic analysis indicates that the ITS1 sequences from *L.* (*L.*) *tropica* DNA in Ghana have close affinity to an African isolate of *L.* (L.) *tropica* from Egypt, they were divergent from reported African strains. *L.* (*L.*) *tropica* is recognized as a very heterogeneous species of *Leishmania* and intra-specific microheterogeneity has been readily demonstrated (Schönian et al., 2001). The lack of information on the existence of *L.* (*L.*) *tropica* in human in this

focus might be largely due to the small numbers of human specimens investigated (Fryauff et al., 2006; Villinski et al., 2008) and difficulties in distinguishing between *L.* (*L.*) *major* and *L.* (*L.*) *tropica*. The emergence of human CL due to *L.* (*L.*) *tropica* as an increasingly important public health problem is now being reported in many foci in Africa such as Libya, Kenya, Egypt and Morocco (Sang et al., 1992; Shehata et al., 2009; Amro et al., 2012; Rhajaoui et al., 2012). Significantly, this study presents the first detection of *L.* (*L.*) *tropica* DNA in *Sergentomyia* species.

The detection of *L.* (*L.*) *tropica* DNA in *S. ingrami* and *S. hamoni* in this area indicates the possibility of the two species participating in the parasite transmission cycle. The conventional wisdom was that there is specificity of *Leishmania* species and the vector hosts. However, it has been demonstrated that most sand fly species can support the development of multiple *Leishmania* species (Volf and Myskova, 2007). The mechanism of *Leishmania* attachment in permissive sand fly vectors as seen in *Lutzomyia longipalpis* and *Phlebotomus arabicus* was shown to be independent of midgut lipophosphoglycan (LPG) (Myskova et al., 2007), whether such reported LPG-independent mechanism will apply to all permissive sand flies remains to be determined.

Another important finding is the infection of *Trypanosoma* DNA within *S. africana africana*. Phylogenetic analysis indicates that the SSU rRNA sequence from *Trypanosoma* DNA determined from *S. africana africana* in Ghana has a closer relationship with anuran trypanosomes, but was significantly divergent from all the reported strains (Ferreira et al., 2008; Kato et al., 2010a). These observations strongly suggest that *S. africana africana* was infected by a novel or an uncharacterized *Trypanosoma* species. Several sand fly species have been reported to transmit *Trypanosoma* species in tropical and subtropical areas and most parasites were anuran

trypanosomes (Lemos et al., 2008; Viola et al., 2008; Kato et al., 2010a). To date, there is no data in the literature about the natural infections of *S. africana africana* by trypanosomes. The result presented in this study reinforces the importance of correct identification of parasitic organisms within insect vectors.

This study characterized for the first time DNA barcodes and 18S rRNA gene of *S. ingrami*, *S. hamoni* and *S. africana africana*. However, the sand fly COI phylogenetic analysis places both *S. ingrami* and *S. hamoni* in the same clade while the 18S rRNA gene tree separates them, several factors may explain species non-monophyly (Jinbo et al., 2011), but the most likely explanation, therefore, is incomplete lineage sorting. Notwithstanding this, both genes confirmed that all the infected sand fly species belonged to the genus *Sergentomyia* and support the evidence for species of this genus as the possible vectors of *Leishmania* in Ghana. These findings question the dogma that *Leishmania* is exclusively transmitted by species of the genus *Phlebotomus* in the Old World. This observation warrants further investigation by microscopical examination of these sand fly species to obtain information on the localization of infection and presence of metacyclics, as well as initiating experimental studies in order to demonstrate their capacity to transmit the parasites. Additionally, surveillance would also be intensified aiming to identify the natural reservoir hosts in order to improve the understanding of the epidemiology of CL in the outbreak area.

Although sand flies of the genus *Sergentomyia* are considered as vectors of reptile *Leishmania* species, non-pathogenic to human (Bates, 2007), and *S. schwetzi* reported to be refractory to human *Leishmania* species (Sadlova et al., 2013), the data presented in this study adds to few reported studies on the detection of human pathogenic *Leishmania* species in some *Sergentomyia* species: *S. garnhami* (Mutinga et al., 1994), *S. babu* (Mukherjee et al., 1997), *S.*

sintoni (Parvizi and Amirkhani, 2008), *S. darlingi* (Berdjane-Brouk et al., 2012), *S. minuta* (Campino et al., 2013) and *S. gemmea* (Kanjanopas et al., 2013), incriminating them as potential vectors of mammalian *Leishmania* species. Definitive conclusion that these *Sergentomyia* species are vectors of human *Leishmania* species awaits confirmation by demonstrating experimentally their capacity to transmit *Leishmania* parasite by biting to mammals. Though it is technically difficult to establish laboratory colonies of some species of sand flies and infection studies therein, it remains highly desirable for vector incrimination.

Taken together, the presence of multiple of *Leishmania* spp. in this CL focus suggests a more complex epidemiology for this outbreak than anticipated. Therefore, a proper understanding of the different parasites' life cycles and parasite-vector-reservoir interplays is needed for predicting the risk and expansion of the disease and applying effective prevention and control strategies in Ghana.

Summary

Leishmania (*L*.) *major* and an uncharacterized species have been reported from human patients in a cutaneous leishmaniasis (CL) outbreak area in Ghana. Reports from the area indicate the presence of anthropophilic *Sergentomyia* species. In this study, *Leishmania* DNA-positive sand fly pools were analyzed by PCR-RFLP and ITS1 gene sequencing. The trypanosome was determined using the small subunit (SSU) rRNA gene sequence. DNAs of *L*. (*L*.) *major*, *L*. (*L*.) *tropica* and *Trypanosoma* species were observed to be associated with the sand fly infections. This study provides the first detection of *L*. (*L*.) *tropica* DNA and *Trypanosoma* species as well as the confirmation of *L*. (*L*.) *major* DNA within *Sergentomyia* sand flies in Ghana and suggests that *S. ingrami* and *S. hamoni* are possible vectors of CL in the study area. The detection of *L*. (*L*.) *tropica* DNA in this CL focus is a novel finding in Ghana as well as West Africa. In addition, the unexpected infection of *Trypanosoma* DNA within *S. africana africana* indicates that more attention is necessary when identifying parasitic organisms by PCR within sand fly vectors in Ghana and other areas where leishmaniasis is endemic.

Chapter III

Development of a loop-mediated isothermal amplification method for rapid mass-screening of sand flies for *Leishmania* infection

1. Introduction

The spread of leishmaniasis in endemic areas largely depends on the distribution of sand fly vectors. Approximately 800 sand fly species have been described, but only a few are medically important (Munstermann, 2004, Bates, 2007; Kato et al., 2010b). The monitoring of natural *Leishmania* infection in sand fly populations is an important epidemiological parameter for predicting the risk and prevalence of disease, the estimation of which depends on the reliable identification of infected sand flies. Estimation of *Leishmania* infection rates in the vector could serve as an indicator of a change in transmission intensity at a given endemic area. Such studies have been conducted either by dissecting individual insects and detecting the parasites under a microscope, or polymerase chain reaction (PCR)-based techniques to detect *Leishmania* DNA in sand flies (Kato et al., 2005). The former is laborious and time-consuming due to the low *Leishmania* infection rate in sand flies in most endemic areas (Aransay et al., 2000; Kato et al., 2005). The latter method has been proved to have both high sensitivity and specificity; however, the PCR methods require specialized instruments and take several hours, which make their use in resource-limited countries and under field conditions unfeasible. There is therefore a need for a simplified method of amplification and amplicon detection that can complement the available surveillance tools and generate information on the distribution or expansion of the disease.

As an alternative, the loop-mediated isothermal amplification (LAMP) technique may provide an answer to vector monitoring needs. This technique has been proven to be an accurate, rapid and simple method, which amplifies the target nucleic acid under isothermal conditions (Notomi et al., 2000). Recently, wide applicability of LAMP in the detection of parasitic protozoa such as *Babesia*, *Plasmodium*, *Leishmania* and *Trypanosoma* in clinical samples has been demonstrated (Ikadai et al., 2004; Poon et al., 2006; Thekisoe et al, 2007; Njiru et al., 2008; Takagi et al., 2009;

Laohasinnarong et al., 2011). Studies have also shown the application of LAMP to survey vectors of infectious diseases (Aonuma et al., 2009; Nakao et al., 2010; Thekisoe et al., 2010); however, these studies used purified DNA as a template for the LAMP assays. The use of a DNA extraction kit and the time-consuming DNA purification process in template preparation reduces the utility of LAMP technique as a surveillance tool for mass screening of infected vectors with pathogens in endemic regions. To date, no reports have been available on the use of the LAMP method for detection of *Leishmania*-infected sand flies. Considering these points, a novel LAMP assay for mass screening of sand fly vectors for *Leishmania* infection was developed and validated. In this study, a newly developed LAMP method for rapid detection of the *Leishmania* DNA within sand fly vectors is described, in addition to the use of a non-fluorescent cationic dye, namely, malachite green (MG) for the first time as a simpler colorimetric assay for the detection of LAMP reactions. The present method was evaluated with field-captured sand fly samples in order to demonstrate its efficacy and reliability as an important potential tool in assessing *Leishmania* infection and/or transmission intensity in endemic areas of Ecuador.

2. Materials and methods

2.1. Sand fly collection

Wild sand flies were collected in March, July and August 2012 in Andean areas of Ecuador; Alausi, Chanchan and Huigra (Province of Chimborazo), where Andean-type cutaneous leishmaniasis caused by *Leishmania* (*Leishmania*) *mexicana* is endemic (Kato et al., 2005, 2008). Sand flies were caught using protected human bait, CDC light traps and Shannon traps. The collected sand flies were dissected and then identified at the species level mainly based on the morphology of their spermathecae (Young and Duncan, 1994). These flies were examined for *Leishmania* infection by microscopy. To validate the newly developed LAMP method, sand fly collections were also made at the same areas using the capture methods mentioned above. These flies were fixed in absolute ethanol and stored at room temperature.

2.2. DNA preparation

DNA samples used in this study were prepared from the following *Leishmania* reference strains: *L.* (*L.*) *major*-like (MHOM/EC/1988/PT-115), *L.* (*L.*) *mexicana* (MNYC/BZ/1962/M379), *L.* (*L.*) *amazonensis* (MHOM/BR/1973/M2269), *L.* (*Viannia*) *guyanensis* (MHOM/BR/1975/M4147), *L.* (*V.*) *braziliensis* (MHOM/BR/1975/M2904) and *L.* (*V.*) *panamensis* (MHOM/PA/1971/LS94) in the New World, and *L.* (*L.*) *major* (MHOM/SU/1973/5ASKH), *L.* (*L.*) *tropica* (MHOM/SU/1958/strainOD), *L.* (*L.*) *infantum* (MHOM/ES/1993/PM1), *L.* (*L.*) *donovani* (MHOM/SU/1962/2S-25M-C2) in the Old World. DNA from *Leishmania*-related parasites capable of infecting sand flies, such as *Endotrypanum* (Kato et al., 2007) and anuran *Trypanosoma* species (Ferreira et al., 2008), and also DNA from the sand flies *Phlebotomus papatasi* and *Lutzomyia ayacuchensis*, were included to evaluate the present LAMP primer specificity.

For sand fly crude DNA extraction, ethanol-fixed sand fly samples were individually lysed in 50 µl of DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH8.0), 10 mM EDTA and 0.1% sodium dodecyl sulfate (SDS)] in the presence of proteinase K (100 µg/ml) without homogenization. The samples were incubated overnight at 37 °C. After 25 µl of distilled water was added, the samples were preheated at 95 °C for 5 min, and 1 µl was directly used as a template for the LAMP method.

2.3. Polymerase chain reaction amplification

Conventional PCR was performed with previously described primers specific for *Leishmania* minicircle kinetoplast DNA (mkDNA) (Kato et al., 2005, 2007). The primer sequences used for amplification were 5´ -CTRGGGGTTGGTGTAAAATAG- 3´ (L.MC-1S) and 5´ -TWTGAACGGGRTTTCTG- 3´ (L.MC-1R) (Kato et al., 2005). The reaction was carried out in a volume of 15 µl using a pair of primers (0.4 µM each) and 2 x Ampli Taq Gold PCR Master Mix (Applied Biosystems, NJ, USA). After an initial denaturation at 95 °C for 10 min, PCR amplification was performed with 30 cycles of denaturation (95 °C, for 1 min), annealing (55 °C, for 1 min), and polymerization (72 °C, 1 min), followed by a final extension at 72 °C for 10 min. The PCR products were resolved on a 2% agarose gel electrophoresis.

To identify sand fly by PCR-restriction fragment length polymorphism (PCR-RFLP), PCR was performed with *Lutzomyia* 18S rRNA gene-specific primer sequences 5´-TGCCAGTAGTTATATGCTTG- 3´ (Lu. 18S 1S) and 5´ -CACCTACGGAAACCTTGTTAC- 3´ (Lu. 18S AR) (Kato et al., 2007, 2008). The reaction was carried out in a volume of 15 µl using the primers (0.4 µM each), Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan), and *Taq* polymerase (*Ex Taq*; Takara Bio, Shiga, Japan). After an initial denaturation at 95 °C for 10 min,

PCR amplification was performed with 40 cycles of denaturation (95 °C, for 1 min), annealing (50 °C, for 1 min), and polymerization (72 °C, 2 min), followed by a final extension at 72 °C for 10 min.

Each PCR product (5 µl) was digested with the restriction enzyme, *Afa* I (Takara Bio) and subsequently resolved on a 2% agarose gel electrophoresis (Kato et al., 2007, 2008).

2.4. Loop-mediated isothermal amplification method

Sets of forward and backward external primers (F3 and B3) and forward and backward internal primers (FIP and BIP) were designed using PrimerExplorer version 4.0 software (http://primerexplorer.jp/elamp4.0.0) based on the conserved region of the *Leishmania* 18S ribosomal RNA (rRNA) gene. This region was considered to be appropriate because of its conservation across *Leishmania* species. Selection of primers used required a number of preliminary LAMP experiments for optimization. Selected LAMP primers are shown in Table 4. The LAMP assay was conducted in 15 µl of a reaction mixture consisting of a 1.6 µM concentration of each inner primer (FIP−Le.rRNA and BIP−Le.rRNA), a 0.2 µM concentration of each outer primer (F3−Le.rRNA and B3−Le.rRNA), 1 x reaction mix (Eiken, Tochigi, Japan), 8 U *Bst* DNA polymerase (Eiken), 0.004% malachite green (MG) dye (dissolved in distilled water), and 1 µl of template DNA. The mixture was incubated at 64 °C for 60 min in a heating block and then heated at 80 °C for 5 min to terminate the reaction. A positive control (DNA from a reference stain: *L.* (*V.*) *braziliensis* - MHOM/BR/1975/M2904) and a negative (water) sample were included in each LAMP assay. At the end of incubation, the amplification of the target DNA was confirmed based on direct visual inspection of the reaction tubes by the naked eye; a positive amplification showed as light blue, whereas in the absence of amplification, the reaction mixture became

colorless. For further confirmation, 2 µl of each LAMP product were subjected to electrophoresis on 2.5% agarose gels and visualized.

To determine the analytical sensitivity of the LAMP test, 10-fold serial dilutions of purified *L.* (*L.*) *mexicana* (MNYC/BZ/62/M379) and *L.* (*L.*) *major*-like (MHOM/EC/88/PT-115) DNA (equivalent to 10^4 to 0.01 parasites) were used as a template. Additionally, 0.1 and 0.01 parasite DNA mixed with crude sand fly extract were used as a template to test the detection sensitivity of the newly developed LAMP method in crude extract. Furthermore, LAMP assay was performed using crude sand fly DNA samples naturally infected with *L. (L.) mexicana* and *L.* (*V.*) *naiffi* to confirm the assay amplification efficiency.

2.5. *DNA sequencing*

The LAMP products were purified using a PCR purification kit (Qiagen, Valencia, CA) and then sequenced with FIP−Le.rRNA and BIP−Le.rRNA primers (Table 4) using a BigDye Terminator version 3.1 Cycle-Sequencing Kit (Applied Biosystems, Foster City, CA). Furthermore, in order to confirm the *Leishmania* species within the LAMP-positive sand flies, PCR amplification was performed with primers specific for *Leishmania* cytochrome *b* (*cyt* b). The primer sequences were 5´-GGTGTAGGTTTTAGTYTAGG-3´ (L.cyt-S) and 5´-CTACAATAAACAAATCATAATATRCAATT- 3´ (L.cyt-R) (Kato et al 2005, 2007). PCR was carried out in a volume of 15 µl using the primers (0.4 µM each), Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan), and BIOTaq HS DNA polymerase (Bioline, London, UK). After an initial denaturation at 95 °C for 10 min, PCR amplification was performed with 40 cycles of denaturation (95 °C, for 1 min), annealing (55 °C, for 1 min), and polymerization (72 °C, 2 min), followed by a final extension at 72 °C for 10 min. The PCR products were analyzed on a 2%

agarose gel and directly cloned into the pGEM-T Easy Vector System (Promega, Madison, WI) and sequences were determined by the dideoxy chain termination method using a BigDye Terminator Cycle-Sequencing Kit (Applied Biosystems, Foster City, CA).

Table 4. Nucleotide sequences for the *Leishmania* 18S rRNA LAMP primers

Name of primer	Sequence (5´ − 3´)
F3−Le.rRNA	GGGTGTTCTCCACTCCAGA
B3−Le.rRNA	CCATGGCAGTCCACTACAC
FIP−Le.rRNA	TACTGCCAGTGAAGGCATTGGTGGCAACCATCGTCGTGAG
BIP−Le.rRNA	TGCGAAAGCCGGCTTGTTCCCATCACCAGCTGATAGGGC

3. Results

3.1. Sensitivity and specificity of LAMP

Successful amplification reactions with I8S rRNA *Leishmania* LAMP primers (Table 4) were achieved in sample tubes containing each target DNA. The LAMP results were identical when a thermal-cycler, water bath and the heating block were used as the source of heat. The sensitivities of the broad range LAMP assay were assessed with serial dilutions of *L*. (*L*.) *mexicana* and *L*. (*L*.) *major*-like DNA (10^4 to 0.01 parasites). The LAMP assay was able to detect all the broad range dilutions tested, while no amplification was detected in the negative control. The results were visually discriminated. All samples containing DNA of *Leishmania* parasites turned light blue, whereas the negative samples turned from green to colorless after the LAMP reaction without adding any reagent (Fig. 11A). In agreement with the new colorimetric LAMP method using DNA intercalating malachite green dye, gel electrophoresis also showed that 0.01 *Leishmania* parasites is sufficient for LAMP detection (Fig. 11B). The results of all the positive samples detected by the LAMP assay were achieved within 60 min. The comparative sensitivities of the LAMP and mkDNA PCR assays revealed that the LAMP assay was more sensitive than the mkDNA PCR assay, which has a detection limit of 0.1−1 parasites (data not shown), corresponding to a previous report (Kato et al., 2005). The sensitivity of the LAMP assay was found to be the same for both the *L*. (*L*.) *mexicana* and *L*. (*L*.) *major*-like strains tested in this study. The LAMP assay was able to amplify all the New and Old World human pathogenic *Leishmania* species tested and no reactivity was recorded from the anuran *Trypanosoma* sp., *P. papatasi* and/or *Lu. ayacuchensis* sand fly vectors, but cross reacted with *Endotrypanum*, a non-pathogenic parasitic protozoa to humans in the New World (Fig. 12A and B).

The LAMP assay could reliably be used to amplify *Leishmania* DNA directly from crude infected sand fly extract, and had high detection sensitivity down to levels of 0.1 and 0.01 parasites (data not shown). This result indicated the ability of the LAMP assay to withstand the inhibitory effects of components present in the crude sand fly extracts and still show high detection sensitivity.

3.2. Evaluation of LAMP for mass screening of sand flies from areas where leishmaniasis is endemic

In order to evaluate the field utility of the newly established LAMP method, the technique was applied to sand flies caught from leishmaniasis endemic areas of Eucuador. A total of 397 sand flies (192, 68 and 137 samples from Huigra, Chanchan and Alausi, respectively) were individually screened for *Leishmania* infection (Table 5). LAMP amplicons were directly detected in 8 samples (3 from Chanchan, 5 from Alausi). No *Leishmania* DNA was detected in any Huigra samples by the LAMP method. This observation corresponded with the results obtained from the microscopic examination of sand flies for *Leishmania* promastigotes in the same areas (Table 5). The LAMP results confirmed the applicability of this new field-based, MG-based-LAMP detection assay using crude extract as templates, and without gel electrophoresis in endemic areas, thereby reducing the cost and turnaround time for the LAMP method. The accuracy of the LAMP amplicons was confirmed by direct sequencing with inner primers wherein the sequences obtained showed greater homologies with 18S rRNA genes from *Leishmania* species registered in GeneBank (data not shown). For further confirmation of the *Leishmania* species within LAMP-positive flies, the *cyt* b gene was analyzed by PCR. The *cyt* b genes from the eight LAMP-positive samples were successfully amplified and the sequences were determined. The gene sequences of all the positive samples (GenBank accession numbers: AB847149–AB847156) had the highest

degree of homology with the *cyt* b gene of *L*. (*L*.) *mexicana* (98.4 – 100%) when compared with those of other species (86.8 – 96.8%). This result indicated that the *Leishmania* DNA detected by the LAMP method within individual sand flies caught from Alausi and Chanchan were all *L*. (*L*.) *mexicana*, corresponding to the previous reports from the same areas (Kato et al, 2005, 2008). In addition, based on PCR-RFLP analysis of sand fly 18S rRNA with *Afa* I endonuclease digestion (Kato et al., 2007, 2008), all the LAMP-positive flies were identified as *Lutzomyia ayacuchensis* (Table 5), a proven and sole vector of *L*. (*L*.) *mexicana* in both Alausi and Chanchan, as well as other Andean endemic areas of Ecuador (Takaoka et al., 1990; Kato et al., 2005, 2008).

Table 5. Mass screening of sand flies from areas endemic for leishmaniasis in Ecuador by LAMP assay or microscopic examination

Location	Sand fly species	LAMP		Microscopic examination	
		No. screened	No. infected (%)	No. examined	No. infected (%)
Huigra	*Lu. ayacuchensis*	190	0	199	0
	Lu. maranonensis	2	0	10	0
	Lu. hartmanni	0	0	4	0
Chanchan	*Lu. ayacuchensis*	68	3 (4.4)	176	5 (2.8)
	Lu. maranonensis	0	0	2	0
	Lu. robusta	0	0	1	0
Alausi	*Lu. ayacuchensis*	137	5 (3.6)	92	2 (2.2)
Total		397	8 (2.0)	484	7 (1.4)

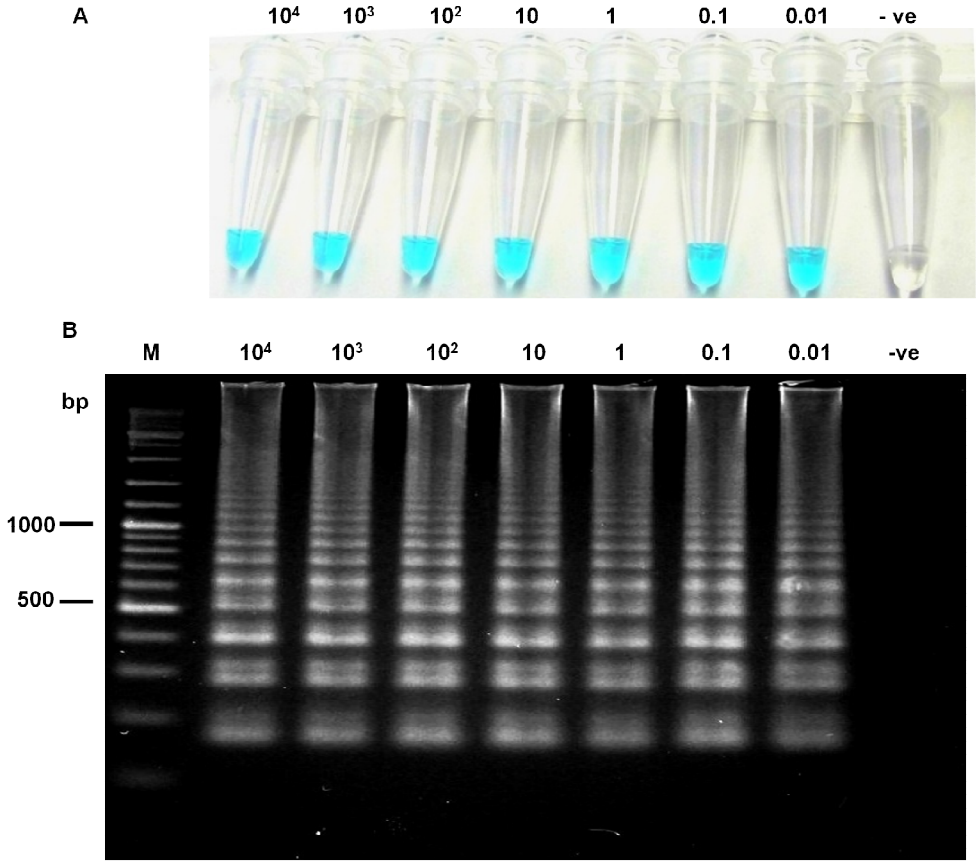

Fig. 11. Sensitivity of LAMP assays for detection of *Leishmania* DNA using serial dilutions of *Leishmania* (*Leishmania*) *mexicana* (MNYC/BZ/1962/M379).

(A) Colorimetric visual detection sensitivity of the LAMP assay, using pre-added malachite green dye in a closed reaction system. After incubation at 64 °C for 60 min, LAMP reactions were inspected visually by the naked eye without a UV illuminator or light box. The light blue color indicates a positive reaction and the colorless product indicates a negative reaction. (B) Agarose gel electrophoresis of LAMP products. M: gene ruler.

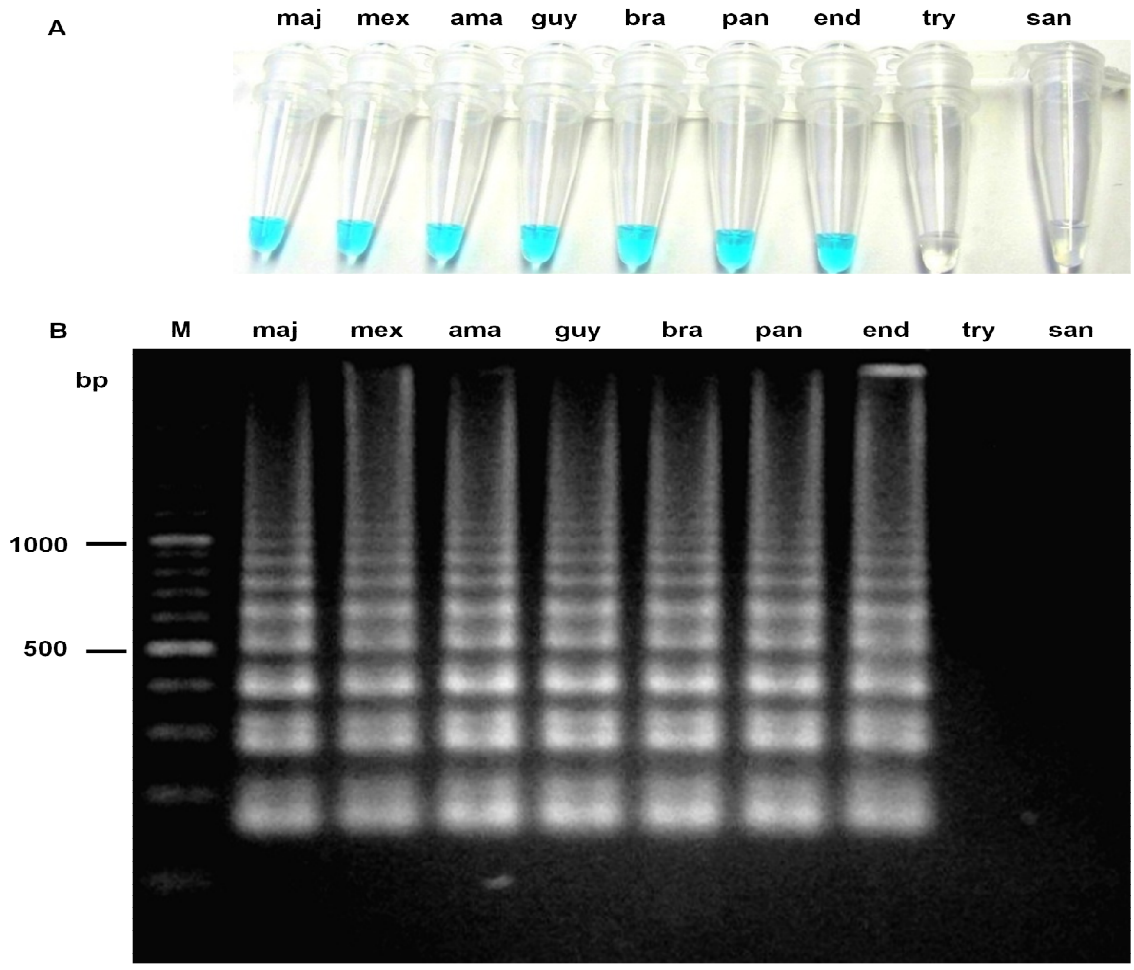

Fig. 12. Specificity of LAMP assay.

(A) Visual appearance of malachite green based-LAMP specificity reaction products inspected by the naked eye without UV or ordinary light. The color changes from light blue in the positive reactions to colorless in the negative reactions. (B) Specificity of the LAMP assay studied by agarose gel electrophoresis. M: gene ruler; maj: *L.* (*L.*) *major*-like; mex: *L.* (*L.*) *mexicana*; ama: *L.* (*L.*) *amazonensis*; guy: *L.* (*V.*) *guyanensis*; bra: *L.* (*V.*) *braziliensis*; pan: *L.* (*V.*) *panamensis*; end: *Endotrypanum* sp.; try: anuran *Trypanosoma* sp.; san: sand fly, *Lu. ayacuchensis*.

4. Discussion

Monitoring of infection in the vector sand fly population is an essential tool for surveillance of infections and identification of the vector species. The mass screening of sand flies for *Leishmania* infection has the potential to provide insight into the current transmission of the infection in endemic areas. In this study, the detection of *Leishmania* DNA within sand fly vectors using a newly developed LAMP method based on the 18S rRNA gene was demonstrated. The LAMP method is rapid, reliable and amplification of all *Leishmania* species in one test is achieved within 60 min using a normal water bath or heating block, obviating the need for a thermal cycler. The recorded analytical sensitivity of 0.01 *Leishmania* parasites indicates the applicability of this method, not only for detection of vector samples, but also that of clinical cases with extremely low parasite burden.

One of the most attractive features of the newly developed LAMP assay is that it has a great advantage in terms of amplicon detection by simple MG dye-mediated naked eye visualization. The detection method can be accomplished in a closed system without opening reaction tubes or post-amplification handling; a contamination prone step is completely avoided due to pre-addition of the MG in the LAMP reaction. As the MG signal recognition is highly sensitive, this system enables visual discrimination of results without costly specialized equipment and the use of a dangerous UV illuminator or light box, while interpretation by an independent observer is not required. The change of color is permanent and thus can be kept for record purposes. This new detection method could be helpful for high-throughput DNA detection in basic research and point-of-care testing. Several studies have used LAMP assays for detecting various pathogens, but mostly using a real-time turbidimeter (Toriniwa and Komiya, 2006; Parida et al., 2007; Yoneyama et al., 2007; Thekisoe et al., 2010), a real-time PCR system (Cai et al., 2008; Ohtsuki et al., 2008)

or fluorescent detection reagents (FDR) (Eiken) (Adams et al., 2010), which require a UV illuminator for LAMP product discrimination. The use of expensive and specialized equipment reduces the versatility of LAMP and greatly limits the wide use of this technique, especially in developing countries. Malachite green is very cheap, easy to prepare and does not require freezing for storage. This study represents the first report on the use of MG dye in the visualization of LAMP products. The visual assessment of LAMP products using MG dye is reproducible, robust and consistent. Significantly, MG does not interfere with DNA synthesis by *Bst* DNA polymerase. This wider working flexibility makes MG an effective, straightforward field colorimetric detection dye in LAMP studies.

The potential usefulness of the LAMP method as a field-friendly molecular surveillance tool is demonstrated by the ability of the new assay to amplify the target DNA from crude DNA extract without the expensive and time-consuming process of DNA purification. More promising is the ability of the LAMP method to overcome potential inhibitors in crude sand fly templates, an issue of particular concern for PCR assays, and still not compromise its detection sensitivity down to levels of 0.1 and 0.01 parasites. It is suggested that a significant amount of parasite DNA is lost during the extraction process, resulting in a lower detection limit when purified DNA is used as a template (Njiru et al., 2011). Other studies have shown superior tolerance of LAMP tests for biological substances (Poon et al., 2006; Kaneko et al., 2007). Uncompromising detection sensitivity of LAMP, as well as amplification consistency of the target DNA from crude sand fly DNA extracts stored at – 20 °C for months was observed, indicating the stability of the target DNA.

In the present study, the applicability of the colorimetric MG-based LAMP assay for detection of *Leishmania* DNA in sand flies as a field diagnostic and surveillance tool was validated with 397

wild-caught sand flies. The mass screening of sand flies from the three endemic areas, Huigra, Chanchan and Alausi, resulted in the detection of eight *Leishmania* DNA-positive sand flies. The eight positives were confirmed to be *Leishmania* DNA by direct sequencing of the LAMP products and further identified to the species level as *L.* (*L.*) *mexicana* based on *cyt* b gene sequencing analysis of the PCR amplicons. Furthermore, all the LAMP *Leishmania* DNA-positive flies were shown to be *Lutzomyia ayacuchensis* by PCR-RFLP of their 18S rRNA gene fragment patterns. This result was consistent with previous reports from the same study areas (Hashiguchi et al., 1991; Kato et al., 2005, 2008). Hence, in this proof-of-concept study, the LAMP method using MG dye was found to be a rapid, sensitive and high-throughput screening tool for the detection of sand flies infected with the *Leishmania* DNA in endemic areas. At best, it allows for an immediate real-time assessment of the presence of *Leishmania* parasites in endemic communities. The test is robust and amplification of *Leishmania* DNA directly from sand fly crude extract can be achieved using a normal water bath or heating block without compromising the test sensitivity.

Taken together, the successful detection of *Leishmania* DNA in field-caught sand flies demonstrated the applicability of the LAMP assay in the surveillance of *Leishmania* parasites in sand fly vectors in areas where leishmaniasis is endemic. The developed colorimetric LAMP method using MG will not only make an excellent surveillance tool, but also a very rapid, reliable and highly sensitive field diagnostic test which will be widely applicable for vectors, reservoirs and humans in resource-limited endemic countries.

Summary

Entomological monitoring of *Leishmania* infection in leishmaniasis endemic areas offers epidemiologic advantages for predicting the risk and expansion of the disease, as well as evaluation of the effectiveness of control programs. In this study, a highly sensitive LAMP method for the mass screening of sand flies for *Leishmania* infection based on the 18S rRNA gene was developed. The LAMP technique could detect 0.01 parasites, which was more sensitive than classical PCR. The method was robust and could amplify the target DNA within 1 hr from a crude sand fly template without DNA purification. Amplicon detection could be accomplished by the newly developed colorimetric malachite green-mediated naked eye visualization. Pre-addition of MG to the LAMP reaction solution did not inhibit amplification efficiency. The field applicability of the colorimetric MG-based LAMP assay was demonstrated with 397 field-caught samples from the endemic areas of Ecuador and eight positive sand flies were detected. The robustness, superior sensitivity, and ability to produce better visual discriminatory reaction products than existing LAMP fluorescence and turbidity assays indicated the field potential usefulness of this new method for surveillance and epidemiological studies of leishmaniasis in developing countries.

Chapter IV

A rapid molecular diagnosis of cutaneous leishmaniasis by colorimetric malachite green-loop-mediated isothermal amplification (LAMP) combined with Flinders Technology Associates (FTA) card as a direct sampling tool

1. Introduction

Leishmaniasis is a wide spectrum of diseases, presenting three main clinically distinct manifestations in the human host; cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). The disease is endemic in 98 endemic countries worldwide, and threatens 350 million people, with some 2 million new cases occuring annually (WHO, 2010; Alvar et al., 2012). However, the actual burden of leishmaniasis is adversely underestimated and most cases are unreported, misdiagnosed or undiagnosed, especially in rural endemic areas (Alvar et al, 2012). A critical component towards an understanding of the epidemiology and proper control/treatment of leishmaniasis is early and accurate diagnosis.

The gold standard for the diagnosis of leishmaniasis is microscopic demonstration of *Leishmania* parasites from skin smear/biopsy samples or aspirates from lesions for CL and MCL, and splenic or bone marrow aspirates for VL (Reithinger and Dujardin, 2007; de Vries et al., 2015). Despite high specificity, these methods are insensitive, invasive, and also require technical expertise (Reithinger and Dujardin, 2007; de Vries et al., 2015). Serological tests such as the direct agglutination test (DAT) and rK39 immunochromatographic tests are sensitive, but are not very useful in CL diagnosis because of undetectable or low antibodies titers (Goto and Lindoso, 2010). In the last three decades, molecular approaches such as polymerase chain reaction (PCR)-based assays have been employed in the diagnosis of leishmaniasis due to their high sensitivity and specificity (Reithinger and Dujardin, 2007; de Ruiter et al., 2014; de Vries et al., 2015). Unfortunately, despite the numerous advancements based on PCR in leishmaniasis diagnosis, the need for expensive specialized equipment, the long time-to-result and lack of field applicability or feasibility have greatly hindered the integration of these techniques into the diagnostic algorithm in endemic areas. Therefore, there is an urgent need for development of a simple and robust field-

and clinic-applicable diagnostic method with high sensitivity for rapid detection of *Leishmania* infection in endemic areas.

In the search for a relatively cheap, rapid and simplified molecular technique, the loop-mediated isothermal amplification (LAMP) method has been shown to be an effective tool in detection of human pathogenic infectious agents such as viruses, bacteria and protozoa (Notomi et al., 2000, Mori et al., 2012; Dhama et al., 2014). Recent studies have also shown the utility of LAMP assays with the aid of fluorescent detection reagents (FDR) and turbidity in the reaction tubes (Takagi et al., 2009; Adams et al., 2010; Khan et al., 2012; Mikita et al., 2014) for the detection of *Leishmania* DNA amplification. Despite the progress made with LAMP in diagnosis of leishmaniasis, a simple and efficient procedure for field and clinic sample collection and storage without the need for liquid handling and refrigerant or cold storage, which are often unavailable in resource-poor endemic rural settings, is necessary. Flinders Technology Associates (FTA) cards (Whatman) lyse spotted cells and pathogens, and protect the nucleic acids from oxidative, nucleases and UV damage, as well as inactivate most microorganisms. The entrapped nucleic acids are preserved intact, while cellular debris can be removed by simple washes of the FTA cards. Studies have shown the utility of FTA cards for nested PCR analyses in epidemiological studies of leishmaniasis (Kato et al., 2010c, 2011b). However, the potential usefulness of FTA cards as a direct sampling tool for diagnosis of leishmaniasis by LAMP assay has not yet been well-explored. Therefore, in this study a quick, one-step, single-tube, sensitive colorimetric malachite green (MG)-based LAMP assay for detection of *Leishmania* DNA directly from FTA cards spotted with tissue materials from patient's lesions was established. The applicability of the described assay for diagnosis and surveillance of leishmaniasis was evaluated with samples collected from patients in endemic areas of Peru.

2. Materials and methods

2.1. Parasites and template preparation

A WHO reference strain of *Leishmania* (*Leishmania*) *major* (MHOM/SU/1973/5ASKH) was cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Cansera International, Etobicoke Ontario, Canada), 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 25ºC. Parasites were harvested in the log phase and suspended in phosphate-buffered saline (PBS), counted using a Neubauer counting chamber (Hirschmann, Germany), and then 10^6 to 1 parasites were prepared from the cultures. Each set of 10^6 to 1 parasites was applied to FTA cards (Whatman, Newton Center, MA, USA), and the coded cards were allowed to air dry and stored at room temperature. Ten to eleven months later, 2.0-mm-diameter discs were punched from each FTA card using a Harris micro-punch tool (Whatman) and washed twice with a FTA purification reagent (Whatman) and once with distilled water. The discs were air-dried and used directly as the DNA template for the LAMP reactions.

In addition to the analytical sensitivity of the FTA-LAMP using live parasites (10^6 to 1) prepared from *L.* (*L.*) *major* (MHOM/SU/1973/5ASKH) as described above, 10-fold serial dilutions of purified *L.* (*L.*) *mexicana* (MNYC/BZ/1962/M379) DNA (equivalent to 10^4 to 0.01 parasites) were individually applied to 2.0 mm pre-punched FTA cards, washed and air-dried. Each FTA card was directly used as a template to verify the detection sensitivity of the LAMP test down to levels of 0.1 and 0.01 parasites. Furthermore, the specificity of the field and clinic-LAMP test against *Leishmania*-related human pathogenic *Trypanosoma* parasites were assessed with DNA prepared from *Trypanosoma cruzi* (both Tulahuen and Y strains) and *T. brucei gambiense* (both IL2343 and Wellcome strains), as well as human and dog genomic DNAs.

2.2. Mice and experimental infection

Female BALB/c mice of 6-10 weeks old were purchased from CLEA Japan, Inc (Tokyo, Japan). The mice were infected by injecting stationary phase promastigotes (4×10^5) of *L.* (*L.*) *major* (MHOM/SU/1973/5ASKH) in 40 µl 0.9% normal saline solution intradermally via a footpad. Formation and progression of lesions were monitored for 4 months. Samples were taken by aspirating the cutaneous lesions on the footpad and spotting them onto an FTA card (Whatman). Discs of 2.0-mm-diameter were punched out from the FTA card and washed two times with a FTA purification reagent (Whatman), rinsed once with distilled water, air-dried and directly used as a template for the LAMP reaction.

2.3. Clinical samples

A total of 122 samples previously collected from patients with CL and MCL who visited the rural health centers at 15 Departments: Piura, Amazonas, Loreto, Lambayeque, Cajamarca, La Libertad, San Martin, Ancash, Lima, Pasco, Junin, Ayacucho, Apurimac, Cusco and Madre de Dios in Peru, for the diagnosis and treatment of leishmaniasis (Kato et al., 2010c) were used for evaluation in this study. Tissue materials were taken by aspirating or scraping the active edge of the lesions of a patient by local physicians and well-trained laboratory technicians and spotted onto an FTA card, coded, air dried and enclosed in self-sealing bag and stored at room temperature (Kato et al., 2010c).

2.4. FTA-loop-mediated isothermal amplification reactions

The LAMP assay was carried out as previously described (Chapter III) using the primers shown in Table 4 (Chapter III). The primer sequences were forward inner primer (FIP−Le.rRNA)

5´ -TACTGCCAGTGAAGGCATTGGTGGCAACCATCGTCGTGAG-3´; backward inner primer (BIP−Le.rRNA) 5´-TGCGAAAGCCGGCTTGTTCCCATCACCAGCTGATAGGGC-3´; forward outer primer (F3−Le.rRNA) 5´-GGGTGTTCTCCACTCCAGA-3´; backward outer primer (B3−Le.rRNA) 5´-CCATGGCAGTCCACTACAC-3´ (Table 4, Chapter III). One punch of an FTA card was used as a template for the LAMP reaction. The mixture was incubated at 64 °C for 60 or 30 min in a heating block and then heated at 80 °C for 5 min to terminate the reaction. A positive control (DNA from a reference stain: *L.* (*V.*) *braziliensis* - MHOM/BR/1975/M2904) and a negative (water) sample were included in each LAMP run. The LAMP test using one punch of an FTA card template per sample was repeated not more than twice for samples negative in the first LAMP reaction in order to achieve gene amplification.

2.5. Sequencing

To confirm that the LAMP products had the target sequence, direct sequencing of the LAMP amplicons was performed. The LAMP products were purified using a PCR purification kit (NIPPON Genetics, Tokyo, Japan) and then sequenced with a FIP−Le.rRNA primer (Table 4, Chapter III) using a BigDye Terminator version 3.1 Cycle-Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. Detection sensitivity and specificity of the FTA-LAMP test

The sensitivity of the FTA-LAMP assay was assessed with 10^6 to 1 *L.* (*L.*) *major* (MHOM/SU/1973/5ASKH) parasites, and genomic DNA of *L.* (*L.*) *mexicana* (MNYC/BZ/1962/M379, equivalent to 10^4 to 0.01 parasites) dilutions applied to FTA cards. The LAMP assay could detect all the *L.* (*L.*) *major* (10^6 to 1 parasites) levels from 2.0 mm FTA cards templates. No amplification was detected in the negative controls. Additionally, the FTA-LAMP test had high detection sensitivity down to a level of 0.01 parasites (Fig. 13A), which agreed with the previous report using purified DNA as a template (Chapter III). All the positive reactions turned light blue while the negative reactions became colorless without adding any reagent after incubation. The colorimetric results were confirmed by gel electrophoresis (Fig. 13B), and there was agreement in the detection of the amplification products by both systems. Furthermore, DNA amplification could also be detected within 30 min of incubation, even down to the level of 0.01 parasites (data not shown). No cross-reactivity was recorded from *Trypanosoma cruzi* (both Tulahuen and Y strains), *T. brucei gambiense* (both IL2343 and Wellcome strains), human and dog genomic DNAs (data not shown).

In addition, the LAMP assay reliably yielded positive reactions from a 2.0 mm punch of an FTA card spotted with cutaneous lesion-materials of the experimentally-infected mice (data not shown). These observations indicated the robustness and efficiency of the present LAMP assay in amplification of the target DNA from FTA card templates. The structures of the LAMP amplicons were confirmed by direct sequencing.

3.2. Evaluation of the FTA-LAMP assay with clinical samples from leishmaniasis endemic areas

Field and clinic-applicability of the developed FTA-LAMP assay were validated with clinical tissue materials spotted on FTA cards. A total of 122 clinical samples were used for the LAMP evaluation study (Kato et al., 2010c). LAMP amplicons were detected in 71/122 (58.2%) samples. All colorimetric results were supported by gel electrophoresis. The accuracy of the LAMP amplicons was confirmed by direct sequencing. Overall, the results indicated that the reliability and robustness of the established LAMP test were as efficient as the nested PCR using an FTA card as a template (Kato et al., 2010c).

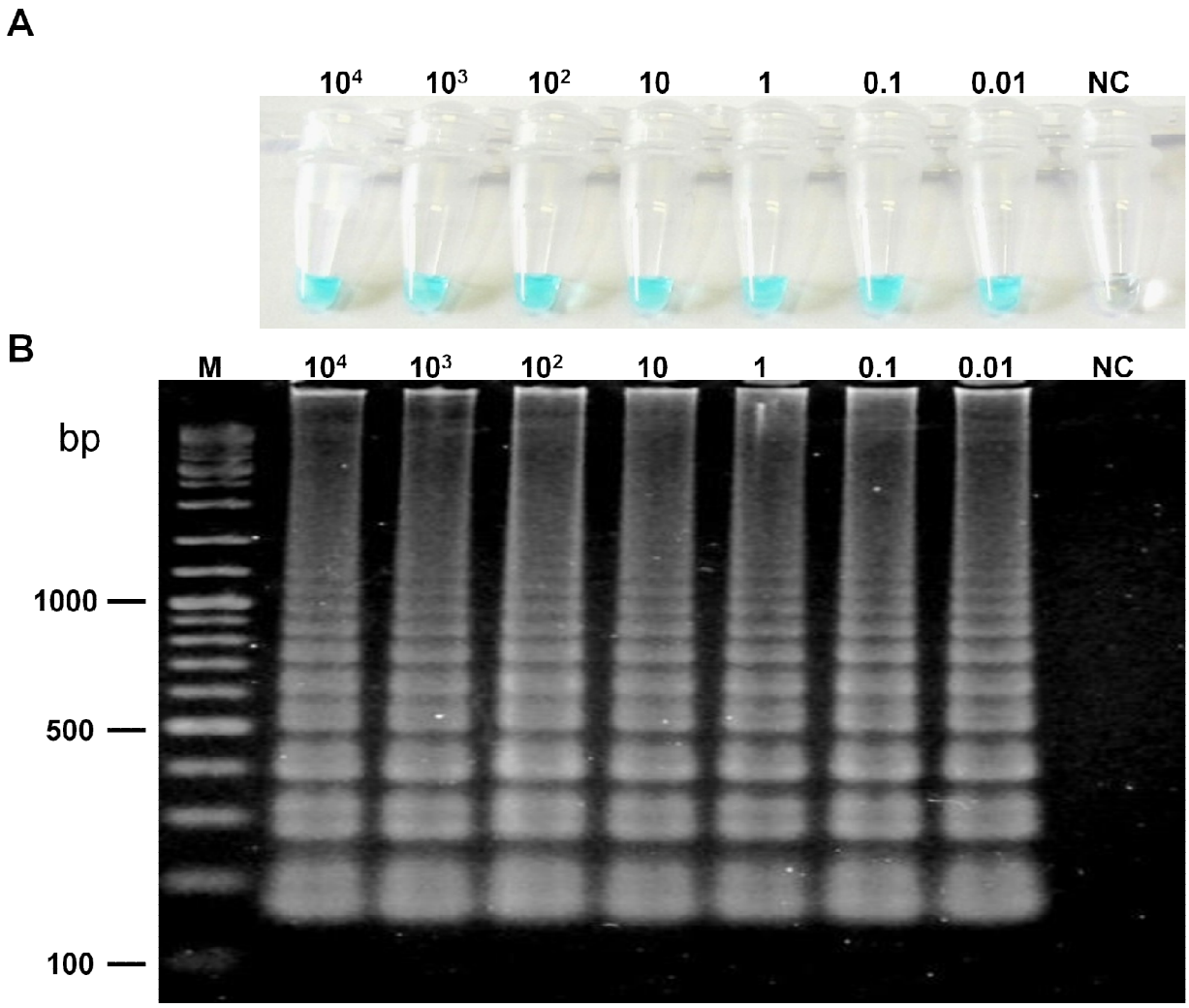

Fig. 13. Colorimetric FTA-LAMP detection sensitivity.

Genome DNAs from 10-fold serially diluted *Leishmania* (*Leishmania*) *mexicana* (MNYC/BZ/1962/M379) applied directly onto FTA cards were used as templates. Each reaction tube for the serial dilutions had 2.0 mm disc of FTA card DNA templates. (A) Visual appearance of malachite green based FTA-LAMP sensitivity reaction products inspected by the naked eye without a UV illuminator or ordinary light immediately after incubation. The color changes from light blue in the positive reactions to colorless in the negative reaction. (B) Sensitivity of the FTA-LAMP assay studied by agarose gel electrophoresis. bp: base pair; M: gene ruler; NC: negative control.

4. Discussion

Effective control of leishmaniasis relies on early diagnosis and treatment of infected patients. In endemic areas, patients are frequently treated on the base of clinical suspicion, because most classical and PCR-based diagnostic tools are often available in central reference laboratories or specialized centers. A test that is rapid, simple and field-applicable would be ideal in routine screening and diagnosis of patients in endemic areas. In this study, a rapid, one-step and single-tube, sensitive colorimetric-LAMP assay for the detection of *Leishmania* DNA in combination with an FTA card as a direct sampling tool was demonstrated. The assay showed high *Leishmania* detection sensitivity and negative results for human-pathogenic trypanosomes such as *Trypanosoma cruzi* and *T. brucei gambiense*. In addition to the LAMP high sensitivity, the test results can be obtained within 60 min using a normal water bath or heating block maintained at 64ºC.

In the present study, the applicability of the colorimetric malachite green (MG)-LAMP assay for detection of *Leishmania* DNA in clinical samples spotted on an FTA card was validated with 122 human tissue materials collected from lesions of patients with CL in Peru. *Leishmania* DNA was detected in 71 of the 122 FTA card samples by the LAMP test and indicated the robustness and reliability of the FTA-LAMP test, which was as efficient as nested PCR using an FTA card as a template (Kato et al., 2010c). Furthermore, the LAMP test could reliably amplify *Leishmania* DNA directly from clinical FTA card DNA templates washed with only distilled water, and the LAMP products were readily distinguished by both colorimetric MG and gel electrophoresis detection systems. Generally, the use of FTA card as a DNA template is critical, especially when parasite density is very low; the probability of a punch containing a parasite DNA is very low since parasite DNA is not evenly spread across the FTA matrix, but localized in areas where

parasites in the tissue materials are fixed. In addition to assay sensitivity, to obtain a successful detection, careful attention is necessary during sample collection to avoid spotting of diluted samples (blood-containing materials) onto FTA cards, which reduces the concentration of parasites on the cards.

Particularly important is the fact that in this study LAMP could reliably amplify *Leishmania* DNA directly from FTA cards templates. Studies have shown that PCR has lower sensitivity for amplification of DNA on FTA cards compared to LAMP (Kuboki et al., 2003), and PCR requires a special reagent, namely, Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan) to get rid of inhibitors in the FTA card templates (Kuboki et al., 2003; Kato et al, 2010c, 2011b), which does not affect the *Bst* DNA polymerase used in the LAMP reaction. In Chapter III, LAMP was shown to reliably amplify *Leishmania* DNA from crude sand fly templates, and previous reports have also shown superior tolerance of LAMP tests for biological substances (Poon et al., 2006; Kaneko et al., 2007). This study represents the first time tissue materials spotted on FTA cards have been used for diagnosis of leishmaniasis by LAMP assay.

The colorimetric FTA-LAMP assay has great potential as a simple molecular diagnostic tool. The closed MG detection system is highly sensitive, which enables visual discrimination of results by the naked eye without the aid of any specialized instrument, UV illuminator and ordinary light or independent observer. Pre-addition of MG eliminates the openings of tubes and completely avoids contamination problems. Aside from its success in discriminating LAMP products, MG is very cheap, easy to prepare and the change of color is permanent and can be kept for record purposes. Collectively, the use of FTA cards for direct sample collection and storage without the need for a cold chain, while malachite green dye which does not require freezing for storage coupled with the LAMP reagents reported to be stable at 25 and 37 ºC for 30 days (Thekisoe et al.,

2009), indicates the assay's potential as a field and clinical diagnostic tool deployable in developing countries. Furthermore, since the described LAMP assay is not species-specific, as the primers can amplify both Old and New World *Leishmania* species (Chapter III), the developed universal-LAMP test allows for the screening and detection of multiple species of the genus *Leishmania* in endemic areas.

In conclusion, this study provided a rapid, simple and highly sensitive colorimetric LAMP test combined with an FTA card as a direct sampling tool for diagnosis of leishmaniasis. The established LAMP test is in line with the recent global trend in seeking rapid, point-of-care tests for the control of infectious diseases. The simplicity, rapidity and sensitivity of the LAMP assay make it an ideal routine molecular diagnostic tool that will strongly support the cooperation of patients and clinicians in endemic areas. With such advantages, the established FTA-LAMP assay will be a good molecular tool for active screening and diagnosis of leishmaniasis in endemic areas.

Summary

Leishmaniasis remains one of the world's most neglected diseases, and early detection of the infectious agent, especially in developing countries, will require a simple and rapid test. In this study, a quick, one-step, single-tube, highly sensitive loop-mediated isothermal amplification assay for rapid detection of *Leishmania* DNA from tissue materials spotted on an FTA card was established. An FTA-LAMP with pre-added malachite green was performed at 64 ºC for 60 min using a heating block and/or water bath and DNA amplification was detected immediately after incubation. The LAMP test had high detection sensitivity down to levels of 1, 0.1 and 0.01 parasites. The field and clinic-applicability of the colorimetric FTA-LAMP assay was demonstrated with 122 clinical samples collected from patients suspected of having cutaneous leishmaniasis in Peru, from which 71 positives were detected. The LAMP assay in combination with an FTA card described here is rapid and sensitive, as well as simple to perform, and has great potential usefulness for diagnosis and surveillance of leishmaniasis in endemic areas.

General Conclusion

Leishmaniasis is a vector-borne parasitic disease caused by intracellular protozoa of the genus *Leishmania*, transmitted by female phlebotomine sand flies. Clinical forms of leishmaniasis are diverse and largely determined by the infecting species of *Leishmania*. Early and accurate diagnosis of infected patients and the identification of *Leishmania* species are crucial for treatment and prognosis. Moreover, effective control and monitoring of leishmaniasis transmission require the identification of circulating sand fly species and vectors in endemic areas.

In Chapter 1, the study provided the cytochrome c oxidase subunit 1 (COI) barcodes for several Peruvian sand flies and showed their effectiveness in discriminating species recognized through prior conventional taxonomic work. Neighbor-joining analysis based on Kimura 2-Parameter genetic distances formed non-overlapping clusters for all species. The levels of intraspecific genetic divergence ranged from 0 to 5.96%, whereas interspecific genetic divergence among different species ranged from 8.39 to 19.08%. The generated COI barcodes could discriminate between all the sand fly taxa. In addition, the COI gene was found to be useful in revealing population differentiation and cryptic diversity, and thus promises to be a valuable tool for vector surveillance.

In Chapter II, the study detected for the first time the DNA of *L.* (*L.*) *tropica*, *L.* (*L.*) *major* and *Trypanosoma* species within *Sergentomyia* sand flies in Ghana, and suggests that *S. ingrami* and *S. hamoni* are potential vectors of cutaneous leishmaniasis (CL) in the outbreak area of Ghana. The detection of *L.* (*L.*) *tropica* DNA in the CL outbreak focus is a novel finding in Ghana as well as West Africa. Additionally, the detection of *Trypanosoma* species in this study also represents the first report on infection of sand fly by trypanosomes other than *Leishmania* in West Africa.

These findings have considerable implications in determining the epidemiology of CL in Ghana. Significantly, the study supports the possibility of *Sergentomyia* sand flies as the vectors of CL in Ghana other than *Phlebotomus*, which contains all currently known vectors for *Leishmania* in the Old World.

In Chapter III, a novel highly sensitive LAMP method for the mass screening of sand flies for *Leishmania* infection based on 18S rRNA gene was developed. The LAMP method is rapid, robust and amplification of all *Leishmania* species in one test is achieved within 60 min using a normal water bath or heating block, obviating the need for a thermal cycler. The recorded analytical sensitivity of 0.01 *Leishmania* parasites indicates the applicability of this method, not only for detection of vector samples, but also that of clinical cases with extremely low parasite burden. One of the most attractive features of the established LAMP assay is that it has a great advantage in terms of amplicon detection by newly developed colorimetric malachite green dye-mediated naked eye visualization. Interestingly, the study represents the first report on the use of malachite green dye in the visualization of LAMP products. The field applicability of the assay was demonstrated using field captured sand fly specimens from Ecuador, and showed to be a reliable surveillance tool for leishmaniasis. At best, it allows for an immediate assessment of the presence of *Leishmania* parasites in endemic communities

In Chapter IV, a one-step, single-tube, field applicable LAMP assay in combination with FTA card as a direct sampling tool for diagnosis of cutaneous leishmaniasis was established. The developed LAMP assay could reliably amplify 0.01 *Leishmania* parasites from FTA card DNA template and showed no cross reactivity with *Leishmania* related human pathogenic trypanosomes. The colormetric FTA-LAMP assay with pre-addition of malachite green is rapid, robust and simple to perform and may provide an important tool for diagnosis of cutaneous leishmaniasis.

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Japanese Abstract

リーシュマニアおよび媒介昆虫サシチョウバエに関する分子疫学的研究

リーシュマニア症は、メスのサシチョウバエに媒介されるリーシュマニア原虫によって引き起こされる節足動物媒介性寄生虫疾患である。リーシュマニア症の臨床症状は多様で、感染原虫種が臨床像を決定する大きな要因となるため、感染者の早期診断や感染種の同定は、本症の治療や予後判定に重要である。また、リーシュマニア症伝播の制御や監視には、流行地に分布するサシチョウバエやベクター種の同定が重要である。

第 1 章では、ペルーに分布するサシチョウバエの COI 遺伝子バーコード領域の塩基配列を決定し、種の鑑別への有用性を明らかにした。Kimura 2-parameter を用いた近隣結合（Neighbor-joining）解析では、解析した全ての種が独立したクラスターを形成していた。遺伝子多型の頻度は、同種内では 0-5.96%、異種間では 8.39-19.08%であり、COI バーコードによって解析した全ての種を区別することが可能であった。COI 遺伝子は、集団解析や隠蔽種の多型解析に有用であることが明らかにされていることから、本遺伝子はサシチョウバエの調査に有用なツールとなると考えられた。

第 2 章では、*Sergentomyia* 属サシチョウバエから初めて *Leishmania* (*Leishmania*) *tropica*、*L.* (*L.*) *major* および *Trypanosoma* sp.の DNA を検出し、ガーナの皮膚型リーシュマニア症の発生地域で、*S. ingrami* や *S. hamoni* がベクターとなっている可能性について報告した。本研究は、ガーナなど西アフリカの皮膚型リーシュマニア症の発生地域で *L.* (*L.*) *tropica* を検出した最初の報告で、西アフリカのサシチョウバエからリーシュマニア属以外のトリパノソーマ科の原虫を検出した最初の報告でもある。これらの知見は、ガーナの皮膚型リーシュマニア症の疫学において、非常に重要な意味を持つものである。また本研究は、ガーナにおける皮膚型リーシュマニア症のベクターが、これまで旧大陸におけるベクター種として報告されている *Phlebotomus* 属のサシチョウバエとは異なり、*Sergentomyia* 属のサシチョウバエである可能性を示唆するものである。

第 3 章では、多検体のサシチョウバエから簡便にリーシュマニア感染の有無を検出することを目的とし、18S rRNA 遺伝子を標的とした高感度の LAMP 法を確立した。確立した LAMP 法は迅速かつ正確で、サーマルサイクラーの必要もなく、一般的なウォーターバスや加熱ブロッ

クを用いて 60 分以内に結果が判定できた。本法は 0.01 匹相当のリーシュマニア DNA を検出できる検出感度を有しており、これはサシチョウバエ検体のみならず、感染原虫数が少ない臨床検体からも原虫を検出できる感度であった。本研究で確立した LAMP 法の特徴の一つは、DNA の増幅をマラカイトグリーン色素により可視化することを可能にしたことで、マラカイトグリーンを LAMP 産物の可視化に用いた初めての報告である。本法をエクアドルの流行地で捕獲したサシチョウバエの調査に応用し、リーシュマニア症の調査ツールとしての有用性を確認した。本法は、疾患流行地におけるリーシュマニア原虫の迅速な調査を可能にするものである。

第 4 章では、皮膚型リーシュマニア症の分子診断を目的とし、FTA カードによるサンプリング法を用いて、ワンステップ、シングルチューブで、流行地で応用可能な LAMP 法の確立を行った。確立した LAMP 法は、FTA カードに添加した 0.01 匹相当のリーシュマニア DNA を検出することができ、リーシュマニアに近縁なヒト感染性トリパノソーマとの交差反応は認められなかった。採材に FTA カードを用いた LAMP 法は、迅速かつ簡便であり、皮膚型リーシュマニア症における有用な診断ツールになると考えられた。